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The Key Role of Homocysteine Thiolactonase Activity in Regulating Antioxidant Status of Cigarette Smokers According to PON1 Q192R Gene Polymorphisms

A Thesis
Submitted to the Council of the College of Science، University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemistry

by

Nasr Jabr Obaid Shanawa

B.Sc. University of Babylon

2007
Supervised by

Prof. Dr. Mahmood Hussein Hadwan

1442 A.H **2021 A.D**

بسم الله الرحمن الرحيم

فَكُلُوا مِمَّا رَزَقَكُمُ اللَّهُ حَلَالاً

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CERTIFICATION

I certify that this thesis entitled (**The Key Role of Homocysteine Thiolactonase Activity in Regulating Antioxidant Status of Cigarette Smokers According to PON1 Q192R Gene Polymorphisms)** was prepared under my supervision at the Department of chemistry/ College of Science / University of Babylon, in partial requirements for the Degree of Master of Science in Biochemistry and this work has never been published anywhere.

Signature:

Name: **Dr. Mahmoud Hussein Hadwan**

Title: Professor

Address: Department of Biochemistry -College of Sciences - University of Babylon

Date: / / 2021

In the view of the available recommendation, I forward this thesis for debate by the Examination Committee.

Signature:

Name: **Dr. Saadon Abdulla Aowda**

Title: Professor

Address: Head of Department of Chemistry-College of Sciences-University of Babylon

Date: / /2021

Examination Committee

We, the examiner committee, certify that we have read the thesis entitled "(**The Key Role of Homocysteine Thiolactonase Activity in Regulating Antioxidant Status of Cigarette Smokers According to PON1 Q192R Gene Polymorphisms)**" and examined the student (**Nasr Jabr Obaid)** in its contents at 22/ 11/ 2021, and that in our opinion it is accepted as a thesis for a **Master‹ s** degree in chemistry with (**Excellent**) estimation.

Signature:

Name: **Dr. Raid M.H. Al- Salih**

Title: Professor

Address: Dhi Qar University

College of Science, Chemistry

Date: / / 2021

(Chairman)

Signature:

Name**: Dr. Anwar Jasib Thaaban**

Title: Professor

Address: Al-Qadisiyah University

College of Medicine

Date: / / 2021

(Member)

Signature:

Name: **Dr. Zeina Abbas Ali**

Title: Professor

Address: Babylon University

College of Medicine

Date: / / 2021

(Member)

Signature:

Name: **Dr. Mahmood Hussein Hadwan**

Title: Professor

Address: Babylon University

College of Science, Chemistry

Date: / / 2021

(Member and supervisor)

Approved for the College Committee of Graduate Studies.

Signature:

Name: **Dr. Enas M. AL-Robayi**

Title: Professor

Address: Dean of the College of Science - University of Babylon

Date: / / 2021

Dedication

To my father

The reason for what it has become today Thank you for your continued support and interest.

I dedicate a special appreciation to my dear mother, my brothers, my friends and my colleagues.

To all the pure souls of the martyrs of Iraq

Nassr

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Praise be to God, Lord of the Worlds, and prayers and peace be upon the most honorable of the prophets and messengers, our Prophet Muhammad and all his family and companions.

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Summary

Cigarette smoke is linked to a number of major health issues, including mouth cancer, throat cancer, stroke, liver cancer, and bladder cancer. Cigarette smoking is a complicated cocktail of around 7,000 chemicals. Any cigarette bowl of smoke contains reactive smoke (ROS) molecules such as superoxide (O_2-), hydrogen peroxide (H_2O_2), hydroxyl (OH), and peroxyl (ROO•) radicals. Any organ that plays a role in cancer growth, such as the lungs, neck, esophagus, pancreas, prostate, and bladder A tar phase and a gas phase have been found in CS, both of which are rich in oxygen-centered, carbon-centered, and nitrogen-centered free radicals as well as non-radical oxidants. These ROS, in turn, can trigger and promote oxidative damage in the form of lipid peroxidation.

In the year 2021, a clinical trial was conducted at the University of Babylon. Blood samples were collected from University of Babylon students. A total of 150 nonsmokers and 150 smokers were enrolled in the study. The following two groups were studied in the current study:
Group I: 150 nonsmoker subjects.

Group II: 150 Cigarette Smokers.

This study deals with five parts: -

1. The individuals' Total oxidant status (TOS) and Total antioxidant status (TAS) levels were assessed. Cigarette had significantly higher serum concentrations of total oxidants species than non-smokers. While cigarette smokers' serum total antioxidant capacity was considerably lower than that of non-smokers. These findings point to

oxidative stress and a smoker's oxidant defense mechanism being compromised.

2. The concentration of lipid peroxidation in the sera of nonsmokers and cigarette smokers was investigated. The current study's findings show a substantial rise (p 0.05) in Malondialdehyde concentration in all study cigarette smokers, as compared to the nonsmokers control group in Homocysteine Thiolactonase concentration in all group of study cigarette smokers and comparable to nonsmokers control group [1.1091ng/ml] in cigarette smokers group more significantly to be [1.2304 ng/ml].
3. The current study describes a simple spectrophotometric protocol for the assessment of Homocysteine Thiolactonase and demonstrates its reproducibility, accuracy, and precision. Result of this study refers to significant increase ($p < 0.05$) in Homocysteine Thiolactonase concentration in all group of study cigarette smokers and comparable to nonsmokers control group [146.393 ng/ml] in cigarette smokers group decreased significantly to be [104.636 ng/ml].
4. The Paraoxonase activity level was studied, and the results of the study indicated to significant decrease ($p < 0.05$) in Paraoxonase activity concentration in all group of study cigarette smokers and comparable to nonsmokers control group [145.33 ng/ml] in cigarette smokers group decreased significantly to be [128.56 ng/ml].
5. This study aims to investigate the effect of cigarette smoking on Homocysteine Thiolactonase (HTLase) according to PON1 Q192R gene polymorphisms and the correlation between this parameter and oxidant/ antioxidant status. The study the following parameters. Assay homocysteine thiolactonase (HTLase) and paraoxonase-1 (PON1) activity. Assessment total antioxidant status and total oxidant concentration.

6. Measure the lipid profile and lipid peroxidation. Study the Homocysteine Thiolactonase (HTLase) Gene Polymorphisms (PON1 Q192R gene polymorphisms). Try to develop a novel precise method for assessment of Homocysteine Thiolactonase (HTLase) activity and compared it with ordinary methods.

The extent of the effect of smoking on the activity of Homocysteine Thiolactonase has been reached, as well as the development of a new method for measuring enzyme activity, and comparing it with the usual methods.

Table of Contents

List of tables

List of Figures

List of Abbreviation

Abbreviation	Details
AD	Alzheimer’s disease
ALA	α-Lipoic acid
BBB	Blood-brain barrier
CAD	coronary artery disease
CAT	Catalase
CBS	cystathionine beta-synthase
CETP	cholesteryl ester transfer protein
CNS	The central nervous system
CO	Carbon mono oxide
COPD	Chronic obstructive pulmonary disease
CRD	Chronic renal disease
CS	Cigarette smoke
CVD	Cardiovascular Disease (CVD)
D.F	dilution factor
DCM	dichloromethane
DHLA	dihydro lipoic acid
DM	Diabetes mellitus
DTT	1,4-dithio-DL-threitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
Ero1	Endoplasmic reticulum oxidoreductin
ESRD	end-stage renal disease
FAS	ferrous ammonium sulfate
FOX	ferrous oxidation-xylenol orange assay
FRAP	ferric reducing antioxidant assay
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
HAT	hydrogen atom transfer
Hb	Hemoglobin
HCTL	Homocysteine thiolactone
HDL	High -density lipoproteins
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHcy	Hyperhomocysteinemia
ICSI	Microinjection

LCAT	lecithin: cholesterol acyltransferase
LDL	Low-density lipoproteins
LOOH	Lipid hydroperoxides
LPO	Lipid peroxidation
MDA	Malondialdehyde
MTHFR	Methylenetetrahydrofolate reductase
NF-Kb	The redox-sensitive transcription factor
NOX	NADPH oxidase
Nrf2	The nuclear factor 2
OD	optical density
O-dianisidine dihydrochloridein	3,3′-Dimethoxybenzidine dihydrochloride
$OH^{\bullet}$	Hydroxyl radical
OP	organophosphate
PCR	polymerase chain reaction
PDB	Protein Data Bank
PDI	protein disulfide-isomerase
PDT	photodynamic therapy
PON1	paraoxonase
Prx	Peroxiredoxin
Prx 4	Peroxiredoxin 4
RCF	Relative centrifugal force
RCT	randomized controlled trial
RFLP	Restriction Fragment Length Polymorphism
RNS	Reactive Nitrogen specious
R-O•	Alkoxyl radical
$ROO^{\cdot}$	peroxyl radical
ROS	Reactive Oxygen specious
SET	single electron transfer
SOD	Super Oxide dismutase
SSA	Sulfosalicylic acid
T2DM	Type 2 diabetes mellitus
TAC	Total antioxidant capacity
TAS	Total antioxidant status
TBA	thiobarbituric acid
TBARS	Thiobarbituric acid-reacting
TBE	Tris borate EDTA
t-BOOH	t-butyl hydro peroxide
TCA	Trichloroacetic
TE	Tris EDTA
TOS	Total oxidants status
TOS	Total oxidant status

Tris	
TRX	Thioredoxin
UPR	unfolded protein response
X	Xanthine
E	extinction coefficient

CHAPTER

ONE

INTRODUCTION

1. Introduction

1.1 Smoking

Despite the fact that smoking has been identified as the largest preventable cause of death worldwide and that its incidence has lately dropped in several countries, it remains a major public health threat [1]. Inhaling cigarette smoke passively in public areas is harmful to one's health [2]. At least one billion adults in the globe are addicted to tobacco, and at least 700 million children breathe cigarette smoke-polluted air [3]. Because adults smoke in locations where children live or play, exposure to cigarette smoke is almost unavoidable. According to the World Health Organization (WHO), the tobacco pandemic is one of the world's most serious public health risks (WHO, 2020). Tobacco-related deaths, which cause significant cardiovascular and respiratory ailments, have increased to more than eight million individuals each year worldwide. Surprisingly, almost seven million of those deaths are linked to direct tobacco use, while 1.2 million are nonsmokers who are exposed to secondhand smoke. All kinds of tobacco are known to be dangerous, and there is no such thing as a safe level of tobacco exposure (WHO, 2020). Cigarette smoking has been shown to increase the risk of atrial and ventricular arrhythmias, sudden mortality, and acute myocardial infarction, as well as create hemodynamic alterations that worsen heart failure [4].

1.1.1 Chemical Compounds of Cigarette Smoker

Inhalation of a number of toxins in cigarette smoke, including nitrosamines, polycyclic aromatic hydrocarbons, volatile organic compounds, and many heavy metals, can cause smoking-related disorders [5]. Arsenic (As), Cadmium (Cd), and Lead (Pb) are some of the most common heavy metals linked to smoking's negative health impacts [6]. Although some heavy metals are required for survival, the majority are deemed non-essential, and some have negative health effects in humans

[7]. Similarly, heavy metals (such as Cd and Pb) have been linked to increased oxidative stress, cell apoptosis, endocrine disruption, and epigenetic damage, and through these mechanisms of toxicity, such elements could have a negative impact on ICSI patient outcomes [8]. Nicotine is the main active element in cigarettes, and it raises catecholamine levels, which causes an increase in heart rate and blood pressure [9]. Smoking is a long-established risk factor for several malignancies, cardiovascular disorders, and chronic obstructive pulmonary disease, according to the World Health Organization [10]. Over 6 million people die each year due to tobacco consumption among the world's 1.3 billion smokers [11]. A number of epidemiological studies have linked cigarette smoking to glaucoma and increased intraocular pressure [12]. By clogging artery lumen with atherosclerotic plaque and intimal thickening, cigarette smoking contributes to vascular diseases. Glaucoma has been linked to damage to retinal ganglion cells and trabecular meshwork cells caused by smoking, which includes inflammation and apoptosis. Because of the oxidizing chemicals that form free radicals, smoking causes a lot of oxidative stress [11]. Tobacco smoking is one of the leading preventable causes of death in the world [13]. Toxins Associated with Smoking was the subject of Figure (1-1).

The Figure. (1-1) Toxins Associated with Smoking is a term that refers to toxins that are produced as a result of smoking.

(https://quittrainblog.com/chemicals-in-cigarettes/)

1.1.2. Types of Tobacco Smoke Exposure

1.1.2.1 The First Hand Smoke Exposure

Tobacco use has been identified as one of the leading preventable causes of mortality and disability in the United States (US). Every year, about 440,000 people die as a result of smoking. This suggests that smoking was responsible for about one out of every five deaths. This is predicted to be 5.4 million each year globally, and it is expected to rise [14,15].

1.1.2.2 The Second Hand Smoke Exposure

This is cigarette smoke inhaled by nonsmokers. It's also known as tobacco's hazardous smoke. Secondhand smoke is divided into two types:

1) Smoke that is emitted from the burning end or pipe of a cigarette.

2) Smoke from a smoker's exhalation in the main stream.

More than 126 million persons are exposed to second-hand smoke, according to a 2011 study by Spiegler. It resulted in 50000 deaths in the United States, implying that the global death toll would be significantly higher. As a result, sitting in the proximity of a smoker is harmful to non-smokers as well [15].

1.1.2.3. The Third Hand Tobacco Exposure

Tobacco residue can be found on appliances, clothing, and other items. It consists of unseen smoke that remains in the air after a cigarette has been extinguished [16]. It has been demonstrated that nicotine residue found on surfaces such as furniture can react with other chemicals in the environment to form carcinogens. Nicotine vapor generated by a lit cigarette adsorbs heavily on indoor surfaces such as walls, carpets, chairs, clothing, and loose household dust. In addition to the recognized negative health effects of first- and second-hand smoking, third-hand smoking may potentially pose a risk to neonates and infants [17].

1.2 Oxidative stress (OS)

Oxidative stress (OS) is described as a change in an organism's redox state, or a disruption in the balance between reactive oxygen species (ROS) and endogenous antioxidant defenses, which causes oxidation of lipids, proteins, and DNA, impairing cellular function [18]. As shown in Figure (1-2). Antioxidants help to delay or prevent oxidative damage caused by reactive oxygen species (ROS). The superoxide dismutase (SODs) antioxidant enzyme family is thought to be the first line of defense against oxygen poisoning. It catalyzes superoxide radical disputation to hydrogen peroxide and molecular oxygen [19]. The most prominent mode of action of tobacco smoke is oxidative damage. Since of the direct impacts of the free radicals found in smoke. It is induced by cigarette smoking. Although the relative importance of supposed smoking-induced psoriasis mechanisms is uncertain, direct oxidant delivery and subsequent enhancement of oxidative stress have been involved in the pathogenesis of smoking-induced psoriasis [20]. Previous investigation was assessed the amount of smoking-induced oxidative stress in psoriatic patients as defined by the lipid peroxidation product MDA and the efficient antioxidant activity represented by SOD, as well as their relationship to disease severity [21].

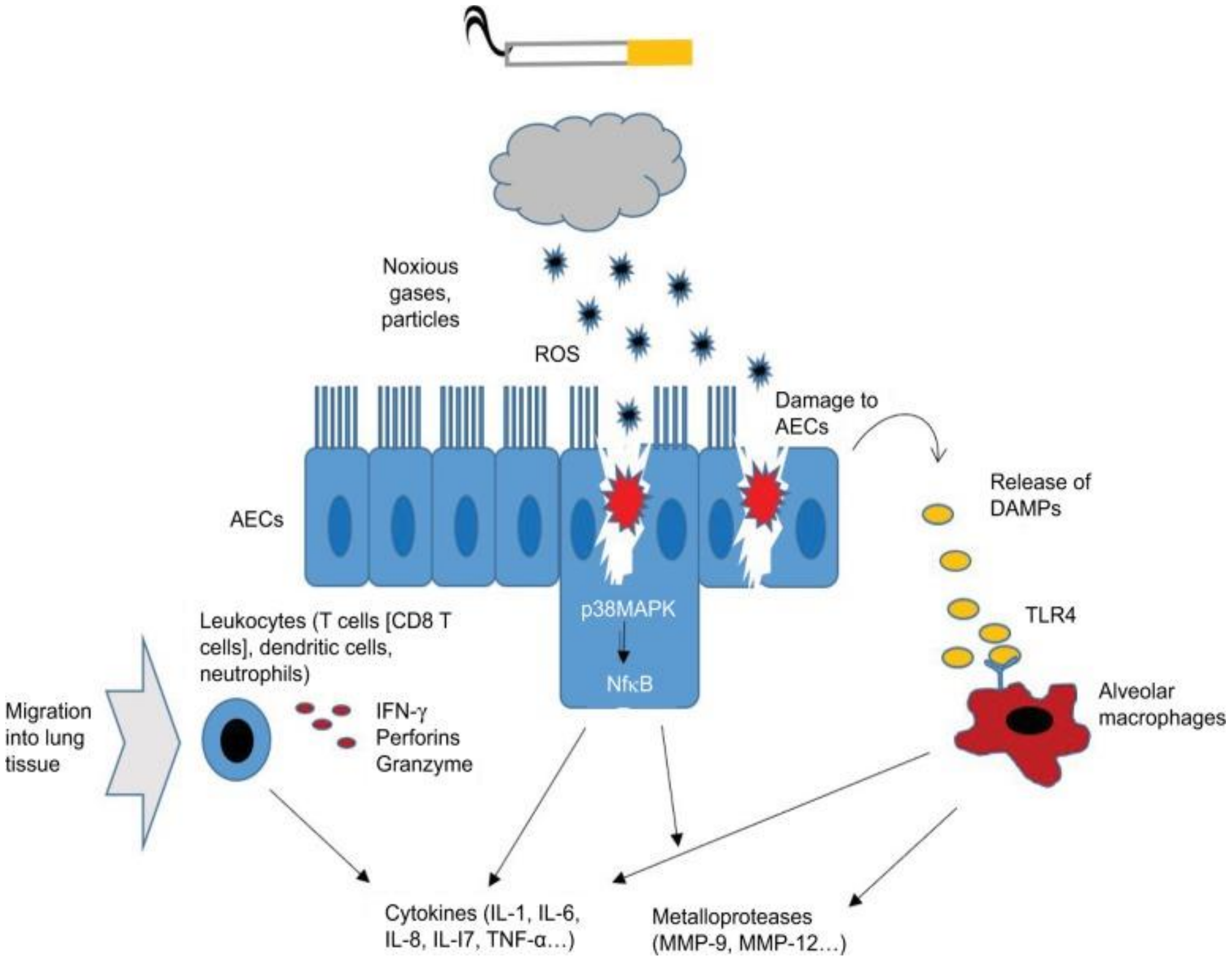


Fig. 1.2 Figure depicting the production of oxidative stress and inflammation in AECs by smoking [22].

reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive sulfur species (RSS) and other species, have the potential to induce oxidative stress as a result of their buildup within the cell to a level that exceeds the capacity to eliminate them [23]. Reactive oxygen species (ROS) are the most important type of reactive species because they contain non-radical forms that do not have unpaired electrons but are nevertheless extremely reactive, such as H_2O_2, $^{-}O_2$, hypochlorous acid (HClO), and ozone, in addition to free radicals (O_3). When an atom or molecule "loses" or "gains" one electron, or during hemolytic cleavage of a covalent link, these molecules with one or more unpaired electrons are created, resulting in high reactivity [24]. While $^{\bullet}OH$ reacts with all biological molecules its surroundings indiscriminately, $O_2^{\bullet}$ and H_2O_2 are highly selective. The most important ROS in terms of pathophysiology and physiological effects are $^{\bullet}OH$, $O_2^{\bullet -}$, organic alkoxy ($RO^{\bullet}$), and organic peroxyl radicals ($ROO^{\bullet}$), as well as non-radical species such as $^{1}O_2$, H_2O_2, and O_3 [25]. Toxicity is not always connected to reactivity. In many circumstances, ROS's prolonged half-life allows for more time for diffusion, giving it the ability to reach sensitive areas within a cell where it can react with biomolecules far away from where it was generated. For example, the relatively long-lived $O_2^{\bullet -}$ created on the mitochondrial membrane (with a half-life of (1– 4) s and a migration distance of 30 nm) diffuses toward the mitochondrial genome and decreases the transition metals within the genome. Hydrogen peroxide has a long half-life of 1 MS and a migration distance of 1 m, allowing it to react with DNA and protein Cys and Met residues distant from their source [26]. $^{\bullet}OH$, on the other hand, has a half-life of about 1 ns and a migration distance of 1 nm, thus it reacts with all nearby biomolecules such as DNA, RNA, lipids, and proteins (Figure 1-3)

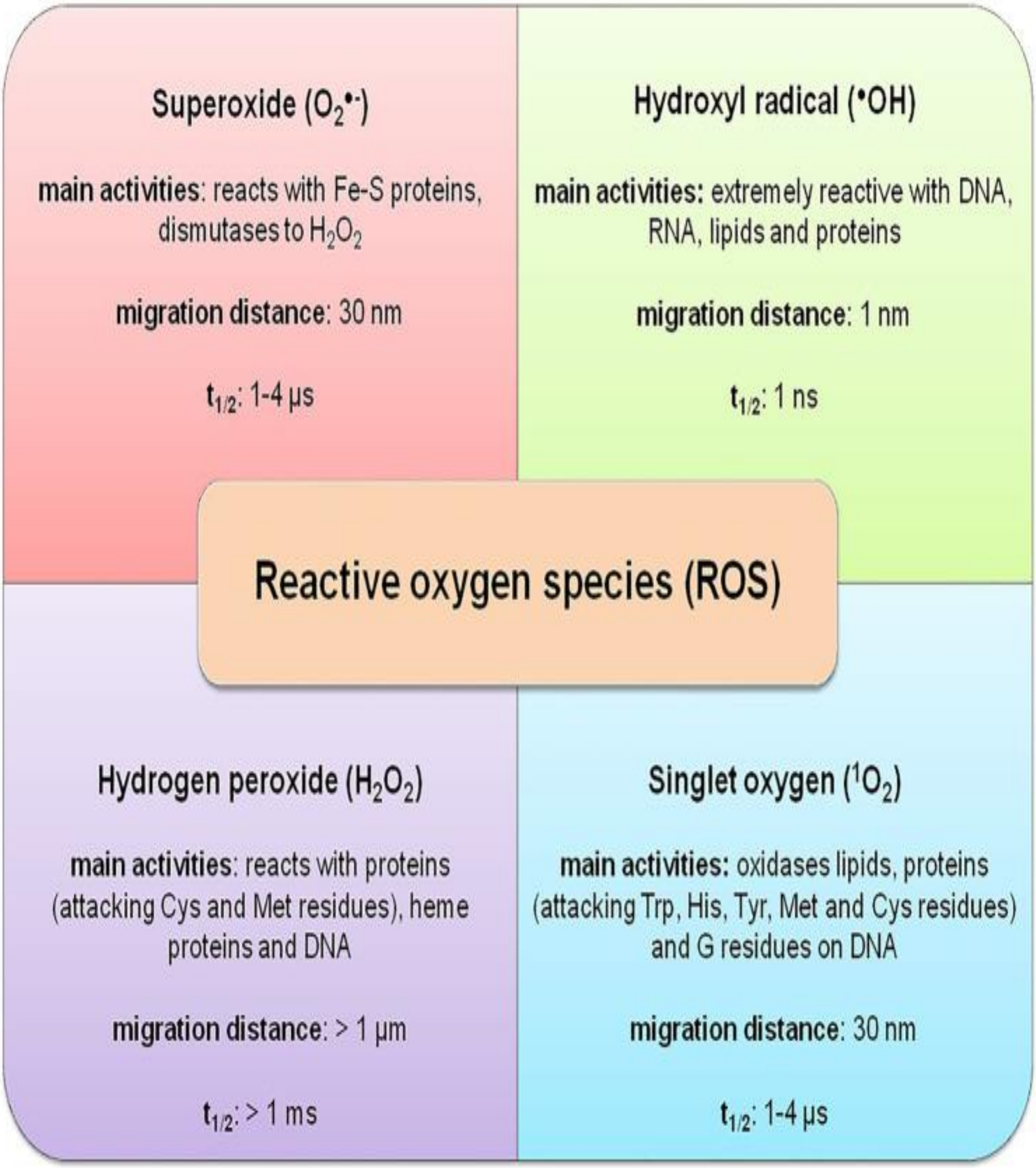


Fig. 1-3**:** Reactive oxygen species (ROS) in a biological cell: fundamental features adapted from: Mittler (2017; https://creativecommons.org/licenses/by-nc-nd/4.0/).

Antioxidants should be close to the radical's source of creation, competing with the free radical for the biological substrate, to prevent interactions between radicals and biological molecules. Oxidation of biomolecules, such as lipid peroxidation, protein damage (carbonylation of aminoalkanoic acid residues), oxidation of single DNA and RNA nucleotides, enzyme inhibition, and apoptosis activation, will occur if an antioxidant is not present in adequate quantities to neutralize ROS [27]. Direct inactivation of enzymes by oxidation of cysteine (–SH) or methionine ($–SCH_3$) residues required for catalysis is the mechanism of its toxicity. H_2O_2 has an indirect effect on DNA molecules by penetrating cellular membranes via peroxiporins and interacting with Fe^{+2} or Cu^{+} ions, resulting in the creation of more potent toxic species like $^{\bullet}OH$. $^{\bullet}OH$ is responsible for the majority of the damage to DNA molecules in H_2O_2-treated cells. Because NADPH oxidase (NOX) (EC 1.6.3.1) is activated when cells are exposed to H_2O_2, this might sometimes result in an increase in $O_2^{\bullet}$ production [28]. The initial cell-generated ROS, superoxide anion radical ($O_2^{\bullet}$), initiates a cascade of reactions and, as a result, the creation of secondary ROS, either directly or via enzymatic and metal-catalyzed processes, depending on the cell compartment. Produced by single-electron reduction of molecular oxygen within the cell, superoxide dismutase activity quickly converts it to H_2O_2, avoiding the build-up of $O_2^{\bullet-}$ and, as a result, damage and inactivation of proteins containing Fe-S clusters [29].

1.3 Antioxidant

Antioxidants are substances that can either suppress or delay the oxidation processes that occur under the presence of ambient oxygen or reactive oxygen species, or ROS 1. It is described as a chemical in meals that significantly inhibits or reduces the detrimental effects of reactive species such as ROS and reactive nitrogen species or normal physiological functioning in humans when present at low concentrations relative to oxidizable substrates [30]. As illustrated in Figure (1-4), natural antioxidants can be separated into two categories: (1) enzymatic antioxidants and (2) non-enzymatic antioxidants [31]. These antioxidants are available as nutritional supplements or pharmaceutical medicines and contain active antioxidant components [30,32]. Many non-enzymatic antioxidants come from dietary sources, and they come in a variety of chemical families. Polyphenols are the most numerous [33]. Antioxidants are divided into two classes based on how they work:(1) hydrogen atom transfer (HAT) components (2) single electron transfer (SET) components. Low solubility, low shelf life, difficulty in packaging and handling, losses due to environmental stresses (light, oxygen, high temperature, and pH), and food processes (pasteurization, mixing, baking, sterilization, storage, home preparation, microwaving, boiling, steaming, and frying) are all obstacles to using natural antioxidants in food systems [34].

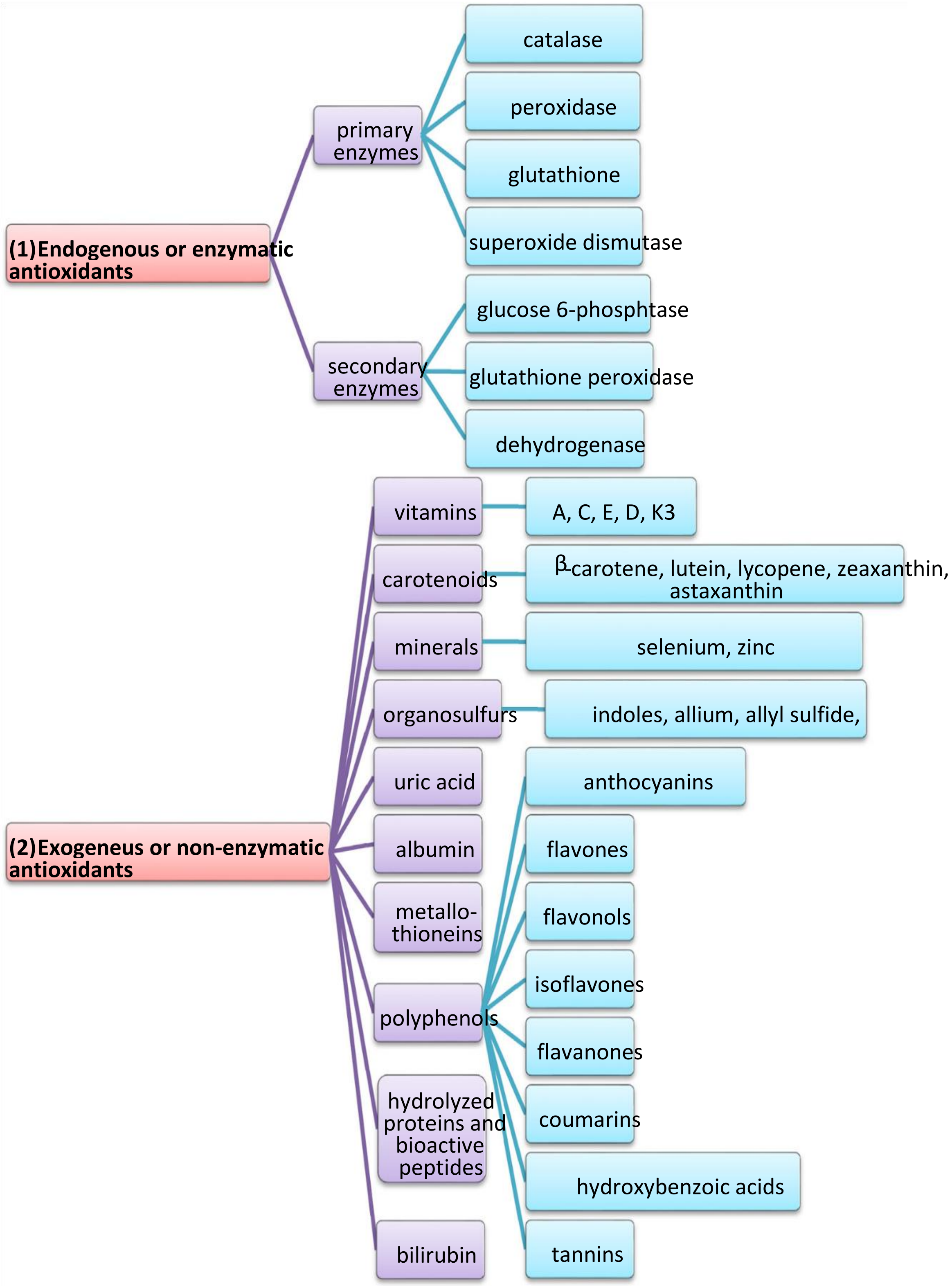


Fig. (1 -4) Natural antioxidants are divided into four categories [31].

Non-enzymatic antioxidants and enzymatic antioxidants are the two types of antioxidants (Fig 1.4). Enzymatic antioxidants provide primary defense, while non-enzymatic antioxidants such as vitamins A, E, and C provide secondary defense [35]. Vitamin A, E, and C deficiency appear to be a possible cause of depression. Humans require reactive oxygen and nitrogen species (ROS/RNS) to maintain homeostasis and health, however uncontrolled and excessive ROS/RNS have been linked to the development of a variety of disorders. A fine defense network protects aerobic organisms from oxidative stress caused by ROS/RNS, in which many antioxidants with multiple functionalities play roles such as hydrogen peroxide and hydro peroxide reduction, metal ion sequestration, reactive species scavenging, and damage repair [36].

1.3.1 Classification of Antioxidants

Non-enzymatic antioxidants and enzymatic antioxidants (Fig 1-4) are the two types of antioxidants [37].

1.3.1.1 Enzymatic antioxidants

1.3.1.1.1 Superoxide dismutase (SOD):

Superoxide dismutase is thought to be the first line of defense against cell harm since it catalyzes the conversion of primary ROS in oxygen metabolism to molecular oxygen and peroxide. The most important aspect is that SOD dissociates superoxide into less hazardous molecules [38].

1.3.1.1.2 Catalase (CAT):

Catalase was the first antioxidant enzyme discovered, and it catalyzes the two-phase conversion of hydrogen peroxide to water and oxygen. Catalases are enzymes that use an iron or manganese cofactor to catalyze the conversion of hydrogen peroxide to water and oxygen. One hydrogen peroxide molecule oxidizes its cofactor, which is then regenerated by transferring the attached oxygen to a second substrate molecule.

$$\text{Catalase-Fe (III)} + H_2O_2 \longrightarrow \text{Compound I}$$

$$\text{Compound I} + H_2O_2 \longrightarrow \text{Catalase-Fe (III)} + H_2O_2 + O_2$$

Each of the four protein subunits of catalase contains a hemi group and a NADPH molecule [39].

1.3.1.1.3 Glutathione Enzymes

Glutathione transferases, glutathione reductase, glutathione peroxidases, and glutathione "s" are all part of the glutathione mechanism. Glutathione peroxidase is a selenium-containing enzyme that catalyzes the degradation of hydrogen peroxide and organic hydro peroxides. These enzymes are particularly abundant in the liver. Glutathione peroxidases are enzymes that catalyze the oxidation of glutathione. Hydro peroxides, such as hydrogen peroxide and lipid hydro peroxides, act as substrates for these enzymes [40].

$$ROOH + 2GSSG \longrightarrow GSSG + H_2O + ROH$$

1.3.1.2 Non-enzymatic antioxidants

Organosulfur compounds (allium and allium sulfur), enzyme cofactors (Q10), nitrogen compounds (uric acid), vitamins (A, C, E, and K), minerals (Zn and Se), peptides (glutathione), and polyphenols (flavonoids and phenolic acid) are among the non-enzymatic antioxidants [41].

1.3.1.2.1 Vitamin A

Vitamin A is a carotenoid generated in the liver as a result of -carotene degradation. It has antioxidant activity because of its capacity to interact with peroxyl radicals before they propagate peroxidation to lipids. The face, hair, and internal organs are all protected by vitamin A [42].

1.3.1.2.2 Coenzyme Q10

Coenzyme Q10 is thought to work by inhibiting the formation of lipid peroxyl radicals. It neutralizes radicals even after they have formed. Vitamin E regeneration is an important function of this coenzyme. This coenzyme can be found in all cells and membranes, and it is essential for the respiratory chain and other metabolic processes in cells [43].

1.3.1.2.3 Uric Acid

Purine nucleotide metabolism produces uric acid in humans. After renal filtration, 90% of uric acid is reabsorbed by the body, demonstrating that it has significant roles inside the body. Uric acid is a potent scavenger of singlet oxygen and hydroxyl radicals and inhibits erythrocyte lysis by peroxidation. It also prevents the overproduction of oxo-heme oxidants caused by the interaction of hemoglobin with peroxides [44].

1.3.1.2.4 Glutathione

Glutathione is a naturally occurring tripeptide that shields cells from free radicals by donating an electron or a hydrogen atom. It's also important for the recovery of numerous antioxidants, including ascorbate. However, the endogenous antioxidant system is insufficient, and humans must rely on dietary antioxidants to reduce free radical levels [45].

1.3.1.2.5 Vitamin C

The generic names for vitamin C and vitamin E are ascorbic acid and tocopherols, respectively. L-ascorbic acid and L-dehydroascorbic acid are two antioxidant compounds made up of ascorbic acid. The gastrointestinal system processes these two compounds, which may be enzymatically interchanged in vivo. The radical anion, hydrogen peroxide, radical hydroxyl, singlet oxygen, and reactive nitrogen oxide are all scavenged by ascorbic acid [46].

1.3.1.2.6 Vitamin E

Vitamin E is the only primary lipid-soluble, chain-breaking antioxidant found in plasma, red cells, and tissues, and it protects lipid structures, particularly membranes. This avoids lipid peroxidation by transferring phenolic hydrogen to peroxyl radicals, which then produce tocopheroxyl radicals, which, despite being radicals themselves, are non-reactive and unable to initiate the oxidative chain reaction. Four tocopherols and four tocotrienols make up vitamin E. [47].

1.3.1.2.7 Vitamin K

Vitamin K1 and K2 are two natural isoforms of this vitamin. Vitamin K is a family of fat-soluble chemicals that are required for the conversion of protein-bound glutamates in a variety of target proteins to carboxyglutamates after translation. The 1, 4- naphthoquinone structure of certain vitamins is responsible for their antioxidant action [48].

1.3.1.2.8 Flavonoids

Flavonoids are a class of chemicals made up of the diphenyl propane skeleton (C6C3C6). Flavonols, flavanols, anthocyanins, isoflavonoids, flavanones, and flavones are all types of flavonoids. Flavanones and flavones are often found together in the same fruits and are related by particular enzymes, but flavones and flavonols are not linked by specific enzymes and are rarely found together. Flavonoids have antioxidant activity because of the phenolic hydroxyl groups linked to ring complexes. They can be used as reducing agents, superoxide radical scavengers, hydrogen donors, single oxygen quenchers, and metal chelators. They boost uric acid levels and low molecular weight molecules, activate antioxidant enzymes, reduce -tocopherol radicals (tocopheroxyls), inhibit oxide, ameliorate nitrosative stress, and activate antioxidant enzymes [48].

1.3.1.2.9 Phenolic Acids

Phenolic acids are used to make hydroxycinnamic and hydroxybenzoic acids. Gallic acid, a type of phenolic acid found in gallnuts, sumac, tea leaves, oak bark, and other plants, is one of the most researched and promising hydroxybenzoic compounds, while cinnamic acid is the precursor of all hydroxycinnamic acid. They're present in plant products and, on rare occasions, as esters and glycosides. They act as chelators and free radical scavengers, with specific effects on hydroxyl and peroxyl radicals, superoxide anions, and peroxynitrites [49].

1.3.1.2.10 Carotenoids

Carotenoids are a group of natural colors produced by plants and microorganisms. They are divided into two categories: carotenoid hydrocarbons known as carotenes with unique end-groups like lycopene and β-carotene, and xanthophyll-known oxygenated carotenoids like zeaxanthin and lutein. Carotenoids have antioxidant activity as a result of singlet oxygen quenching, which results in excited carotenoids dispelling the newly gained energy through a series of rotational and vibrational interactions with the solvent, allowing more radical species to quench themselves [50].

1.3.1.2.11 Minerals

Minerals are found in tiny amounts in animals and make up a minor portion of dietary antioxidants, but they play a critical role in their metabolism. Minerals with antioxidant action include selenium and zinc. Selenium is found in both organic (selenocysteine and selenomethionine) and inorganic (selenite and selenate) forms in the human body. It doesn't work directly on free radicals, but it's an essential component of the majority of antioxidant enzymes (metalloenzymes, glutathione peroxidase, and thioredoxin reductase), without which it wouldn't do much good [51].

1.4 Lipoprotein

Because cholesterol and triglycerides are water insoluble. Lipoproteins are complex particles with a central core of cholesterol esters and triglycerides surrounded by free cholesterol, phospholipids, and Apo-lipoproteins, which explain lipoprotein synthesis and function, as shown in Fig (1-5).

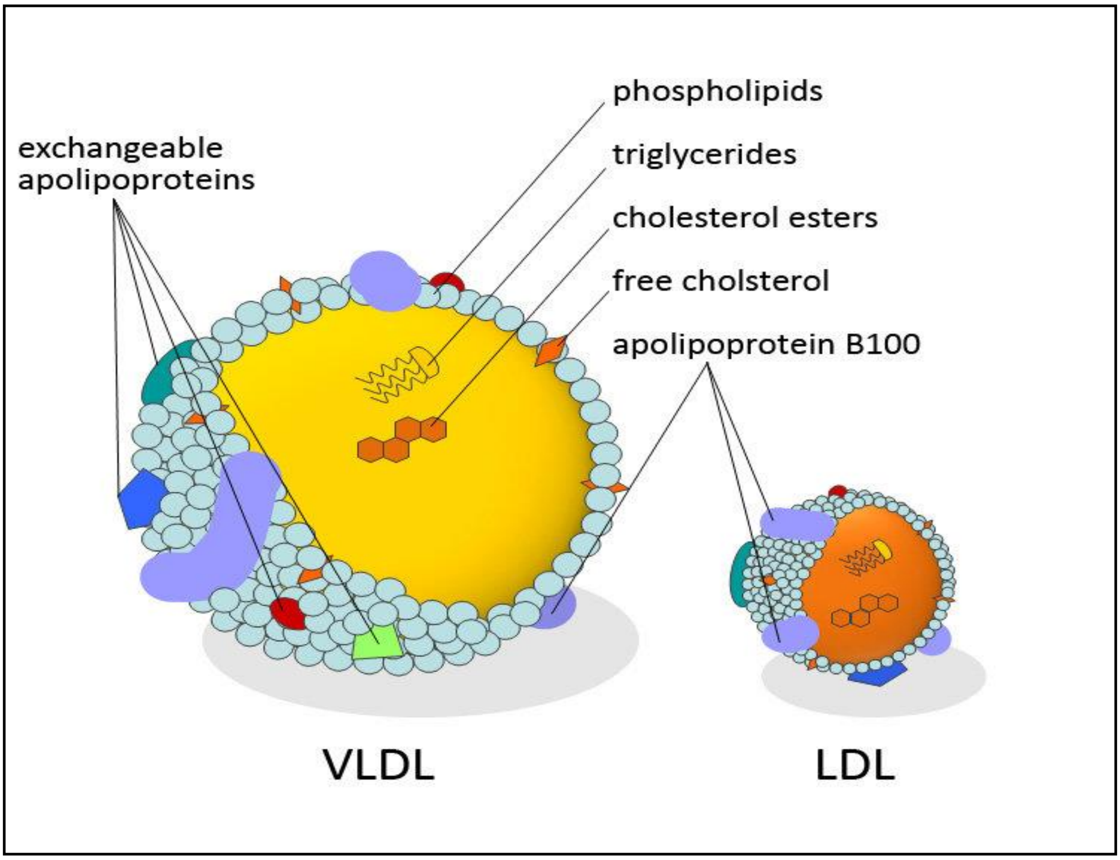


Figure (1-5): Structure of Lipoproteins [52].

Plasma lipoproteins are divided into seven categories based on their size, lipid composition, and Apo-lipoproteins (HDL, LDL, IDL, VLDL, Lp (a), chylomicrons, and chylomicron remnants), as shown in (Fig 1-6) [53]. Chylomicron remnants, IDL, LDL, and Lp (a) are all pro-atherogenic, whereas HDL is anti-atherogenic [52], as shown in Fig (1-6).

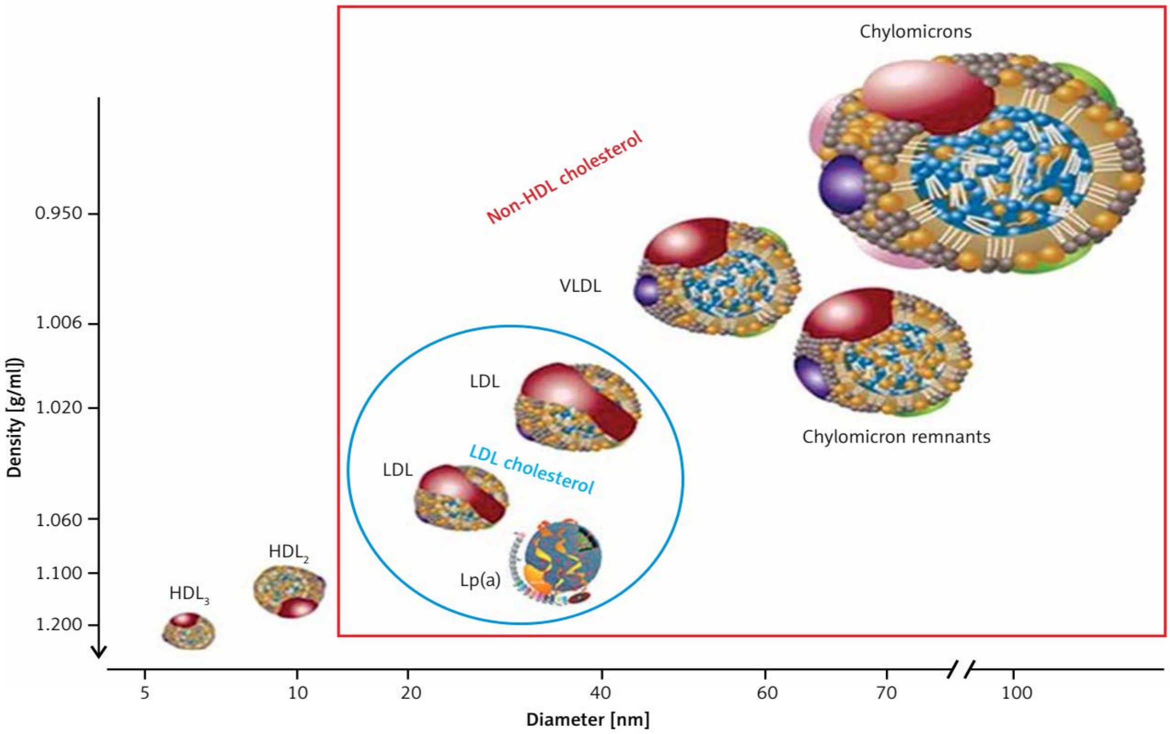


Figure (1-6): Lipoprotein Classes [53].

1.4.1 HDL characteristics and PON1 activity – a bridge to HDL functionality

Because the association between PON1 and High -density lipoproteins (HDL) is more complex than a simple ligand-carrier interaction, it warrants special consideration. Changes in HDL structure, particularly PON1 activity, have been shown in numerous studies to alter HDL functionality. PON1's affinity for various HDL particles is a fascinating subject. Gugliucci recently proposed that PON1 was found in all HDL subclasses, but had the strongest affinity for small HDL 3 particles [54]. Small HDL 3 subtypes are now known to play a dual role in atherogenesis. A growing body of research suggests that functioning tiny HDL particles are more atheroprotective than their bigger counterparts. However, HDL is particularly vulnerable to structural changes under a variety of circumstances. Atherogenic dyslipidemia, oxidative stress, and inflammation modify HDL composition, which can significantly alter or reduce their antiatherogenic capability, resulting in the formation of dysfunctional HDL particles [55]. Small HDL particles, in particular, have been identified as important indicators of Cardiovascular Disease (CVD) morbidity. s well as mortality. These findings show that reduced PON1 activity is at least partly responsible for the decreased antiatherogenic potential of tiny HDL particles. Oxidative changes are especially dangerous to small HDL particles. Specific amino acids in Apo AI can be modified by pro-oxidant enzymes such myeloperoxidase. Furthermore, oxidative stress might cause a decrease in lecithin: cholesterol acyltransferase (LCAT) activity in vitro. As a result, randomized controlled trial (RCT) capacity may be reduced. The potential of oxidized HDL to promote PON1 activity is significantly reduced [56]. Figure (1-7) shows the pathological processes that linked to HDL dysfunctionality

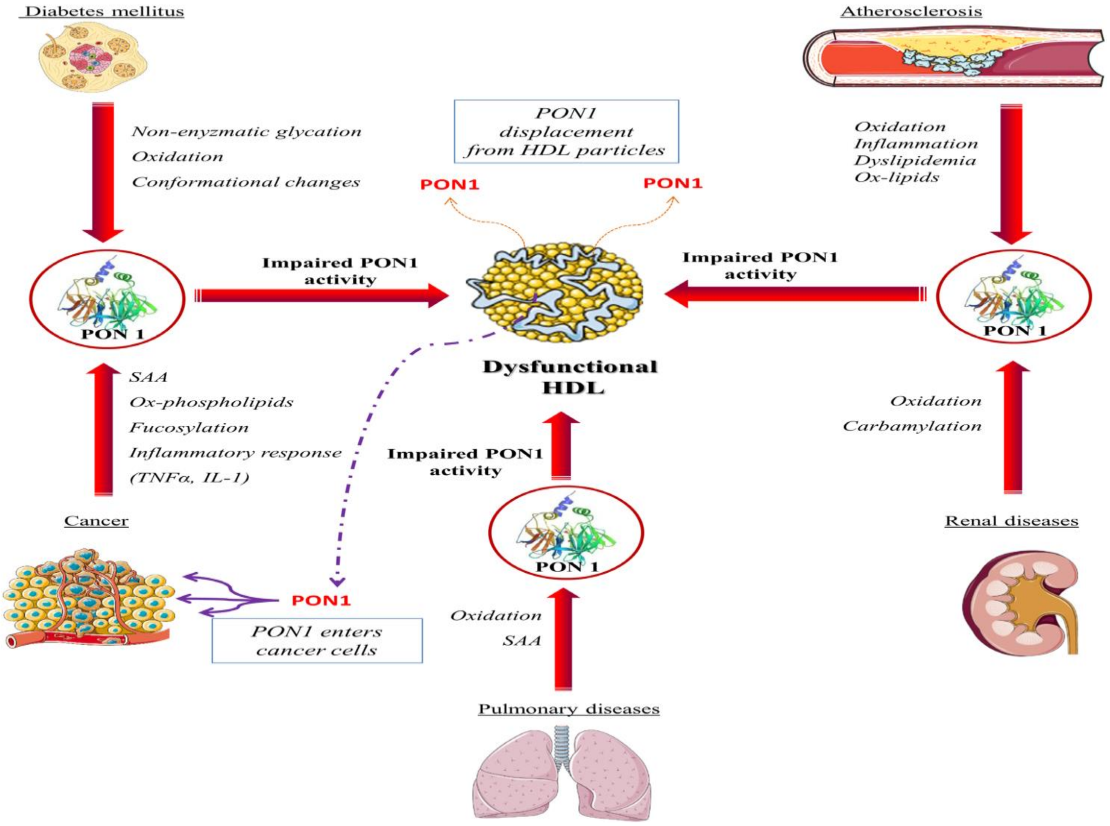


Figure (1-7) Different pathological processes, such as HDL dysfunctionality (possible methods by which PON1 activity/concentration is reduced as a result of various diseases), have an impact on PON1 deterioration [57].

The favorable relationships between increasing proportions of small HDL particles and oxidative stress and inflammatory indicators was documented [58]. Several study groups have verified the relationships between HDL-cholesterol level and size heterogeneity, PON1 activity, and/or markers of inflammation seen in our studies [59]. Despite the fact that PON1 was more numerous on HDL 3 subtypes in Chronic renal disease (CRD) and end-stage renal disease (ESRD) patients. This finding backs up the theory that HDL3 particles have a strong ant oxidative capacity in healthy people, but not in people who are at high risk for CVD. All of the foregoing findings suggest the intriguing idea that differences in PON1 distribution among HDL subclasses could signify changes in HDL functionality. However, more research is needed to confirm such assumptions and to investigate the possible contribution of PON1 activity determination in different HDL subtypes to total CVD risk assessment [60]. Exogenous lipoproteins are formed in the colon by combining dietary lipids into chylomicrons. The triglycerides carried in chylomicrons are digested by lipoprotein lipase in adipose tissue and muscle in the circulation, generating free fatty acids. Following that, adipose tissue and muscle metabolize free fatty acids, and chylomicron remnants are generated. Treatment of chylomicron remains is one of the liver's duties. VLDL is formed via the endogenous lipoprotein route, which begins in the liver [61]. IDL is converted to LDL, which is then taken up by the LDL receptor in the liver and other tissues, which is the primary site of uptake. The production of nascent HDL by the liver and gut is the first step in reverse cholesterol transport. The ATP-binding cassette transporter A1 (ABCA1) then facilitates the acquisition of phospholipids and cholesterol that are refluxed from cells, leading in the creation of mature HDL. The cholesterol is subsequently transported to the liver by HDL, either directly by interacting with hepatic scavenger receptor class B type 1(SR-B1) or indirectly by transferring the cholesterol to VLDL or LDL, a process aided by cholesteryl ester transfer protein (CETP)." "Cholesterol efflux from macrophages to HDL plays a critical role in the prevention of atherosclerosis" [62]. Low density lipoproteins (LDL) are particles that are formed from very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL) and are abundant in cholesterol. LDL is responsible for transporting the majority of cholesterol through the circulatory system. Because small compact LDL particles have a lower affinity for the LDL receptor, they spend more time in the circulation. They also enter the artery wall more easily and bind to intra-arterial proteoglycans more enthusiastically, trapping them in the arterial wall. Finally, small dense LDL particles are more prone to oxidation, which could lead to increased macrophage absorption." [63]. The crucial involvement of HDL particles in reverse cholesterol transportation from peripheral tissues to the liver elucidated the potential mechanism of HDL's role and its connection with anti-atherogenic agents. HDL particles also exhibit anti-oxidant, anti-thrombotic, anti-inflammatory, and anti-apoptotic capabilities, all of which may contribute to their ability to prevent atherosclerosis [64]. HDL particles are highly varied in nature and can be classified based on density, Apo-lipoprotein composition, size, or charge (Table 1-1) [65].

Table 1-1. Classifications of HDL

Method of classifications	**Types of HDL**
Density gradient ultracentrifugation	HDL_2 , HDL_3, Very high density HDL
Nuclear magnetic resonance	Large , medium, and small
Gradient gel electrophoresis	HDL 2a, 2b,3a,3b,3c
2-dimensional gel electrophoresis	Pre-beta 1 and 2, alpha 1 , 2 , 3, 4
Apolipoprotein composition	A-Iparticles , A-I:A-II particles I:E particles

1.5 Paraoxonase PON

Paraoxonase are three enzymes that have been identified: paraoxonase-1 (PON1, EC 3.1.1.2, 3.1.1.81, 3.1.8.1), paraoxonase-2 (EC 3.1.1.2, 3.1.1.81), and paraoxonase-3 (EC 3.1.1.2, 3.1.1.81). (PON3, EC 3.1.1.2, 3.1.1.81, 3.1.8.1). PON1 is the one that has been examined the most. However, it is not always seen as the most significant. PON2 looks to be the earliest component, from which PON3 and eventually PON1 emerged. PONs have different activities and are found in different places, despite their identical amino acid sequences. PON1 and PON3 are located predominantly in high-density lipoproteins (HDLs) in the circulatory system, hydrolyzing organophosphates and bulky statin lactones, respectively, but PON2 is found in numerous organs and primarily works as an intracellular oxidative stress defender. PON1 isoforms are involved in drug metabolism as well as the prevention of cardiovascular and neurological disorders. In a single active site, the three PONs show a wide spectrum of enzymatic activity toward diverse substrates. Lactonase and arylesterase activities are both low in PON2. PON3 has a strong lactonase activity, a poor arylesterase activity, and little paraoxonase activity. PON1 demonstrates all three functions [66].

1.5.1 Paraoxonase 1: An Evolutionary Highlight with Many Enzymatic Activities

PON1 is a 355-amino-acid-residue,a calcium-dependent glycoprotein. PON1 is generated in the liver and released into the bloodstream, where it is present primarily in HDLs and to a lesser level in VLDLs and chylomicrons [66,67]. PON1 is transported from the liver to a variety of organs, where it attaches to cell membranes and shields lipids from oxidation [68]. PON1 also inhibits the oxidation of low-density lipoprotein (LDL) and the inflammatory response. PON1's second critical function is to defend against particular organophosphate (OP) exposure. PON1 is a key defender against exposure to the OPs chlorpyrifos oxon and diazoxon, but not paraoxon, [67]. Homocysteine thiolactone (HCTL) is a toxic metabolite that binds with lysines in proteins, causing them to become inactive and dysfunctional. HCTL levels in the blood have been linked to an increased risk of cardiovascular, neurological, and immunological illnesses, as well as cancer. However, given to its low specific enzyme activity, the physiological importance of PON1 for HCTL is debatable. At amino acid residues 55 and 192, PON1 possesses two significant polymorphism sites. The substitution of methionine for leucine at position 55 (L55M) boosts paraoxonase activity and resulting in isoform-specific enzyme concentrations. Furthermore, mutations at locations 55 and 192 affect PON1's thiolactonase activity [69,70].

1.5.2 Structural Insight into the Active Site of PON1

Calcium-binding enzymes are known as PONs. The two calcium ions serve different purposes; the structural calcium ion (Ca_2) is the more closely bound ion, and its removal causes irreversible structural disturbance. The other ion is the so-called catalytic calcium ion (Ca1), whose reversible removal results in a loss of catalytic activity that is restored when calcium ions are added [71]. Ca1 operates as an oxyanion that stabilizes the substrate and is required for optimal hydrolytic activity by interacting with the side-chain oxygens of N224, N270, N168, D269, and E53 [66]. PON1 is likewise heavily glycosylated, making recombinant production difficult [72]. Figure (8.1A) shows a six-bladed propeller with a distinctive active site lid, which has been shown to be involved in the enzyme's HDL binding [73]. Because there were no analogous proteins to utilize as a model for molecular replacement, the structure was solved using Se-Met protein and isomorphous replacement. The structure lacks the N-terminal portion, i.e. the signal peptide, despite the fact that the signal peptide is not cleaved. In addition, one surface loop is absent. More than four-bladed beta-blade propellers are uncommon, although they are found across the PON family, and each blade has four strands. The disulfide bridge connecting the protein's N- and C-terminals (C42 and C353) completes the fold's distinctive closure, and replacing these two cysteine’s causes PON1 inactivation. PON1 possesses an extra free cysteine residue (C284), which when replaced with alanine reduces paraoxonase and arylesterase activity slightly. C284 is also required for PON1's function in protecting LDLs from oxidation [66,74]. Both calcium ions are stacked on top of each other in the propeller's primary tunnel. [66,75]. PON1's arylesterase and paraoxonase activities are mediated by histidine’s (H115, H134, H155, H243, and H285), tryptophan (W281), and aspartic acid/glutamic acid residues (D54, D169, D183, D269, D279, E53, and E195) [66]. Both lactonase and arylesterase activities are mediated by the H115/H134 dyad. Both actions are reduced or totally eliminated when one or both histidine’s are mutated. Surprisingly, replacing H115 with tryptophan increases paraoxonase activity in the presence of paraoxon and the nerve toxin VX [66,75]. Ca1 appears to interact with N224, N270, N168, N269, and E53, all of which are within a 2.5-mile radius of each other [66]. Ca1 has a higher solvent accessibility than Ca2, indicating that Ca2 is the higher-affinity calcium ion (Figure 8.1B). This is consistent with the finding that eliminating this ion causes irreversible protein structural destruction [76]. Figure (1-8) clarifies the structure of paraoxonase enzyme.

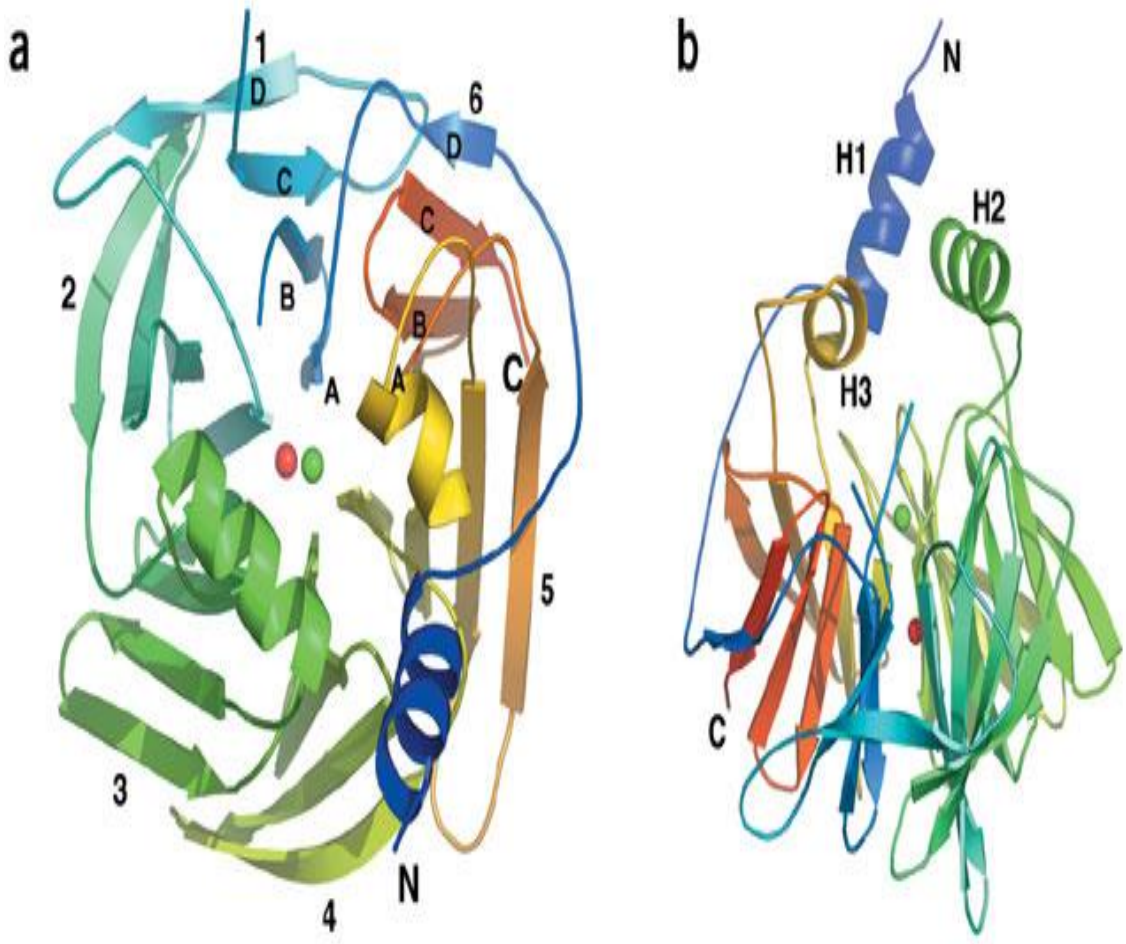


Figure (1-8): Structure of paraoxonase 1 and insight into its active location (AB) Wild-type protein (PDB id. 1V04 [77] in the protein database). (C) A wild-type protein with 2HQ attached to it (PDB id. 3SRG [78]). The backbone of the active site residues is shown, along with the kind and sequence number. The inhibitor is depicted in magenta.

Other HDL-bound proteins, such as apoA-I and cholesteryl ester transfer protein, have shown the similar behavior. PON1 oligomerization is most likely the result of its anchoring to detergent micelles in a similar manner to how it anchors to HDLs. PON1's N-terminal region, which resembles a transmembrane helix, is important for HDL binding. The residue 192, which is linked to the two most prevalent R192Q isoforms, is found in the active site, which explains its considerable influence on protein activity and function. Furthermore, the mutation L55M may affect protein stability and, as a result, protein function [66,79]. Most proteins with a _propeller structure have only the loops connecting the _strands that dictate the active site architecture and anchoring to HDLs. Several PON1 structures have been discovered so far. One of them is linked to 2HQ, a competitive inhibitor Figure (1-9.C). 2HQ lactam is a nonhydrolyzable lactone analogue with NH instead of steric oxygen. PON1 usually hydrolyzes the homologous lactone DHC, which is employed as a substrate to assess protein activity [80]. 2HQ interacts with the side chains of H115, D269, E53, and N168 in addition to Ca1 in the active site Figure (1-9.C) [66]. Based on the position of the loop region, crystal structures indicated that PON1 has at least three different active site conformations: I closed conformation with bound inhibitor Figure (1-9.C), in which the active site loop is structured and anchored to the enzyme's surface, and Y71 points into the active site; (ii) opened—unbound conformation (structure determined at pH 4.5, Figure (1-9.A,B), in which the active site loop is flexible (and thus not visible in the crystal structure), but Y71 is fixed and points outside the active site. All of the identified conformations have different interaction potentials and could be used to mediate different catalytic activity [81]. Ca1 has some flexibility and can reorganize itself within the active site. The various modes of Ca1 have been linked to lipo-lactonase catalytic promiscuity and PON divergence [66]. The substitution of tryptophan for H115 (an active site residue) Figure (1-9. A) reduces lactonase activity while increasing paraoxonase activity [82]. The mutation of H115 into tryptophan causes Ca1 to be displaced 1.8 times closer to the enzyme surface, according to the crystal structure. When both H115 and H134 Figure (1-9. B) are changed to glutamine, the identical Ca1 translocation occurs [66]. These mutations prevent 2HQ from binding to the enzyme, resulting in a reduction of lactonase function. When PON1 connects with HDLs, its stability and catalytic activity are greatly enhanced. Even though there are >15 hydrogen bonds between the binding site (which binds to HDLs) and the active site, this is likely due to the network of hydrogen bonds between them (Figure 1-9.C). The lactone substrate is aligned with Ca1 by this network of hydrogen bonds [66,83]. Three residues have been identified as allowing HDLs and PON1 L9, Y185, and Y293 to form complexes [66]. PON1 was utilized to investigate the evolution of enzyme functions. Ancient functions are frequently lost as a result of evolutionary changes. The histones of lactonase and paraoxonase activity were investigated using active site mutations; both showed tendencies toward die rent substrates and reactions but started with the identical residue, H115, in the active site. Lactonase activity was lost as a result of residue loss, whereas paraoxonase activity was elevated [84]. The paraoxonase activity is amplified by the neofunctionalization pathway, whereas the lactonase activity is restored and amplified by the re-functionalization pathway. The latter produces lactonases that are devoid of H115 active site residues. L69S, H115W, and F222S mutations increase paraoxonase activity (lactonase activity is reduced by >5000-fold) Figure (9.2D). The PON variation with five mutations in the active region (L69S, H115W, H134R, F222S, and T332S) is the most active Figure (1-9. F). Reverting W115 back to the original histidine and using the enzyme with five mutations (the most active enzyme) resulted in the loss of both lactonase and paraoxonase activity Figure (1-9. E) [82].

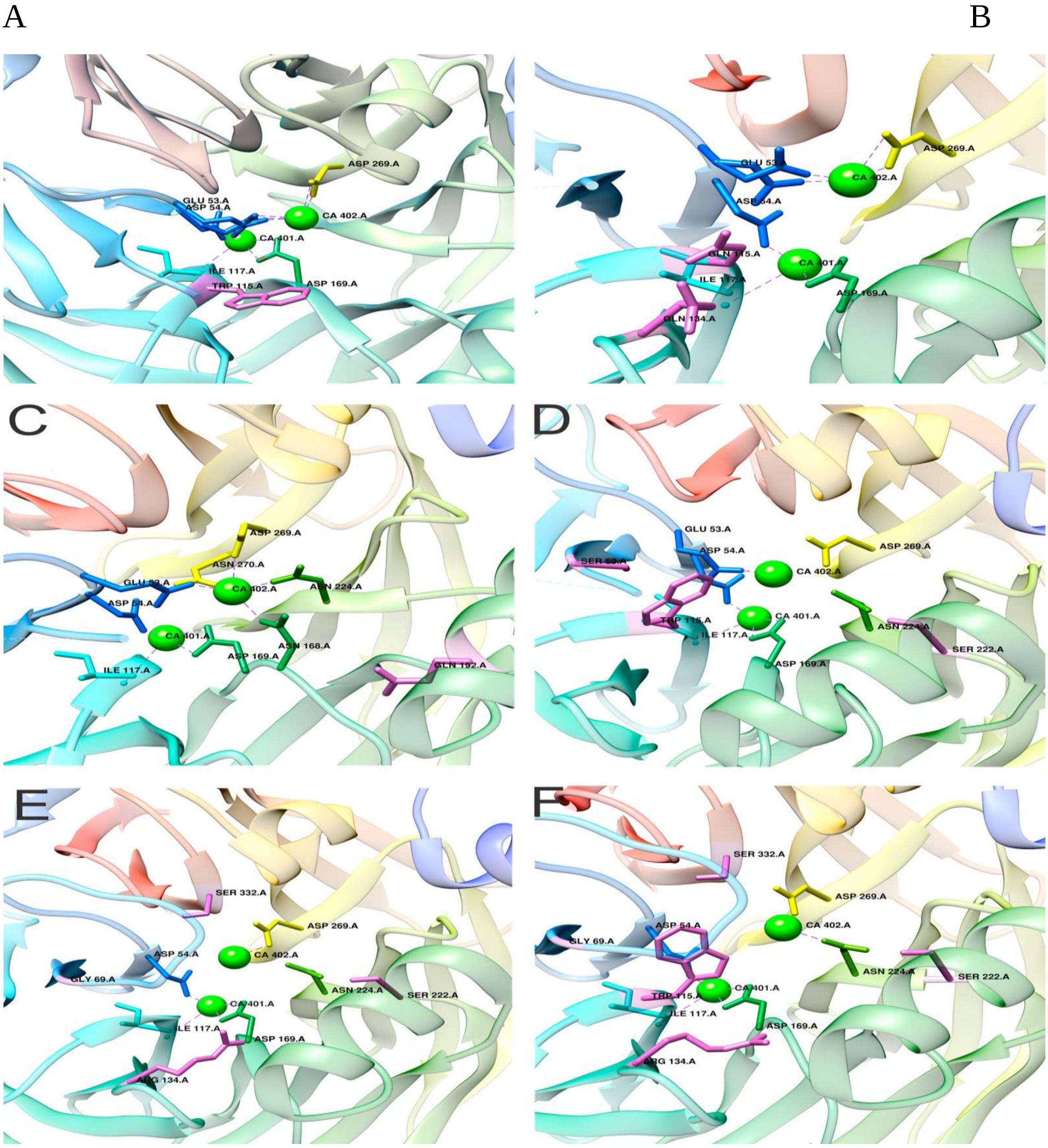


Figure (1-9). The structure of paraoxonase 1 provides insight into the active site of altered proteins. Protein specificity, activity, and evolution are all affected by introduced mutations. Protein mutations that affect enzymatic activity (A–C). (A) A mutant that exclusively has OP activity (H115W; PDB id. 4HHO [82].). (B) A mutant with OP and lactonase activity convergence (H115Q and H134Q; PDB id. 4HHQ, (C) A mutation that affects HDL binding as well as active site shape (K192Q; PDB id. 4Q1U [66].). (D–F) Mutations connected to evolutionary paths finding. L69S, H115W, and F222S (PDB id. 6G82); L69S, H134R, F222S, and T332S (PDB id. 6GMU [66]); L69S, H115W, H134R, F22S, and T332S (PDB id. 6GMU [66]); L69S, H115W, H134R, F22S, and T332S (PDB (PDB id. 6H0A [66].

1.5.3. PON1 and Diseases Connected with Atherosclerosis Development

1.5.3.1 PON1 and vascular diseases

Researchers have spent the previous two decades studying the interplay between dyslipidemia, inflammation, and oxidative stress in vascular disease pathologies. The above-mentioned PON1 polymorphism's relationship with vascular disease was concealed by inconsistencies in research findings. Different biolactones were proved to be endogenous substrates for PON enzymes by Draganov and colleagues [85,86]. The lactone substrate is being researched by several research organizations, including Teiber's. The natural PON1 substrate could be a variety of endogenous biomolecules having a lactone structure of polyunsaturated fatty acids. This is, of course, more evidence that the enzyme is involved in lipid metabolism [87].

1.5.3.2 PON 1 and diabetes mellitus

Diabetes mellitus (DM) is a multifactorial disease characterized by Hyperglycemia, a changed lipid profile, oxidative stress, and an increased risk of CVD development are all linked to hyperglycemia. Type 2 diabetes mellitus (T2DM) patients manifest with insulin resistance and β-cell insufficiency with reduced insulin production, accounting for up to 95 percent of all diagnosed cases of diabetes in the adult population. Given that cardiovascular disease is the leading cause of death in diabetes patients, it's no surprise that PON1 activity and polymorphism have been explored in diabetic pathophysiology over the last two decades. Reduced PON1 activity was linked to DM and the development of chronic DM complications in a recent meta-analysis [88,89].

1.5.3.3 PON1 and renal disease

Previous research has shown that uremic toxins, in combination with increased oxidative stress and inflammation, can alter HDL structure and, as a result, decrease PON1 activity in renal patients [90]. Because the kidneys are involved in the metabolism and excretion of uremic toxins, poor renal function and buildup of these compounds could have a considerable impact on PON1 activity [91]. Low albumin concentration was linked to cardiovascular and overall mortality in hemodialysis patients, according to Chen and colleagues [92,93]. Because atherosclerosis develops early in children with ESRD, prior studies of PON1 status in these patients revealed dramatically lower PON1 activity, particularly in children on hemodialysis [87,94].

1.6 Homocysteine thiolactone

Methionyl-tRNA syntheses produces homocysteine thiolactone, an intramolecular thioester of homocysteine, in an error-editing mechanism that prevents homocysteine from being incorporated into proteins during translation. Thiolactone is synthesized in all of the human cell types studied. In human cells, an increase in homocysteine causes an increase in thiolactone levels. Homocysteine thiolactone reacts with proteins in cultured human cells and human serum by homocysteinylation of protein lysine residues. Protein damage is caused by homocysteinylation. By detoxifying thiolactone, a calcium-dependent homocysteine thiolactonase closely linked with HDL in human serum may prevent protein degradation [95]. Hcy is a sulfur-containing amino acid generated primarily from methionine metabolism. Plasma Hcy levels higher than normal conditions are caused by genetic mutations of enzymes and/or dietary inadequacy in vitamin B necessary for Hcy metabolism, resulting in Hyperhomocysteinemia (HHcy). Hyperlipidemia is linked to a higher risk of cardiovascular and cerebrovascular illness [96,97]. The major enzymes involved in "1-carbon metabolism" may disrupt the metabolic cycle, Increased homocysteine levels in biological fluids have been linked to a variety of illnesses, including neuropsychiatric disorders and autism. The enzymes involved in homocysteine metabolism require various B vitamins as co-factors, including B6 (pyridoxine), B12 (cobalamin), and B9 (folic acid) [98]. Hyperhomocysteinemia, or high plasma total homocysteine (tHcy), has been shown in clinical research to be a risk factor for cardiovascular disease (CVD) and stroke [99]. The list of the 20 traditional protein genic amino acids includes two sulfur amino acids. Cysteine residues are involved in protein folding because of their capacity to generate disulfide bridges. They're also crucial in many catalytic sites [99], particularly catalytic triads, and they're frequently linked to metal cations, as in zinc fingers and iron-sulfur clusters. Even if the initiation codon should code for another residue, a methionine residue is added at the start of protein synthesis [100]. Increased physiological concentrations of Hcy and Homocysteine thiolactone (HCTL) can be caused by deficiencies in the enzymes that metabolize these metabolites, which might have negative consequences. Hcy is recycled back to amino acids through Methylenetetrahydrofolate reductase (MTHFR), methionine synthase, and cystathionine beta-synthase (CBS). MTHFR is essential for the formation of 5-methyl tetrahydrofolate, a metabolite required for Hcy to methionine conversion. To convert Hcy to cysteine, CBS is necessary. Increased levels of Hcy and HCTL can be caused by deficiencies in either of these enzymes [101]. Hcy thiolactone is hydrolyzed to Hcy by human serum Hcy thiolactonase (HTase), a component of HDL. The organophosphate paraoxon is hydrolyzed by HTase, which is identical to paraoxonase, a product of the PON1 gene

[102]. Paraoxonase is also vulnerable to oxidized phospholipids in low-density lipoproteins. In human PON1, there are several genetic polymorphisms, two of which cause amino acid alterations in PON1 proteins at positions 55 and 192 [103]. Homocysteine is a higher homolog of cysteine than cysteine because it has an extra methylene (–CH2–) group in its side chain. Hcy is a lesser homolog of methionine because it lacks a methyl (CH3–) group compared to methionine. Methionine and cysteine are two conventional coded amino acids that are integrated into polypeptide chains of protein by the ribosome biosynthetic apparatus at sites designated by the AUG and UGU/UGC codons, respectively [95]. In reality, the active site of methionyl-tRNA syntheses (MetRS) activates Hcy and generates homocysteinyl adenylate in addition to activating the corresponding substrate methionine and creating Met-AMP (Hcy- AMP). Met-AMP then forms Met-tRNA, which then supplies Met to the ribosomal protein biosynthetic apparatus's developing peptide chains. The Hcy-AMP intermediate, on the other hand, is transformed to Hcy-thiolacton by a MetRS editing process, which inhibits Hcy from attaching to tRNAMet and thus preventing Hcy from entering the genetic code. MetRS has a universal editing mechanism that is involved in the production of the thioester Hcy-thiolacton in all species, from bacteria to human [104]. The only source of Hcy in the human body is methionine, one of the eight necessary amino acids that must be given in the form of dietary protein (Fig. 1.10) [104].

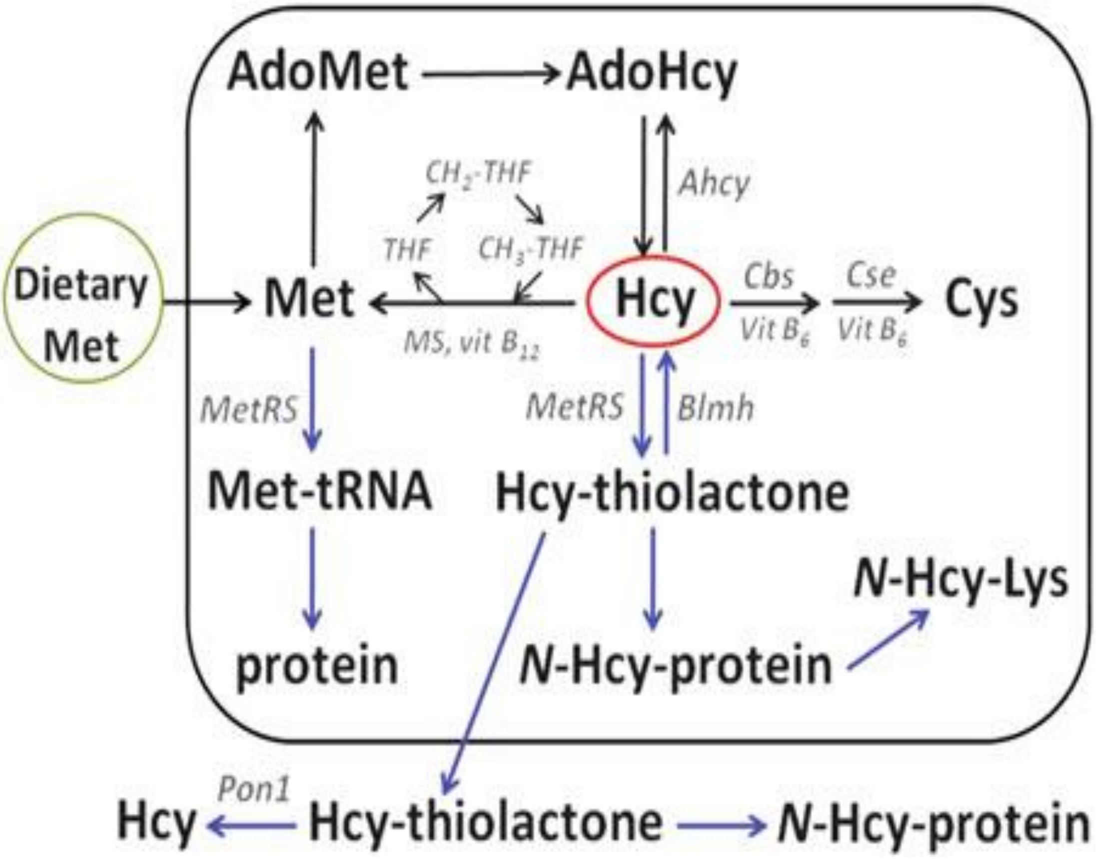


Fig. (1-10) The metabolism of homocysteine in humans is depicted in this

However, when certain genetic or nutritional inadequacies prevent Hcy from being metabolized via the traditional demethylation and transculturation processes, Hcy accumulates, which is linked to a variety of human clinical diseases. The goal to comprehend Hcy's role in cardiovascular and neurological illnesses in humans has fuelled exceptional growth in Hcy research during the last two decades [105]. Hcy is one of the most reactive amino acids in biological systems, taking part in at least seven processes [106]. Hcy is biologically transformed to additional, potentially hazardous metabolites (Fig.1.10), such as Hcy-thiolactone, N-Hcy-protein, and N-Hcy-Lys [107], in addition to its removal via demethylation to methionine, transculturation to cysteine (through cystathionine), and oxidation to disulfides. In genetic or nutritional deficits in Hcy metabolism, these conversions can be substantially accelerated to AdoHcy, homocysteic acid, or S-nitroso-Hcy. Two of these metabolites, Hcy-thiolactone and S-nitroso-Hcy, mediate Hcy incorporation into protein, as identified at the end of the 1990s [107].

1.7 Genotyping of PON

Many investigations have found that the presence of a paraoxonase genetic variation is a factor of its activity. This polymorphism occurs as a result of an amino acid substitution in the enzyme's active site, resulting in low- and high-activity isoenzymes [108]. Player et al. were the first to investigate the genetic basis of the paraoxonase polymorphism (1976). They came to the conclusion that two alleles at a single autosomal locus governed high and low serum paraoxonase activity [109]. The two enzymes are thought to be the result of a gene on chromosome 7's long arm [110]. The DNA basis for the paraoxonase polymorphism was determined through molecular investigations on human paraoxonase genes. In the mature enzyme protein, protein sequencing revealed the presence of an intramolecular disulfide bond, the position of one asparagine-linked carbohydrate, and the first amino acids [111]. Two frequent polymorphism sites have been discovered at amino acids "55" and "192 [112]." The first polymorphism affects amino acid 192 and includes the substitution of glutamine (Q allele) for arginine (R allele), whereas the second polymorphism affects amino acid 55 and involves the substitution of leucine (L allele) for methionine (M-allele) [113]. The PON1 genetic polymorphisms have been found to be an independent risk factor for coronary artery disease (CAD); both the 55L and 192Q genotypes have been linked to increased CAD susceptibility [114]. Between the two polymorphisms, the PON1 192 polymorphism is the most prevalent and has a significant impact on PON1 activity. The level of the enzyme in blood and tissues, as well as the specific isozymes present, will determine protection against organophosphates. Although the B-type, now known as the R-isozyme, is several times more efficient than the A-type (Q-isozyme) in hydrolyzing paraoxon, the Q-isozyme hydrolyzes most organophosphates significantly better than the R-isozyme. As a result, both the quantity and type of PON1 must be considered when assessing PON1's protective role against substances that may be catalytically inactivated by the enzyme [115]. The two polymorphic sites at positions 55 and 192, the internal disulfide bond between cysteine residues 42 and 353, the free cysteine at position 284, the location of potential sugar chains, and the hydrophobic retained leader sequence at the amino terminal end are all shown in the human PON1 structure. The salting-out approach was used to extract genomic DNA from venous blood. The procedure published earlier by Motti et al. with some modifications was used to study the Q192R and L55M polymorphisms of the PON1 gene at the same time. In a nutshell, a multiplex polymerase chain reaction (PCR) approach was used with specific primers corresponding to the PON1 nucleotide sequence, and the PCR products were digested with HinfI and electrophoresed on 4% agarose gels stained with ethidium bromide [116].

1.8 Phenotypes of PON

Unlike serum cholinesterase’s, PON is an aromatic esterase that requires calcium for activity and is not irreversibly blocked by organophosphates. Serum paraoxonase activity is bimodality distributed in Caucasian populations, with the degree of activity inherited as a straightforward dominant Mendelian characteristic dictated by two alleles at one autosomal locus [117]. The "high" and "low" allel are referred to as esterase B and esterase A, respectively, to underline the qualitative differences between the two isozymes. NaCl stimulates the B isozyme more strongly, and it usually has more activity than the A isozyme. The apparent Km values, calcium needs, and pH optima of the two isozymes are likewise varied [118]. Aromatic esterase is another name for AREase, which is found in human serum. The most common substrate used to evaluate AREase activity is phenyl acetate. Aromatic substrates containing an acetate moiety have the highest AREase activity. AREase, like PON, is activated by calcium and is unaffected by cholinesterase inhibitors like serine and organophosphates [110]. Despite these similarities, there has been some debate about whether the activities of human serum PON and AREase are features of the same enzyme or two separate enzymes. In a Caucasian population, aAREase activity has a single mode, whereas PON activity is bimodal [110]. Based on the ratio of paraoxonase activity in the presence of 1 M sodium chloride (NaCl) to arylesterase activity, the phenotypes for the PON1 192 polymorphism can be classified as A, AB, or B [119]. Phenotype A has low paraoxon activity, phenotype B has strong paraoxon activity, and phenotype AB has an intermediate level of activity [120]. Based on the ratio of PON activity in the presence of 1 M NaCl to AREase activity, the phenotypes for the PON1 192 polymorphism are A, AB, and B [121]. PON-BB homozygotes' HDL is less effective at protecting LDL from oxidation than PON-AA homozygotes' HDL, implying that the B all enzyme of PON1 is less active in metabolizing lipid peroxides than the A all enzyme [110,122].

1.9. A quantitative method for assessment of homocysteine thiolactonase enzyme activity

Homocysteine thiolacton is hydrolyzed to homocysteine by human serum homocysteine thiolactonase (HTase) [123]. Homocysteine thiolacton is the natural substrate for HTase/paraoxonase [124]. HTase/paraoxonase, a component of HDL, is a multifunctional enzymatic antioxidant that detoxifies the homocysteine metabolite, homocysteine thiolacton, and destroys Ox-LDL. Consequently, HTase lowers the risk of atherosclerosis that arise from the protein damage by homocysteinylation of the lysine residues [125]. Human blood contains homocysteine thiolacton (HTL) and N-homocysteinylated forms of proteins such as albumin, LDL, fibrinogen, HDL, and hemoglobin [126]. HTL has been recognized by Jakubowski et al.as an active metabolite, which mediates the damaging and toxic effects of homocysteine [127]. HTLase is one product that arise from the polymorphic paraoxonase-1 gene. The protection of protein from homocysteinylation associated with high HTLase activity [128]. Based on what was showed above of the importance of homocysteine thiolactonase enzyme in clinical chemistry; three types of analytical methods have been developed to measure the homocysteine thiolactonase enzyme activity. The first type is radio-enzymatic assay, which monitor the hydrolysis rates of [^{35}S] Hcy thiolacton [124]. The 1U of HTase refers to the quantity of enzyme capable of efficiently converting 100 nmol [35S] Hcy thiolacton to [35S] Hcy in 30 minutes at 37°C. This type of methods has high specificity and high sensitivity, but the disadvantages include presence radiation hazards that necessitate using labs with special license to handle radioactive material. Also, the method requires analysts with high skills. The second method depends on measuring the amount of hydrogen ions (H^+)

generated during thiolacton hydrolysis to homocysteine as a function to the homocysteine thiolactonase activity. The assay solution is contained L-Hcy-thiolacton, BSA, calcium chloride, and phenol red [129]. The HTase hydrolyzes homocysteine thiolacton (HTL) back to homocysteine that reduce phenol red. Hcy-thiolactonase activity was expressed in µmol H^+ liberated/L serum per min and calculated from standard HCl titration curve. The third method was measured HTase activity spectrophotometrically [130]. Thiolacton hydrolysis was measured at 37℃ using Elman's reagent to monitor the accumulation of free sulfhydryl groups reacting with DTNB at 412 nm. Enzymatic activity was calculated from the molar extinction coefficient of DTNB (C-412 =13600 M^{-1} cm^{-1}) and corrected for non-enzymatic hydrolysis. One unit of HTase activity is defined as 1µmol of substrate hydrolyzed per min under the defined assay conditions. In the present method, the activity of HTLase enzyme was assessed by incubating enzyme containing samples in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, which contains adequate amounts of substrate γ-thiobutyrolactone. CUPRAC reagent ($Cu(Nc)_2^{2+}$) was added at the end of the enzymatic reaction. The reduction of Cu(II)-neocuproine complex ($Cu(Nc)_2^{2+}$) to highly colored Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$) by the produced 4-mercaptobutyric acid was determined at 450 nm (CUPRAC method). The HTLase activity being associated with the increment of absorbance of colored Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$). The current method is specific, efficient, and reliable. Amino acids, sugars and other types of biomolecules do not affect the results of the current protocol. Concentrated strong bases or acids not applied to stop the enzymatic reaction, and steps of the protocol can be completed within few minutes.

1.10. Aims of the study:

This study aims to investigate the effect of cigarette smoking on homocysteine thiolactonase (HTLase) according to PON1 Q192R gene polymorphisms and the correlation between this parameter and oxidant/ antioxidant status. The study was concerned with the following parameters:

1. Assay homocysteine thiolactonase (HTLase) and paraoxonase-1 (PON1) activity.
2. Assessment of total antioxidant status and total oxidant concentration.
3. Measurement of the lipid profile and lipid peroxidation.
4. Study of the homocysteine thiolactonase (HTLase) Gene Polymorphisms (PON1 Q192R gene polymorphisms).
5. Develop a novel precise method for assessment of homocysteine thiolactonase (HTLase) activity and compared it with ordinary methods.

CHAPTER
TWO
MATERIALS
AND
METHODS

2.1 Materials

2.1.1. Chemicals

All chemical and biochemical reagents were supplied from analytical grade and were purchased from standard chemical commercial providers, then consumed without any addition purification, as shown in Table (2.1):

Table (2.1) The Chemicals Used in this Study.

Chemicals	Purity %	Supplied company
Paraoxon	90	Sigma-Aldrich
CaCl2	99.0	BDH
Sodium chloride (NaCl)	99.0	BDH
Hydrochloric acid (HCl)	99.0	BDH
Tris buffer		
HEPES	99.0	BDH
Sodium hydroxide (NaOH)	99.0	BDH
Sodium azide(NaN3)	99.0	BDH
Bovine serum albumin (BSA)	--	Sigma-Aldrich
CaCl2	99.0	BDH
γ-thiobutyrolactone	99.0	BDH
Phenol red	99.0	BDH
Copper (II) chloride $CuCL_2.2H_2O$	99.0	Sigma-Aldrich
2,9-dimethyl-1,10-phenanthroline (Neocuproine) $C_{14}H_{12}N_2$	98	Sigma-Aldrich
Ammonium acetate (NH_4Ac).	99	BDH
Glutathione (GSH)	99.0	BDH
Ascorbic acid	99.0	BDH

α-Tocopherol	99.0	BDH
Dichloromethane(DCM)		
Trichloroacetatic acid. (TCA). $C_2HCl_3O_2$	99.0	BDH
1,1,3,3- Tetra methoxypropane $C_7H_{16}O_4$	99.0	BDH
Sodium dodecyl sulfate. (SDS). $NaC_{12}H_{25}SO_4$	99.0	BDH
Hydrochloric acid (HCl)	99.0	BDH
Thio barbituric acid. (TBA). $C_4H_4N_2O_2S$	99.0	Sigma-Aldrich
Ferrous ammonium sulfate $Fe(SO_4)(NH4)_2(SO_4)$	99.0	BDH
O-dianisidine dihydrochloride $C_{14}H_{16}N_2O_2.2HCl$	99.0	Sigma-Aldrich
Glycerol ($C_3H_8O_3$)	99.0	BDH
Hydrogen peroxide (H_2O_2)	---------	Fluka
Sulfuric acid (H_2SO_4)	98	BDH
Xylenol orange ($C_{31}H_{32}N_2O_{13}S$)	99.0	BDH
Sodium chloride (NaCl)	99.0	BDH
Sodium cyanide NaCN	99.0	BDH
Uric acid	99.0	BDH
Tris 90%	99.0	BDH
Methanol 10%	99.0	BDH
EDTA	99.0	BDH
D. W		

Sodium acetate		
Ethanol	99.98	Fluke
Agarose	-----	Conda (spain)
TBE buffer		
Tris base	————	Himedia(india)
Boric acid		Thomas baker(india)
EDTA	99.0	BDH
Ethidium bromide	-------	Promega, USA
Boric acid	99.0	BDH
Iso propanol	99.0	BDH
Mgcl2	99.0	BDH
PCR Master Mix Kit	-----	Promega, USA
Primer		
DNA Extraction kit	-----	Favorgen Biotech, Taiwan
100 bp DNA ladder	-----	Russian(cyntol)
Ethylene diamine tetra acetic di-hydrate (EDTA).2 H_2O	99.5	Thomas baker(india)
Loading dye (bromophenole blue)	99	Promega, USA
PCR Master Mix Kit	-----	Russian(cyntol)
Restriction enzyme Sau3AI	------	Biolabs, England

2.1.2. Instrument Analysis and Equipment:

Table (2-2): Instrument Used in this Study.

Instrument	Supplied company
Centrifuge	Heraeus (Germany)
Deep Freez	GFL / Germany
Horizontal Gel electrophoresis unit	Biorad(jappan)
Horizontal Gel electrophoresis unit	Cleaver scientific / UK
Magnetic stirrer	Gallin kamp (England)
Micro-centrifuge	Hettich / Germany
Microplate reader	BioTek (USA)
Nano drop	Analytik jena
Oven	Hearson (England)
PG T80+ Spectrophotometer	Shimadzu / (USA)
pH meter	Jenway (Germany)
Photo-documentation	Cleaver Scientific / UK
Sensitive balance	Stanton 461 AN (Germany)
Spectrofluorometer shimadzu RF-5301pc	Shimadzu / (USA)
Spectrophotometer	PG T80+ Spectrophotometer
Spectrophotometer UV -1601	Shimadzu / (USA)
Thrmocycler PCR(Biometra)	Germany
Vortex mixer	Karlkole (Germany)
Water bath	Karlkole (Germany)

2.2. Methods

This study was performed on the smoker and nonsmoker students in the University of Babylon, all the blood sample were collected between from November 2020 to January 2021

2.2.1. Collection of Blood and Sampling

The vein on the front of the elbow or forearm is almost employed. The arm must be temperate to develop the circulation and distend the vein. When five ml of blood has been drawn into the needle was inserted, and the needle is withdrawn. This pad is firmly pressed onto until the bleeding stops. The needle is removed from the syringe and the blood slowly transferred to a Gel tube without anticoagulant. The blood permitted to clot for 15 minutes; the clot shrinks, and serum can be taken by centrifuging for approximately 10 minutes at a relative centrifugal force (RCF) of 1500 revolutions per minute (rpm) to 2000 rpm.

$$\mathbf{g = (1.118 \times 10^{-5})\ R\ S^2}$$

Where g is the relative centrifugal force, R is the radius of the rotor in centimeters, and S is the speed of the centrifuge in revolutions per minute.

2.2.2. Subjects:

A clinical study was performed in University of Babylon between from November 2020 to January 2021. Blood samples were drawn from the students of the University of Babylon.

A total of 300 male subjects ,150 subjects with non-smoking, 150 subjects with cigarette smoking.

The participants classified in to:

- Group1: Nonsmokers.
- Group2: Smokers of cigarette.

2.2.3 Exclusion Criteria

Any chronic disease such as diabetes mellitus, thyroid diseases, malignancies, those who had been on an antioxidant therapy during the past 2 months, and those with renal and liver failure were not included.

2.2.4 Collection of Data

The survey that questioned the sociodemographic characteristics of the participants (age, marital status, another occupation, place of residence, and starting age of smoking) was filled through face to face interview.

2.3. Determination of Total Oxidant Status (TOS).

2.3.1. Principle

The Total Oxidant Status **(TOS**) of serum was measured using a method, developed by Erel. Oxidants existing in the serum oxidize the ferrous ion–o-dianisidine complex to ferric ion. The oxidation reaction is improved by glycerol molecules, which are abundantly found in the reaction medium. The ferric ion creates a colored complex with xylenol orange in an acidic medium. The color intensity, which can be determined spectrophotometrically, is associated with the total amount of oxidant molecules existing in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micro molar hydrogen peroxide equivalent per liter (µmol H_2O_2 Eq/L) [131].

2.3.2. Assay Reagents

Reagent 1: was prepared by dissolving 1.96 g of ferrous ammonium sulfate and 3.17 g of O-dianisidine dihydrochloridein in 1000 mL of H_2SO_4 solution, 25 mM. The final reagent was consisting of 5 mM ferrous ammonium sulfate and 10 mM O-dianisidine dihydrochloride.

Reagent 2: was prepared by dissolving 114 mg of xylenol orange and 8.18 g of NaCl in 900 mL of 25 mM H_2SO_4 solution. One hundred milliliters of glycerol were added to the solution. The final reagent was composed of 150 µM xylenol orange, 140 mM NaCl and 1.35 M glycerol. The pH value of the reagent was 1.7. This reagent is stable for at least 6 months at 4°C.

Hydrogen Peroxide (STD): (100 µmol/L) was freshly diluted and standardized daily using a molar extinction coefficient of 43.6 $M^{-}1$ $cm^{-}1$ at 240 nm.

2.3.3 Procedure: shown in Table (2-3). [131].

Table (2-3) Shows the Details of the Present Method.

	Blank	**Standard**	**Sample**
Distilled water	25µl	-----	------
Serum	------	-----	25µl
Hydrogen peroxide	------	25µl	------
R1	1ml	1ml	1ml
Test tubes was mixed by vortex, then added:			
R2	0.25ml	0.25ml	0.25ml
Gently mix the content of every tube after addition, let to stand at room temperature for 30 minute, read spectrophotometrically at 560 nm.			

2.3.4. Calculation

$$\text{Total oxidants status} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD$$

2.4. Total Antioxidants Capacity Assay: The CUPRAC Method.

2.4.1. Principle

Antioxidants + Cu^{+2} $\xrightarrow{\hspace{3cm}}$ Cu^{+}

Cu^+ + 2, 9-dimethyl-1, 10-phenanthroline $\longrightarrow$ complex (λ_{max} at 450 nm) [132,133].

2.4.2.Reagents

1. Copper(II) chloride solution at a concentration of 10^{-2}M was prepared from $CuCl_2 \cdot 2.\ H_2O$ weighing 0.4262 g, dissolving in H_2O and diluting to 250 ml with water.
2. Ammonium acetate (NH_4Ac) buffer pH = 7.0 was prepared by dissolving 19.27 g of NH_4Ac in water and completed the volume to 250 ml.
3. Neocuproine (Nc){2,9-dimethyl-1,10-phenanthroline} solution at a concentration of 7.5 * 10^{-3}M was prepared by dissolving 0.039 g Nc in 96% EtOH, the volume was completed to 25 ml with ethanol.
4. The standard solutions of sample antioxidants were prepared at 1.0 * 10^{-3}M concentration α-Tocopherol was dissolved in dichloromethane (DCM). Ascorbic acid and glutathione (GSH) solutions were prepared in distilled water.
5. Working standard solution: composed of three equal volumes of 1.0 * 10^{-3}M of α-Tocopherol (0.0402 gm/100ml dichloromethane), ascorbic acid (0.1761gm/100ml D.W) and glutathione (GSH) (0.0307gm/100ml D.W).

2.4.3.Procedure: shown in Table (2-4) [132,133].

Table (2-4) Shows the Details of the CUPRRAC Method.

Reagents	Test	STD	Blank
Copper(II) chloride solution	1ml	1ml	1ml
Serum	50 µl	------	-------
Working standard solution	-------	50 µl	-------
D.W	-------	-------	50 µl
Neocuproine (Nc) solution	1ml	1ml	1ml
Ammonium acetate (NH_4Ac) buffer	1ml	1ml	1ml
Test tubes was mixed by vortex and incubated for 30 minutes at 37°C, after that the absorbance was read on a spectrophotometer at 450 nm.			

2.4.4.Calculation

$$\text{Total antioxidants levels} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD \ (\mu mol/l)$$

2.5. Determination of Serum Lipid Peroxidation

2.5.1 Principle

Lipid peroxidation in sera was evaluated by thiobarbituric acid reactive substances (TBARS). TBARS test gives a basic, reproducible, and standardized tool for measuring lipid peroxidation in serum. The malondialdehyde- thiobarbituric acid (MDA-TBA) adduct designed by the response of MDA and 1,3-Diethyl-2-thiobarbituric acid (DETBA) under high temperature (90-100°C) at acidic conditions is measured calorimetrically at 530-540 nm or fluorometrically at an excitation wavelength of 515 nm and an emission wavelength of 555 nm. This reaction has a much higher sensitivity when measured fluorometrically [134].

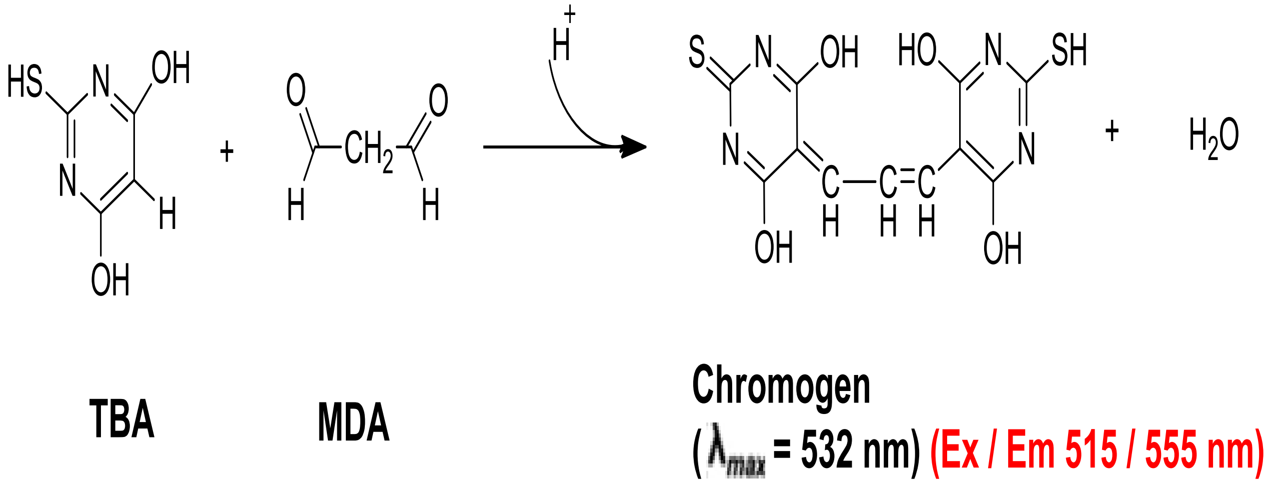


Fig. (2-1). Scheme of the Adduct MDA–$(TBA)_2$ [135].

2.5.2 Reagents

The working solution was prepared by dissolving 0.514 g of thiobarbituric acid (TBA) in distilled water (D.W) then added 25 g of trichloroacetic acid (TCA), thereafter added 0.5 mL of HCL, subsequently complete the volume to 190 ml by using D.W then added 0.5 g of Sodium dodecyl sulfate (SDS) then completed the volume to 200 ml and mixed well.

2.5.3. Procedure: Shown in the Table (2-5) [135].

Table (2-5) Shows the Details of the Present Method.

<table>
<tr><th>Reagents</th><th>Test</th><th>Blank</th></tr>
<tr><td>Serum</td><td>100 µl</td><td>----</td></tr>
<tr><td>D.W</td><td>----</td><td>100 µl</td></tr>
<tr><td>Working solution</td><td>2 ml</td><td>2ml</td></tr>
<tr><td colspan="3">The sample was vortexed and heated in a 90º C water bath for 50 min, and then allowed to cool.</td></tr>
<tr><td colspan="3">The sample was then centrifuged for 5 min at 5000 rpm then measure the absorbance spectrophotometrically of the supernatant at the wave length 532 nm against a reagent blank. The preparation of reagent blank was the same procedure above except change the sample with DW.</td></tr>
</table>

$$serumMDA = \frac{Absorbance}{d \; X \; \zeta} \; X \; D.F$$

d = 1cm, ε =extinction coefficient = $1.56x\ 10^{5}\ M^{-1}cm^{-1}$

D.F = dilution factor

2.6.1. Determination of Serum Total Cholesterol

Total cholesterol in the serum was measured by enzymatic method, with the biomerux kit, France [136].

2.6.1.1. Principle

The principle of this method was lysis the cholesterol ester to produce cholesterol and fatty acids, then oxidized to produce the quinoemine: -

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

$$\text{Cholesterol} \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest - 4 - en - 3 - one} + \mathrm{H}_2O_2$$

$$2\mathrm{H}_2O_2 + Phenol + 4 - a\,\mathrm{min}\,o\,antipyrine \xrightarrow{peroxidase} quino\,e\,\mathrm{min}\,e + 4\mathrm{H}_2O_2$$

2.6.1.2. Reagents

The reagent used in test is a mixture of: -

1- phosphate buffer 0.1 mol/l.

2- phenol 15 mmol/l.

3- sodium cholate surfactant 3.74 mmol/l.

4- 4 amino antipyrine 0.5 mmol/l.

5- peroxidase ≥ 1000 U/l.

6- Cholesterol oxidase ≥ 200 U/l.

7- Cholesterol esterase ≥ 125 U/l.

2.6.1.3. Procedure

Table (2.6) The procedure for this method as the following: -

	Reagent blank	Standard	Sample
Standard 200 mg/dl	----	10 µl	
Sample	---	----	10 µl
Working reagent	1 ml	1 ml	1 ml

The content of every tube is gently mixed after addition, then it should be allowed to stand at room temperature for 10 minutes or incubated at

37 C^0 for 5 minutes, and absorbance is read by spectrophotometer at 500 nm.

The absorbance is directly proportional to the total cholesterol concentration in the sample.

$$\text{Total cholesterol}\,(mmol/l) = \frac{A_{bs}\text{ of sample}}{A_{bs}\text{ of standard}}\;X\;5.17$$

2.6.2. Determination of Serum Triglyceride

Total triglycerides in the serum were measured by enzymatic with the (biomerieux kit, France).

2.6.2.1. Principle

Total triglyceride determination depends on formation of quinoemine by using a group of enzyme as fallow: -

$$\text{Triglyceride} \xrightarrow{lipase} \text{Glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + ATP \xrightarrow{Glycerol\,kinase} \text{Glycerol} - 3 - \text{phate} + ADP$$

$$\text{Glycerol} - 3 - \text{phosphate} \xrightarrow{Glycerol-3-phosphate} \text{Dihydroxy acetone phosphate} + H_2O$$

$$2H_2O_2 + \text{Pchlorophenol} + 4 - \text{aminoantipyrine} \xrightarrow{peroxidase} \text{Quinonemine} + 4H_2O$$

2.6.2.2. Reagents

The reagent used in this test is a mixture of:

1- Tris buffer pH 7.6	100 mmol/l.
2- p-chlorophenol	2.7 mmol/l.
3- Magnesium	4 mmol/l.
4- 4 aminoantipyrine	0.4 mmol/l.
5- lipase	≥ 1000 U/l.
6- Gltcerokinase	≥ 200 U/l.
7- Glycerol-3-phosphate oxidase	≥ 2000 U/l.
8- Peroxidase	≥ 200 U/l.
9- ATP	0.8 mmol/l.

2.6.2.3. Procedure Table (2-7)

	Reagent blank	Standard	Sample
Standard 200 mg/dl	----	10µl	-----
Sample	-----	------	10 µl
Working reagent	1 ml	1 ml	1 ml

The content of every tube is gently mixed. It is allowed to stand at room temperature for. 10 minute or incubate at $37C^{\circ}$ for 5 minute and read spectrophotometrically at 505 nm. The absorbance is directly to the total triglyceride in the sample.

$$\text{Total serum triglyceride (mmol/l)} = \frac{Abs.of\ sample}{Abs.of\ s\tan dard} X\ 2.26$$

2.6.3. Determination of Serum High Density Lipoprotein-Cholesterol (HDL-C)

HDL-Cholesterol in the serum was measured by enzymatic method using biomerieux kit, France.

2.6.3.1. Principle

The addition of the phosphotungestic in the presence of magnesium ions-due to precipitate the chylomicrons and
lipoproteins of very low density (VLDL) and low density (LDL) contained in the sample.

The supernatant obtained after centrifugation contains high-density lipoprotein HDL was measured by enzymatic method.

2.6.3.2. Reagents Table (2-8)

Reagent (1) Precipitation Reagent	Phosphotingestialic acid MgCl2.6HO pH 6.2	40 g/l 100 g/l
Reagent (2) HDL Ch. Calibrating solution	Free esterified Cholesterol	1.30mmd /l 0.5 g/l

2.6.3.3. Procedure table (2-9)

	Reagent blank	Standard	Sample
Distilled water	50 µl	-----	------
HDL-calibrator	-----	50	-----
Supernatant	----	-----	----
Working reagent	1 ml	1 ml	1 ml

The working solution is the cholesterol enzymatic solution. The content of every tube is gently mixed after addition, then it is allowed to stand at room temperature for 10 minute or incubate for 5 minutes at 37C° and read spectrophotometrically at 500 nm.

The concentration of HDL- cholesterol

$$HDL-(mmol/l)=\frac{Abs.of\ sample}{Abs.of\ s\tan dard}X\,1.42$$

1.42(the concentration of standard)

2.6.4. Determination of Serum Low Density Lipoprotein-Cholesterol LDL: -

In lipoprotein fractionation the widely accepted method for determination LDL-cholesterol is the beta quantification procedure. Very low-density lipoprotein (VLDL) is separated by ultracentrifugation and high-density lipoprotein (HDL), by precipitation. Because of ultra-centrifugation is unavailable in most routine laboratories and the procedure is expensive, time consuming and technically demanding, the nearly universal approach in clinical laboratories has been to estimate LDL-cholesterol from the formula of (friedewaid etal.1972). After measurement of total cholesterol, triglycerides and HDL-cholesterol, LDL-cholesterolis calculated as total cholesterol minus very high-density lipoprotein (HDL) (estimated as triglyceride ÷2.2)-with those obtained by lipoprotein fractionation. When triglyceride was <0.2 g/dl. 90% estimated LDL-cholesterol value acceptable, with ∓10% of measured values [136].

2.7. Paraoxonase Activity

2.7.1. The Principle

Essentially toxicologists focused on the PON1protection role against the organophosphate poisoning. Nevertheless, a significant relation between the present of PON1 and LDL oxidation inhibition [137]. Paraoxonase where it associates with high-density lipoprotein (HDL) particles in serum, it is hydrolyzing highly toxic organophosphorus compounds such as the insecticide paraoxon **Fig (2-2)** and various nerve agents used as chemical weapons [138]. Serum butyryl cholinesterase activity which is elevated in smoker and non-smoker from influence on paraoxonase enzyme activity therefor, serum from controls smoker and non-smoker was incubated with 5 mmol/ l Eserine for 10 min at room temperature initially to inhibit serum butyryl cholinesterase activity and would otherwise interfere with the determination of paraoxonase activity.

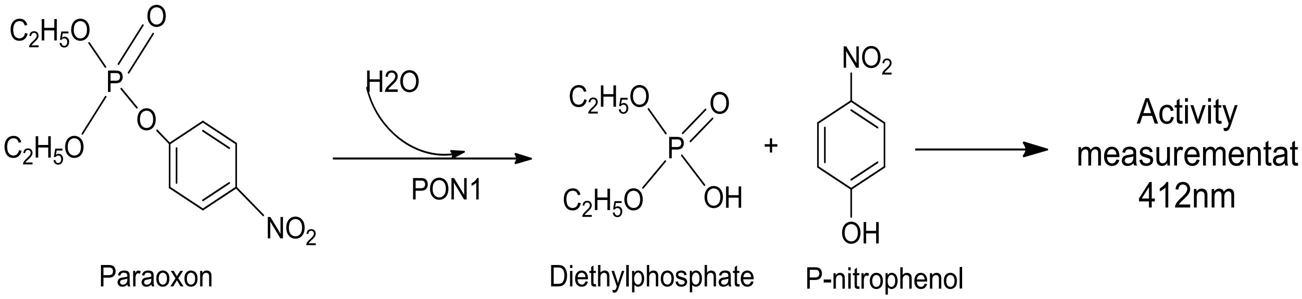


Figure (2-2): Scheme of hydrolases of Paraoxon by PON1 [139].

2.7.2. The Reagents Preparation:

1. Tris Buffer Solution composed of 1.21gm Tris base that dissolved in 90 ml of distilled water. The final pH adjusted to 8.0 with 1 M HCl.
2. Working Buffer Solution WBS: composed from mixing 100ml of stock solution with 900 ml of distilled water.

3. The reagent was prepared by weighted 10.0gm of NaCl and 0.06 gm $CaCl_2$ were dissolved by 500 ml of working Tris buffer solution at last added 200 µL of Paraoxon.

2.7.3. Procedure: shown in Table (2-10).

Sample, blank and standard test tubes were prepared as the instructions below:

Table (2-10) Steps of the procedures used for assessing the activity of Paraoxonase activity.

Sample	Test	Blank
Serum	10 µL	-----
Distilled water	-------	10 µL
Reagent	190 µL	190 µL
Mixed and incubated at 37 °C, for 10 minutes then measured at 405nm in Micro Plate Reader		
Repeat the measuring after 5 minutes		

2.7.4. The Calculation:

$$\text{Activity} = \frac{\left(\frac{\Delta A}{min}\right) \times assay\ valume}{\varepsilon \times sample\ volume \times path\ length}$$

The enzyme activity was calculated from the molar extinction coefficient (ε) of 18700 M^{-}. cm^{-1} at 405 nm, pH equal to 8.0

The activity was expressed as catalytic concentration which corresponds to the product of 1µM of p-nitro phenol/min/L of serum [139, 140].

2.8. Homocysteine thiolactonase enzyme

2.8.1. Homocysteine thiolactonase enzyme activity

PON1 activity toward Hcy-thiolactone was determined by measuring the amount of hydrogen ions generated during thiolactone hydrolysis to homocysteine in the assay mixture (800 mL) containing 5 mM substrate, 1 mM CaCl2, 0.0005% bovine serum albumin, 0.004% phenol red, and 20 µL of serum in 5 mM HEPES buffer (pH 7.0) [141]. The absorbance was monitored for 4 min at 412 nm, and the results were expressed in nmol min^{-1} mL^{-1}. The blank sample containing incubation mixture without serum was simultaneously assayed to correct for spontaneous substrate breakdown. PON1 activity toward Hcy-thiolactone was calculated from the standard curve prepared by titrating the assay medium with the different HCl concentrations.

2.8.2 Reagents:

1. HEPES buffer (25 mM; pH 7.2) was prepared by dissolving 1.2 g of HEPES in 160 ml of deionized water (DW), in a suitable container. pH of the buffer was adjusted to 7.2, using 1.0 M sodium hydroxide. The buffer was brought to a total volume of 200 mL by adding DW. A

0.01g of sodium azide, 0.01g of bovine serum albumin and 22.2mg of $CaCl_2$ (1mM) were added to the buffer.

2. Substrate solution was prepared by dissolving 51.075 mg of γ-thiobutyrolactone (5 mM) in 100 ml of HEPES buffer (25 mM; pH 7.2). 0.004% phenyl red was added.

2.8.3. The Procedures:

Sample, blank and standard test tubes were prepared as the instructions below:

Table (2-11) Steps of the procedures used for assessing the activity of Lactonase

	Test	**Blank**
Serum	10 µL	-----
Distilled water	-------	10 µL
Reagent	200 µL	200 µL
Mixed and incubated at 37 °C, for 5 minutes then measured at 405nm in Micro Plate Reader		
Repeat the measuring after 5 minutes		

2.8.4. The Calculation:

PON1 activity toward Hcy-thiolactone was calculated from the standard curve prepared by titrating the assay medium with the different HCl concentrations.

2.9. The Genetics Study

2.9.1. The solutions used for extraction and methods of preparation [142].

(1) Prepare a wash solution (20) mM (90% Tris + 10% methanol)

to prepare (100 ml) Tris at a concentration of 20 mM .We dissolved

0.31 g of Tris + HCl) in (90 mL) DW, set the pH to 7.5 and completed the volume to (100 mL) DW.

(2) To prepare the extraction solution (lyses)

(3) It was taken (Tris-HCL) (0.3 g) + (0.06 g) EDTA + (0.2 g) from SDS by dissolving a volume of 90 ml distilled water (DW). Adjust pH 8 and complete the volume to 100 ml.

(4) Solution EDTA: to prepare the solution (50) ml EDTA by dissolving(0.03g) of EDTA in volume (40 ml) distilled water. Adjust pH 8 and complete the volume to 50 ml distilled water.

(5) Sodium acetate solution: to prepare (20) ml of the solution at a concentration of (3) M by dissolving (4.9g) of Sodium acetate in volume (15 ml) distilled water Adjust PH 4.5 and complete the volume to 20 ml distilled water.

(6) Buffer solution (TE) consists (Tris + EDTA): to prepare the solution (100) ml TE buffer by dissolving (0.157g) of Tris and (0.029g) of EDTA in volume (90 ml) miliQ water adjust pH 8 and complete the volume to 100 ml distilled water Put in or auto cleave for sterilization at a temperature (121 C) and pressure (0.1) for a quarter of an hour.

2.9.2. Procedure Extraction

1. It was taken 500 microliters of the EDTA treated blood and put it in a volume of one and 1.5 ml a half ml Eppendorf tube then add 1 ml of

washing solution 10% Methanol + 90% Tris (18mM) put on the Rolar for 10 minutes, then Center Fugue 10000 for 1 minute. The filtrate is discarded and the precipitate is taken.

2. The precipitate is suspended by the washing solution to reach full diffusion, then Center Fuge 10,000 cycles for a minute. The filtrate is neglected.
3. The precipitate was suspended by 200 µl of 2 mM EDTA solution.
4. Four hundred microliters of the extraction solution ware added and suspended well until complete dispersal, at 60 C for 15 minutes.
5. Then cool to room temperature, then add 100 µl of sodium acetate (4.5 ph M3) VORTEX for a minute.
6. Center Fuge 10000 cycles for 10 minutes The sediment is neglected and the filter is taken and placed in the silca column.
7. Center Fusion 10000 cycles for 30 seconds.
8. Wash twice with 600 µl of 70% ethanol + 30% Tris (pH and molarity)
9. Five mM (600 µl of column wash solution is added, then Centrifuge 10,000 cycles for 30 seconds and the process is repeated twice).
10. The column is dried after getting rid of the filter by placing it in Center Fugue 10000 cycles for three minutes to get rid of the largest amount of washing solution.
11. The collection tube is discarded and the column is transferred to a sterile Eppendorf one or 0.5 ml volume.
12. One hundred µl of Elution buffer solution is added to the center of the column and left for 5 minutes. After addition, it is confirmed that all the solution is absorbed from the column (and the solution is not left until complete absorption).

13. Then Fuge Center 10,000 cycles for a minute by taking the filter that represents pure DNA and neglecting the column.

2.9.3. Extracted DNA Quantity and Quality assessment.

The extracted DNA was assessed by spectrophotometry method, (scan drop, analytical jena, Germany) sample quantity were ranged from 35-56 ng /µl and the quality (260/280 ratio) ranged from 1.7-1.85, each sample that retrieve a 260/280 ratio lesser than 1.7 would be re extracted, on the other hand the extracted DNA was checked by 1% agarose gel electrophoresis.

2.9.4. Agarose Gel Electrophoresis.

Preparation of TBE (5X) Stock Solution/Liter.

take (Tris base) (27 g) +(14 g) baric acid+ (1.86 g) of EDTA by dissolving volume of 500 ml of distilled water (DW). put the package on the device hot plate stirrer.

2.9.4.1. Gel Electrophoresis Protocol.

1. The gel-casting tray was placed in plastic tray and checked that the teeth of the comb are 0.5mm above the gel bottom.
2. Position the comb 1.5 cm from the edge of the gel.
3. TBE (0.5X) (80ml) was placed into a 250ml flask and 0.8g or 1.6 gram of agarose to prepare 1% and 2% concentration respectively agarose was added and then melt the agarose by heating the solution on microwae for approximately 2 min. The agarose solution was swirled carefully to ensure that the agarose was completely dissolved and the solution became clear.
4. The agarose solution was cooled to approximately 60ºC and adds 4 microliter of 1mg/ml ethidium bromide, and slowly pours the

agarose into the gel-casting tray. If there are any air bubbles must be removed by tip.

5. Agarose was left solidify for about 20-30 minutes. After the agarose has been solidified the comb has been removed with the back and forth stirring, careful to prevent rupture of the gel.
6. The gel-casting tray was removed and placed the tray on the central supporting platform of the gel box.
7. Electrophoresis buffer was added to the buffer chamber until it reaches a level of 3-5 mm above the surface of the gel.
8. Five microliters of pcr product were mixed with 2 µl of loading dye and carefully loaded by mechanical pipet to the gel wells.
9. Ladder molecular weight marker (5 µl) was loaded to one side of the gel (flanking and sample line) and 5 micro of DNA specimen and 5micro of pcr product in the other well.
10. The lid was placed on the gel box and connected the electrodes. DNA will travel towards the positive (red) electrode positioned away from the well. Turn on the power supply.
11. Electrophoresis was continued in constant voltage 5V/Cm until the tracking dye moves at least 10 cm of the gel length.

2.9.4.2. Photo Documentation.

Agarose gel was visualized in a UV trans illuminator provided with gel documentation unit, agarose gel was placed above the UV trans illuminator device, the gel was exposed to UV light and the photo was captured using canon digital camera.

2.9.4.3. Reconstituting and diluting primers.

Primers were commonly shipped in a lyophilized state. The units of a lyophilized primer were given as a mass, in pico-moles. In order to create a stock of primers, one would reconstitute the primer in sterile, nuclease free H_2O to be added to each primer to obtain a master stock that would be used again to obtain a working stock.The following steps were followed for reconstituting and diluting the primers:

1. The tube was spined down before opening the cap.
2. The desired amount of water was added according to the oligos. Manufacturer to obtain a 100Pmoles/µl (Master Stock).
3. The primers were vortexes properly to re-suspend evenly.
4. A 10µl of the master stock was transferred to a 0.2ml Eppendorf tube that contained 90µl of sterile, nuclease-free H_2O (Working Stock).
5. The master stock and working stock were stored at -20 ºC.
6. The working stock was thawed on ice and vortex before using in PCR and then stored at -20 ºC.

Sequences of primers used for PCR amplification this study were shown in table (2-12).

Table (2-12) : Sequences of primers used for PCR amplification of Praoxonase Gene.

Primer gene name	**Sequences**
Praoxonase Gene **Rs 662**	**F:5'-ATGATATTGTTGCTGTGGGACCT -3'**
	R: 5'- CTGCCACCACTCGAACTTCA -3'

2.9.5. Amplification by Polymerase Chain Reaction (PCR)

Table (2-13): Optimization reaction Thermocycling conditions for **(PCR)**

No.	**Step**	**Temp.(Cº)**	Time **(min)**	No. of cycles
1	Initial denaturation	94	5 min	1
2	DNA Denaturation	94	30 sec	35
	Primer annealing	55-66	30 sec	
	Extension	72	30 sec	
3	final elongation	72	5 min	1

Table (2-14) The proportion of mixing materials are Shown below

	Composition	Concentration	Volume
1	Master mix(Russian(cyntol)	100ppm	8 µl
2	Forward primer	10pmol\ml	1 µl
3	Revers primer	10	1 µl
4	DNA sample	30-55ng/ml	2 µl
5	Nucleases free water		7.5 µl
6	Mgcl2	25mM	0.5 µl
	Total volume		20 µl

2.9.6. Restriction Fragment Length Polymorphism (RFLP) for study the genotyping of Arachidonate 12 Lipoxygenase

2.9.7. Principle

After digestion of the DNA tests in question with certain restriction endonucleases, RFLP is a distinction in homologous DNA sequences that can be determined by the proximity of fragments of varying lengths. As a molecular marker, RFLP is specific to a single restriction enzyme/clone combination [143]. Most RFLP markers are co-dominant (in heterozygous cases, the two alleles will be detected) and locus-specific. A RFLP probe is a DNA sequence that, after being extracted by gel electrophoresis, hybridizes with at least one fragment of the processed DNA test, revealing a distinct blotting pattern diagnostic of a certain genotype at a specific locus. RFLP probes are usually short, single- or low-duplicate genomic DNA or cDNA clones. The RFLP tests are frequently used in genome mapping and variety investigation (genotyping, crime scene investigation, paternity tests, and hereditary illness diagnostics, among other things) [144].

2.9.8. Restriction Enzyme

Restriction Enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded DNA at restriction sites, which have unique recognition nucleotide sequences. These enzymes, which are prevalent in bacteria and archaea, are hypothesized to have developed as a virus defense mechanism [145]. Restriction enzyme (also known as restriction endonuclease) is a particular enzyme that cuts double-stranded DNA at restriction sites, which have unique recognition nucleotide sequences.

2.9.9. Procedures

For genotyping based on restriction endunuclase cleavage, the polymerase chain reaction–restriction fragment length polymorphisms (PCR-RFLP) method was utilized. This approach can be used to genotype SNPs that change the restriction sequence. PCR amplification of the region surrounding the restriction enzyme site, followed by digestion with the appropriate enzyme for genotyping [146]. BanII digests endunuclase [147]. Preparation was used to carry out these processes. Five liter are of each amplified product, along with 0.5 micrograms of enzyme, 1.5 buffer, and eight liters of water [147], were placed in tubes and incubated overnight at 37 degrees Celsius. Digested amplified DNA fragments were electrophoresed on 2% agarose for 1 hour at 100 V, and the bands were seen under UV illumination before staining with ethidium bromide. For fragment size estimation, a 100 base-pair ladder (England Biolab - UK) was utilized as a size marker.

2.10. Programs:

The following programs and webs were used to validate the concentration of solutions that used in the current study:

1. SPSS 24 for Windows (SPSS Inc., Chicago, IL, USA).
2. Quickcals-graphpad.
   (https://www.graphpad.com/quickcalcs/Molarityform.cfm).
3. Buffer preparation
   (https://www.cusabio.com/m-296.html).
4. Molarity calculator
   (https://www.graphpad.com/quickcalcs/Molarityform.cfm).

2.11 Statistical analysis

Data analysis was performed using SPSS 24for Windows (SPSS Inc., Chicago, IL, USA). Data were expressed as mean, SD, and range, and were evaluated by one-way analysis of variance (ANOVA) followed by LSD test. The Kolmogorov Smirnov test was used to verify if data followed normal distribution. The statistical significance level was considered at $P < 0.05$.

2.12. A quantitative method for assessment of homocysteine thiolactonase enzyme activity

2.12. 1. Sample preparation

Five ml of blood from a researcher at the Advanced Biochemistry Lab/ University of Babylon/ Iraq were poured into gel tubes. The gel tubes were centrifuged at 2000 × g for 15 minutes, then the sera were collected and stored at -20˚C until analysis.

2.12.2. Purification of HTase

Human HTase was purified according to Jakubowski's method [124]. The resulting HTase activity was assessed according to Billecke *et al.*'s method [129] (see 2.6). The obtained HTase enzyme has specific activity equal to approximately 4 U.mg^{-1} protein.

2.12.3. Standard method to assess homocysteine thiolactonase (HTLase) activity

A modified pH-metric method was applied to assay HTLase activity. The method quantifies the amount of H$^+$ ions that were liberated as a result of hydrolysis of γ-thiobutyrolactone to 4-mercaptobutyric acid [129]. Although alkaline pH is ideal for HTLase activity, HTLase activity was obtained at pH 7.0 because thiolactone is unstable in alkaline solution. Serum (20 µl) was mixed with 800 µl of the reaction mixture containing a cocktail of substrate and reagents that dissolved in 5 mM HEPES (pH 7.0). The mixture contains 0.004% phenol red (106 mM), 5 mM thiolactone, 1.0 mM $CaCl_2$, and 0.005% BSA. The increase in absorbance at 422 nm for 4 min was utilized to obtain HTase activity from the standard HCl titration curve. Final HTLase activity was stated as µmol H$^+$ liberated/l plasma per min.

2.12.4. Accuracy and selectivity

To study the effect of biomolecules on the current protocol, 4-mercaptobutyric acid concentration was determined in four volumetric flasks that contain a suitable concentration of different types of biomolecules. The first consists of HEPES buffer (25 mM; pH 7.2) containing 100 µM 4-mercaptobutyric acid. The second consists of 5 mM of each of the following sugars: lactose, glucose, and fructose. These sugars dissolved in HEPES buffer (25 mM; pH 7.2) contain 100 µM 4-mercaptobutyric acid. The third consists of 5 mM of each of the following amino acids: lysine, aspartic acid, histidine, and methionine, which dissolved in HEPES buffer (25 mM; pH 7.2) containing 100 µM 4-mercaptobutyric acid. The fourth was composed of 3% bovine serum albumin and 3% casein, which dissolved in HEPES buffer (25 mM; pH 7.2) containing 100 µM 4-mercaptobutyric acid. Interfering biomolecules were prepared in HEPES buffer (25 mM; pH 7.2).

2.12.5. Sensitivity and linearity

The linearity and sensitivity of the present protocol were evaluated with a series of HTLase activities (0, 25, 50, 100, 200, 300, 400, and 500 U. L^{-1}). A web-based program for bias estimation and analytical method comparison [148] was utilized to find the linearity of the current method by comparing the results of the current method with that obtained by the H^+ ions liberating method [129]. The limits of quantitation (LOQ) and detection (LOD) were estimated to determine the sensitivity of the CUPRAC assay [149].

3.12.6. Validation and reproducibility

The present protocol was confirmed against the H^+ ions liberating method as described by Billecke *et al*. [129] using Bland-Altman analysis by using a series of HTLase enzyme activity (10 to 1000 U. L^{-1}).

2.12.7. Procedure

HTLase enzyme activity procedure was explained in Table 2-15.

Reagents	**Test**	**Control**	**STD**	**Blank**
Substrate solution	2000µL	1500µL	2000µL	2000µL
D.W.	-----	500 µL	50 µL	50 µL
4-mercaptobutyric acid	------	------	500 µL	------
TCA	------	500µL	------	------
The reaction was initiated by adding serum:				
Serum	50 µL	50 µL	-------	------
Mix by vortex and incubate for 5 minutes at 37°C, after that, the reaction was terminated with 0.5 ml of 8% TCA and Centrifuge for 10 minutes at 3000 xg, then remove 1 ml of supernatant in a clean tube , and add				
Working reagent	3ml	3ml	3ml	3ml

The color developed was read at 450 nm after 30 min.

2.12.8. Calculation

One unit of HTase activity is defined as 1 µmol of γ-thiobutyrolactone formed per liter per min under the defined assay conditions.

The following equation was applied to calculate HTase activity in each test tube:

$$\text{HTase activity (U/L)} = \left(\frac{A\,Test - A\,Control}{\text{A STD}}\right) \times \text{Conc. of STD}$$

Concentration of STD is equal to 1000 µM.

Also, HTase activity can be calculated from the thiol standard curve prepared by 4-mercaptobutyric acid (fig. 2-3).

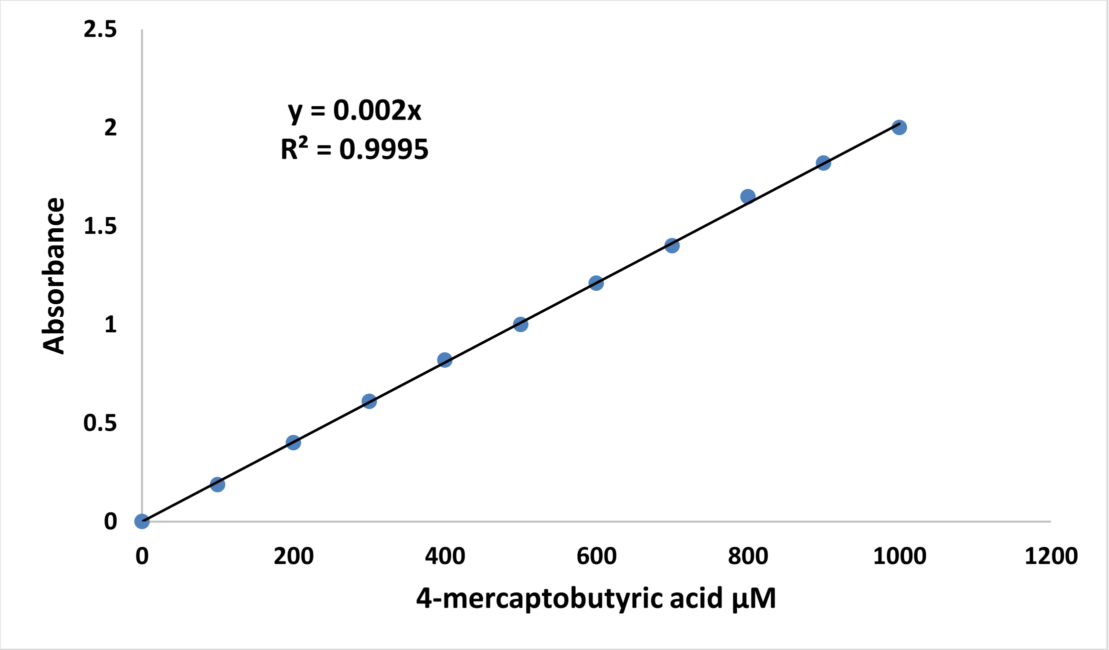


Fig. 2-3. The standard curve of 4-mercaptobutyric acid obtained by utilizing the current protocol.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1. Characteristics of the Study Subjects

This study was performed to the smoker and non – smoker students in the University of Babylon. All the blood samples were collected between from November 2020 to January 2021. Three hundred samples were collected from men. The samples were divided to non-smoker (NS) and cigarettes smoker (CS). There were no significant differences in age, body height, and body mass between the non-smokers, smokers (Table 3-1).

Table (3-1). Participants' characteristics non-smokers, cigarette smokers.

Group / Parameter	Nonsmokers (NS)	Cigarette Smokers (CS)
Number	150	150
Age (Years)	20±2	20±2
BMI (kg/m^2)	21.5±1.5	21±2.5

3.2. The Effect of Smoking on Oxidant/ Antioxidant Status

3.2.1. Total Oxidant Status Concentrations in Sera of Nonsmokers and Cigarette Smokers

The results in Table (3-2) showed the concentrations of total oxidant status (TOS) that found in sera of non-smoker (NS)was 12.612 µmol/l; while, in cigarette smoker (CS) was 14.957 µmol/l. Furthermore, the standard deviation in non-smoker, cigarette smoker (4.4626, 5.8218 µmol/l) respectively.

Table 3-2: Total Oxidants Status Concentration in Sera (µmol/l) of Non-smokers and Cigarette Smokers Groups

Group	N	Mean	Std. Deviation (SD)	Std. Error	95% Confidence Interval for Mean		Sign.
					Lower Bound	Upper Bound	
NS	150	12.612	4.4626	0.3643	11.8922	13.3322	0.001*
CS	150	14.957	5.8218	0.4753	14.0181	15.8967	

*. The mean difference is significant at the 0.05 level.

Free radicals are extremely reactive and unstable chemicals. Their electrons interact with other molecules within cells, causing damage to proteins, lipids, DNA, and nucleotide coenzymes, among other biological materials [150]. Antioxidants are substances that inhibit or postpone the oxidation of biological elements found in living cells, and this process is known as antioxidant defense [151]. In an organism, there is a balance between the rate at which free radicals are produced and the rate at which they are destroyed. This state, known as "oxidative equilibrium," protects the organism by counteracting the harmful effects of free radicals. “Oxidative stress” is defined as an imbalance between free radical generation and antioxidant defense system that favors free radicals [152]. Cigarettes contain a high number of free radicals, which contribute to the disruption of oxidative balance and cell damage. This is the foundation of many smoking-related health issues, such as cancer, cardiovascular disease, and pulmonary disease [153]. Total antioxidant status (TAS) indicates the total effect of all antioxidants in plasma and body fluids, whereas entire oxidant status (TOS) reflects the total effect of all oxidants in plasma and body fluids [154]. Free radicals and reactive oxygen species (ROS) found in tissues can harm biologically vital components like DNA, proteins, carbohydrates, and lipids. ROS are created during metabolic and physiological activities, and in organisms that remove them by enzymatic and nonenzymatic antoxidative mechanisms, damaging oxidative reactions might occur. Under certain circumstances, a rise in oxidants and a decrease in antioxidants is unavoidable, and the oxidative/antioxidative balance switches to the oxidative position. As a result, oxidative stress develops [155], which has been linked to over 100 illnesses.

3.2.2 Total Antioxidant Status (µmol/l) in Sera of Nonsmokers, Cigarette Smokers

The result in Table (3-3), shown the measuring of total antioxidant status (TAS) in smokers and non-smokers. The present study found the mean of antioxidant levels in non-smoker was 0.7248 µmol/l, in cigarette smoker was 0.6702 µmol/l Furthermore, the standard deviation in non-smoker, cigarette smokers were (0.21678, 0.20179 µmol/l) respectively.

Table (3-3): Total Antioxidant Status in Sera (µmol/l) of smokers and non-smokers Groups

Group	N	Mean	Std. Deviation (SD)	Std. Error	95% Confidence Interval for Mean		Sign.
					Lower Bound	Upper Bound	
NS	150	0.7248	0.21678	0.01770	0.6898	0.7597	0.025*
CS	150	0.6702	0.20179	0.01648	0.6377	0.7028	

*. The mean difference is significant at the 0.05 level.

Free radicals are reactive molecules that develop in the environment when nutrients are burnt in the presence of oxygen. These compounds, known as reactive oxygen metabolites, damage cell components, resulting in oxidative stress [156]. To fight the detrimental effects of free oxygen radicals, the body possesses a self-defense system. The antioxidant defense system protects the body intracellularly and extracellularly by acting as a shield against free oxygen metabolites that occur physiologically or in the environment [157]. The formation and removal of free radicals from the organism are in perfect balance, and free radicals have no effect on this delicate environment as long as the oxidative balance is maintained. When one of the steps working to maintain this equilibrium fails, oxidative stress occurs, which can result in cell harm in the organism. Many diseases, such as atherosclerosis, diabetes, and cancer, are etiopathogenesis of oxidative stress [157, 158, 159].

3.3. Lipid and Lipid Peroxidation in Sera of Nonsmokers and Cigarette Smokers

lipid profile concentration was changed according to the state of smoking, as shown in table (3-4)

Table (3-4) Lipid profile in sera of cigarette smokers and nonsmokers' groups.

	Smokers (n = 150)	Nonsmokers (n = 150)	P
TC (mmol/L)	4.37 ± 1.2	4.05 ± 1.15	0.15
TG (mmol/L)	2.87 ± 0.97	3.40 ± 1.16	0.001
HDLc (mmol/L)	1.12 ± 0.21	1.75 ± 0.19	0.03
LDLc (mmol/L)	1.75 ± 0.38	2.27 ± 0.61	0.01

The results in (Table 3-5) found that the level of MDA in sera of non-smoker was 1.1091 µmol/l, and in cigarette smoker was 1.2304 µmol/l. Furthermore, the standard deviation in non-smoker and cigarette smoker were (0.29671, 0.47006 µmol/l) respectively.

Table (3-5): Lipid Peroxidation (MDA Concentration (µmol/l)) in Sera of Cigarette Smokers and Nonsmokers Groups.

Group	N	Mean	Std. Deviation (SD)	Std. Error	95% Confidence Interval for Mean		Sign.
					Lower Bound	Upper Bound	
NS	150	1.1091	0.29671	0.02423	1.0612	1.1569	0.008*
CS	150	1.2304	0.47006	0.03838	1.1546	1.3063	

*. The mean difference is significant at the 0.05 level.

Malondialdehyde (MDA), one of the indicators that used to assess oxidative stress, was shown to be higher in smokers. Consider the evidence for the role of free radicals in human disease while increasing the concentration of lipid peroxidation end products. As a result, increased oxidative damage is likely to occur in most human disorders, with special of them playing substantial pathogenic roles [160], as shown in Fig (3-1).

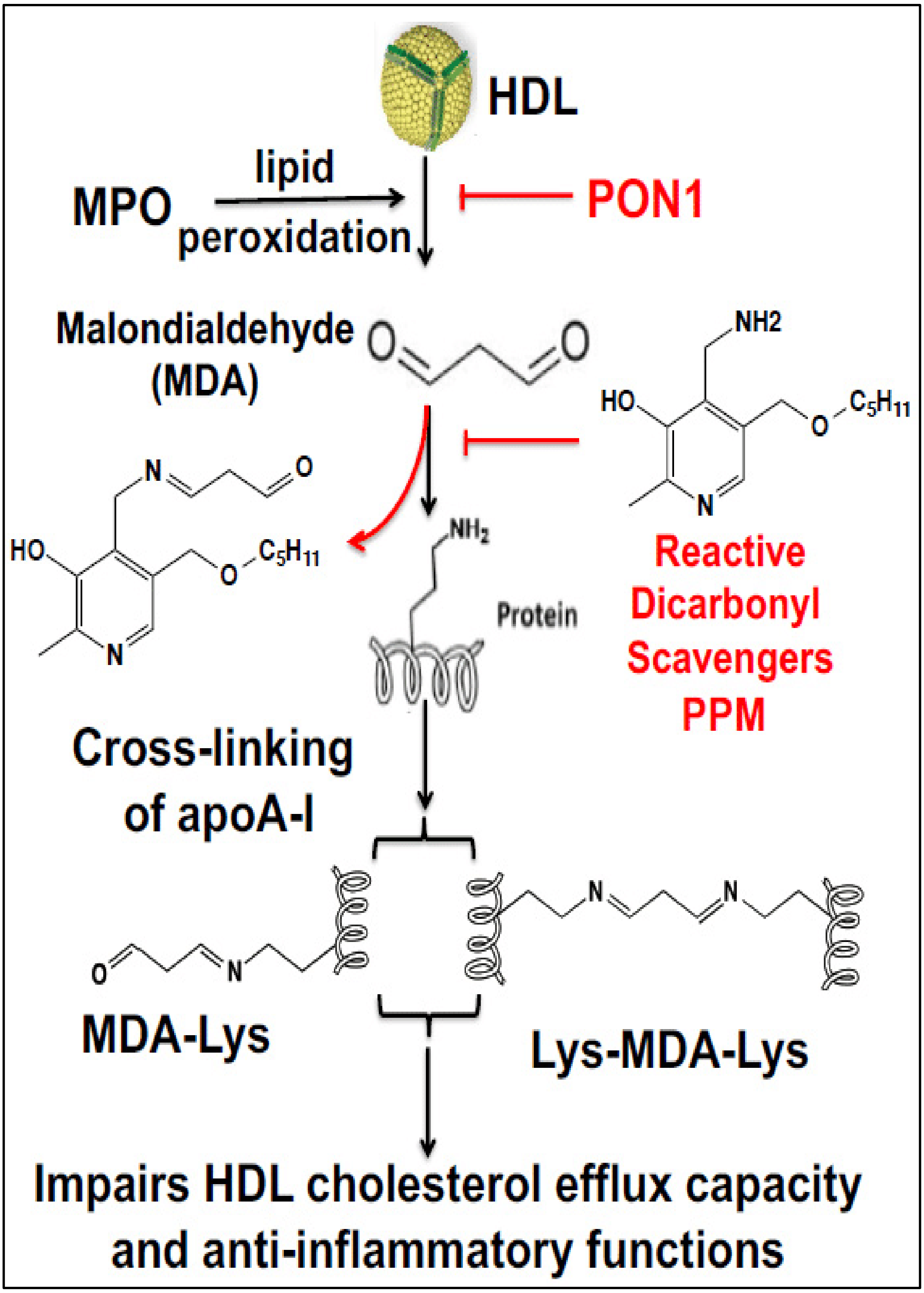


Figure (3-1): Malondialdehyde (MDA) generation from HDL peroxidation, its subsequent crosslinking of proteins, and the impact of reactive dicarbonyl scavenging on HDL cholesterol efflux ability and anti-inflammatory function [161].

3.4. Paraoxonase PON1 enzymatic activity

The results in Table (3-6), showed the activity of paraoxonase sera of non-smoker (NS) was 145.33 U/L. While, in cigarette smoker (CS) was 128.56 U/L. Furthermore, the standard deviation in non-smoker and cigarette smoker were 72.031, 55.211 U/L respectively.

Table (3-6): Paraoxonase activity (U/L) in Sera of Cigarette Smokers and Nonsmokers Groups.

Group	N	Mean	Std. Deviation (SD)	Std. Error	95% Confidence Interval for Mean		Sign.
					Lower Bound	Upper Bound	
NS	150	145.33	72.031	5.881	133.715	156.958	0.024*
CS	150	128.56	55.211	4.508	119.652	137.468	

*. The mean difference is significant at the 0.05 level.

Several studies have found that smokers' low-density lipoprotein (LDL) is more susceptible to oxidation and has higher levels of oxidized LDL. Given the various pathogenic effects of oxidized LDL, this would provide an essential causal pathway linking smoking to vascular disease. Smoking may increase oxidative stress by reducing antioxidant defense mechanisms as well as producing reactive oxygen radicals in the smoke. In this regard, a recent study found that cigarette smoke decreased paraoxonase enzyme activity (PON). This could also contribute to elevate LDL oxidation in smokers due to its antioxidant activity [162]. Table (3-6) shows that there was a significant difference in PON1 enzymatic activity between nonsmokers and smokers. The enzyme activity is high, and it's linked to HDL, as we've seen. The ability of HDL to attach to PON1 under specific experimental settings, such as the influence of oxidative stress or other variables, has been linked to PON1 enzymatic activity [163]. PON1 facts as side to have a key function in maintaining normal renal balance. It's also been suggested that it's a hydrolase agent delivered in the bloodstream by HDL and involved in homocysteine metabolism. As a result, any problems with this enzyme's activity are hazardous to kidney function because they disrupt metabolic pathways that are essential for normal kidney homeostasis [164]. Furthermore, PON1 activity in DM patients has been linked to microvascular problems [165]. Finally, PON1 activity link directly to the xenobiotic concentrations, as shown in figure (3-2).

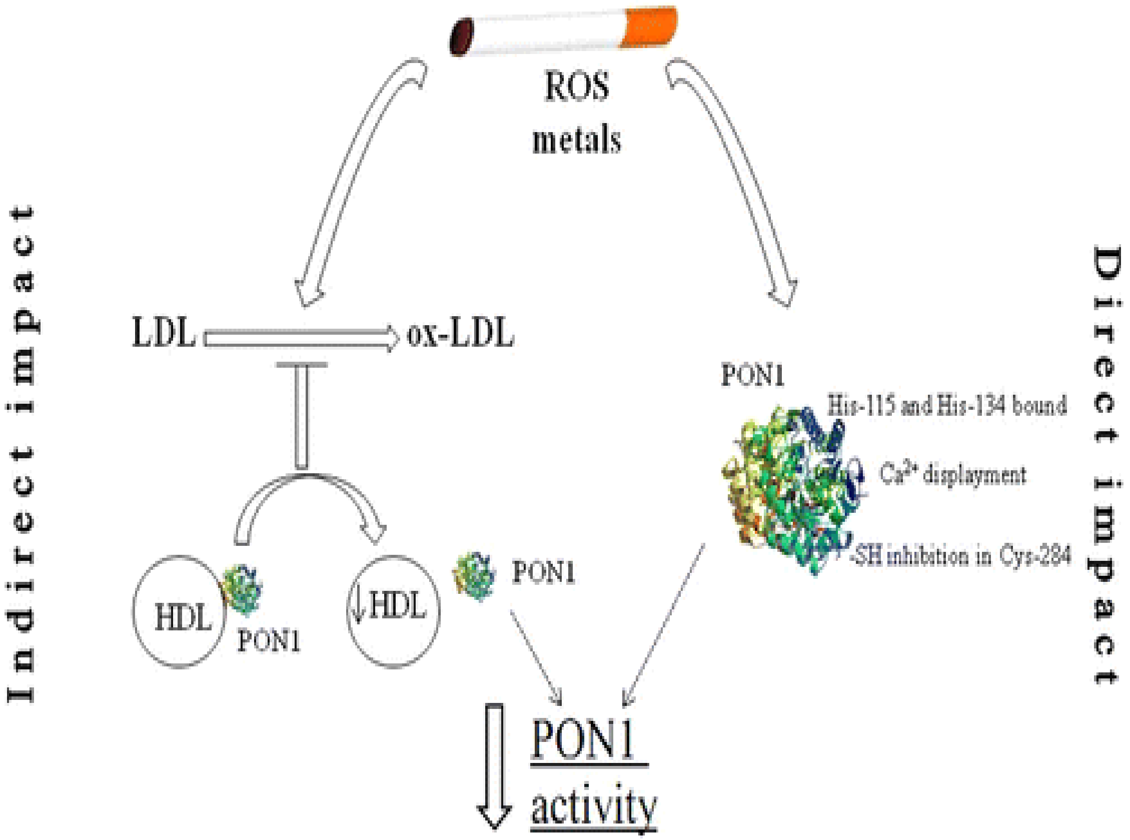


Figure out (3-2). Mechanism of xenobiotic in tobacco smoke interacting with PON1 activity. Cigarette smoking inhibits the activity of PON enzymes both directly and indirectly. Metals in tobacco smoke can interact with histidine's (His-115 and His-134), show calcium ions, and change free thiol groups in a direct way. Indirect effect: cigarette smoking lowers HDL levels, which in turn lowers PON1 activity, which is conjugated to HDL. PON stands for paraoxonase; HDL stands for high-density lipoprotein; LDL stands for low-density lipoprotein; ox-LDL stands for oxidized low-density lipoprotein; ROS stands for reactive oxygen species [166].

3.5. Homocysteine thiolactonase enzyme activity

The results in Table (3-7) is showed the concentrations of homocysteine thiolactonase activity that found in sera of non-smoker (NS) was 146.393 U/L, while in cigarette smoker (CS) was 104.636 U/L. Furthermore, the standard deviation in non-smoker, and cigarette smoker were 16.7082, 11.4193 U/L respectively.

Table (3-7): Homocysteine thiolactonase activity (U/L) in Sera of Cigarette Smokers and Nonsmokers Groups.

Group	N	Mean	Std. Deviation (SD)	Std. Error	95% Confidence Interval for Mean		Sign.
					Lower Bound	Upper Bound	
NS	150	146.393	16.7082	1.3642	119.436	173.35	0.024*
CS	150	104.636	11.4193	0.9323	86.212	123.06	

*. The mean difference is significant at the 0.05 level.

Hcy-thiolactone production and protein homocysteinylation in human vascular endothelial cells are influenced by hcy, methionine, folate, and high-density lipoprotein (HDL), all of which have been associated to vascular disease [167]. Hcy thiolacton is hydrolyzed to Hcy by Hcy thiolactonase (HTase), a component of HDL [168]. Hcy levels in the blood have also been shown to alter the stability and oxidability of low-density lipoprotein (LDL). Furthermore, macrophage-rich plaques have been found to contain larger quantities of oxidized LDL (ox-LDL) than macrophage-poor plaques, and have been linked to heightened levels of ox-LDL in plasma [169]. Paraoxonase is a multifunctional antioxidant enzyme that can detoxify paraoxon, degrade oxidized low-density lipoprotein (ox-LDL), and hydrolyze homocysteine thiolacton in addition to detoxifying paraoxon. Human paraoxonase, in fact, has a thiolactonase (HTase) activity that hydrolyzes Hcy thiolacton to Hcy [170]. The activity of Homocysteine thiolactose was shown to be strong in nonsmokers, but it was found to be less effective in smokers due to oxidative stress [171, 172].

3.6 Genotyping of Paraoxonase enzyme.

3.6.1 Amplification of Paraoxonase gene and designed primers using temperature gradient of PCR reaction.

Temperature is one of the most important factor in PCR because it has the limit of binding and denaturation of DNA amplification process, therefor this step recorded for discovering of the optimize condition of PCR **Fig (3-3).**

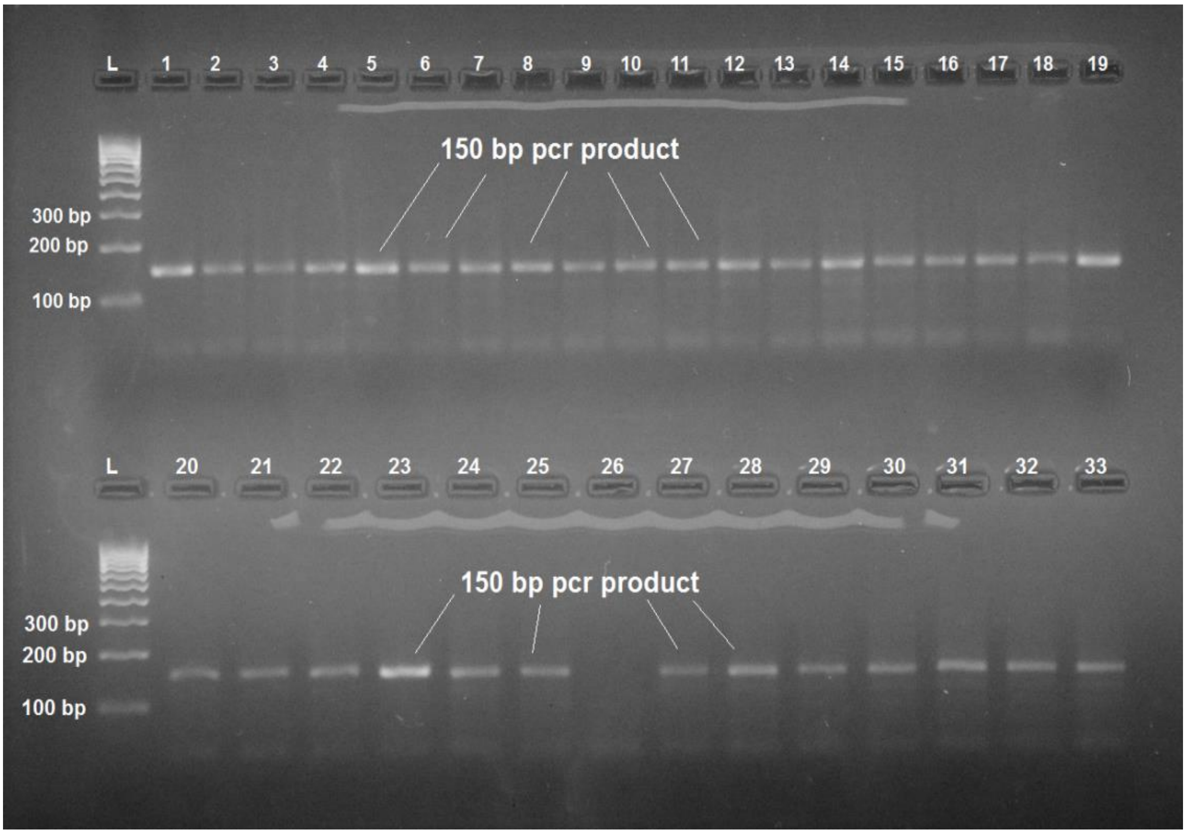


Figure (3-3): The electrophoresis pattern of PCR product for Paraoxonase gene.

3.6.2 Distribution of genotype and allele frequencies of Paraoxonase enzyme gene polymorphisms

The results showed that the present of different genotype, **A**= (74 +85) bp and **G** = (48+17+48+28) bp as revealed in **Fig** (3-4**).**

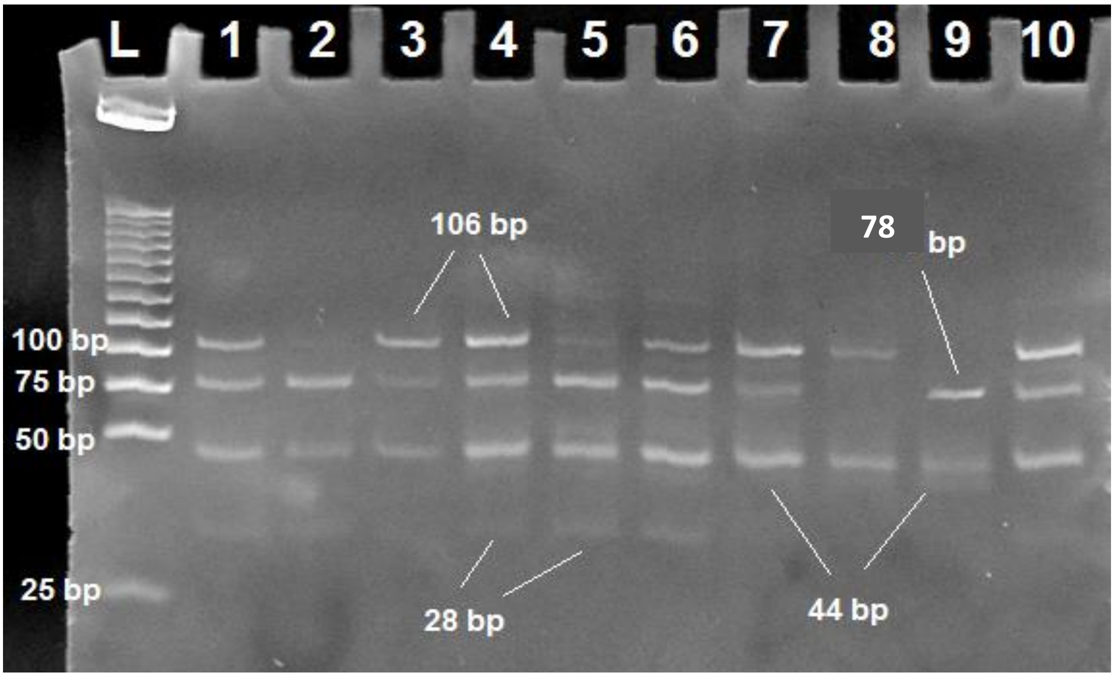


Figure (3-4): RFLP Patterns that electrophoresed on polyacrylamide gel electrophoresis for Paraoxonase gene. The (44) bp length fragment acting as a positive control for restriction enzyme.

Table (3-8) shows the distribution of genetic makeup and allele frequencies for the paraoxonase polymorphism (rs662) in smokers, non-smokers and in healthy controls. The genotype frequencies of HWE in both patients and healthy controls were for paraoxonase (rs662); There was the (Hardy-Weinberg equilibrium) which showed that the principle that genetic variation in a population will remain constant from generation to generation in the absence of disturbing factors, for example, mutations disrupt the balance of allele frequencies by introducing new alleles into the population. Significant differences were observed in Paraoxonase (rs662) polymorphism (Table 3-8 to 3-10).

Table (3-8) Allelic frequency of paraoxonase polymorphisms (rs662) and their associations with risks of cigarette smokers and non-smokers Groups.

	All subjects		non-smokers		cigarette smokers	
Allele	**Count**	**Proportion**	**Count**	**Proportion**	**Count**	**Proportion**
G	141	0.47	65	0.43	76	0.50
A	159	0.53	85	0.56	74	0.49

Table (3-8) shows the distribution of the genetic makeup and allele frequencies of the paraoxonase polymorphism (rs662) in smokers and non-smokers. The genotype frequencies of HWE in both patients and healthy controls were for paraoxonase (rs662); There was a Hardy-Weinberg equilibrium of paraoxonase (rs662). Non-significant differences were observed for the paraoxonase polymorphism (rs662) (Table 3-9) (Table 3-10).

Table (3-9) Genotypic association of Paraoxonase (rs662) in cigarette smokers and non-smokers Groups.

Model	Genotype	non-smokers	cigarette smokers	OR (95% CI)	P-value
Codominant	A/A	85 (56.7%)	74 (49.3%)	1.00	0.18
	A/G	48 (32%)	48 (32%)	1.15 (0.69-1.91)	
	G/G	17 (11.3%)	28 (18.7%)	1.89 (0.96-3.73)	
Dominant	A/A	85 (56.7%)	74 (49.3%)	1.00	0.2
	A/G-G/G	65 (43.3%)	76 (50.7%)	1.34 (0.85-2.12)	
Recessive	A/A-A/G	133 (88.7%)	122 (81.3%)	1.00	0.074
	G/G	17 (11.3%)	28 (18.7%)	1.80 (0.94-3.44)	
Overdominant	A/A-G/G	102 (68%)	102 (68%)	1.00	NA
	A/G	48 (32%)	48 (32%)	1.00 (0.62-1.62)	

Table (3-10) Hardy-Weinberg equilibrium test of Paraoxonase polymorphisms and their associations in cigarette smokers and non-smokers Groups.

	AA	**AG**	**GG**	**A**	**G**	**P-value***
All subjects	**159**	**96**	**45**	**414**	**186**	**<0.0001**
NS=1 non smoker	**85**	**48**	**17**	**218**	**82**	**0.023**
CS=2 smoker	**74**	**48**	**28**	**196**	**104**	**0.00051**

* two tailed p value of fisher's exact test.

To get a better understanding about the pathophysiological function of PON1, we analyzed the relationships between the Q192R polymorphism, serum paraoxonase activity and serum biomarkers important for diabetic nephropathy**.** Finally, table (3-11) shows the biochemical characteristics of subject studied according to the paraoxonase gene SNP (rs662) genotype. The results revealed that there are no significant associations between AA, AG and GG polymorphisms for diabetic patients, diabetic patients with nephropathy and control group. Additionally, the results of table (3-11) show a positive correlation between paraoxonase activity and antioxidants levels. On the other hand, there are a negative correlation with oxidative stress products.

Table (3-11) biochemical characteristics of the biochemical characteristics of subject studied according to the paraoxonase gene SNP (rs662) genotype.

Biochemical Characteristics	PON1 Q192R genotype	Smokers	Nonsmokers
PON1 activity (IU/L)	AA	121.07 ± 54.5089	138.783 ± 71.8056
	AG	142.248 ± 47.0081	138.992 ± 57.1336
	GG	124.903 ± 64.0146	196.039 ± 78.8191
	p	0.035	0.033
HDLc (mmol/L)	AA	0.90 ± 0.25a	1.00 ± 0.16
	AG	0.96 ± 0.23a	1.03 ± 0.33
	GG	0.98 ± 0.28	1.08 ± 0.26
	p	0.6	0.5
TAS	AA	0.63257 ± 0.19514	0.734 ± 0.24122
	AG	0.69667 ± 0.21846	0.70604 ± 0.18705
	GG	0.72214 ± 0.16271	0.73353 ± 0.19465
	p	0.23	0.17
TOS	AA	14.7565 ± 5.7048	12.3705 ± 4.62105
	AG	15.4381 ± 5.75318	13.0146 ± 4.22873
	GG	14.6636 ± 6.08487	12.71335 ± 3.71335
	p	0.6	0.31
HCTL	AA	101.362 ± 107.58	148.181 ± 178.086
	AG	124.488 ± 138.493	147.021 ± 150.693
	GG	79.2571 ± 67.6473	135.682 ± 107.142
	p	0.022	0.11

Single change in the location of amino acids (glutamine Q to arginine R) in the peptide sequence, without a doubt will alter the capability of substrate to bind with PON1 enzyme thus, a significant variation in PON1 enzymatic activities levels have been shown in this current study, as it shown in Fig (3-5).

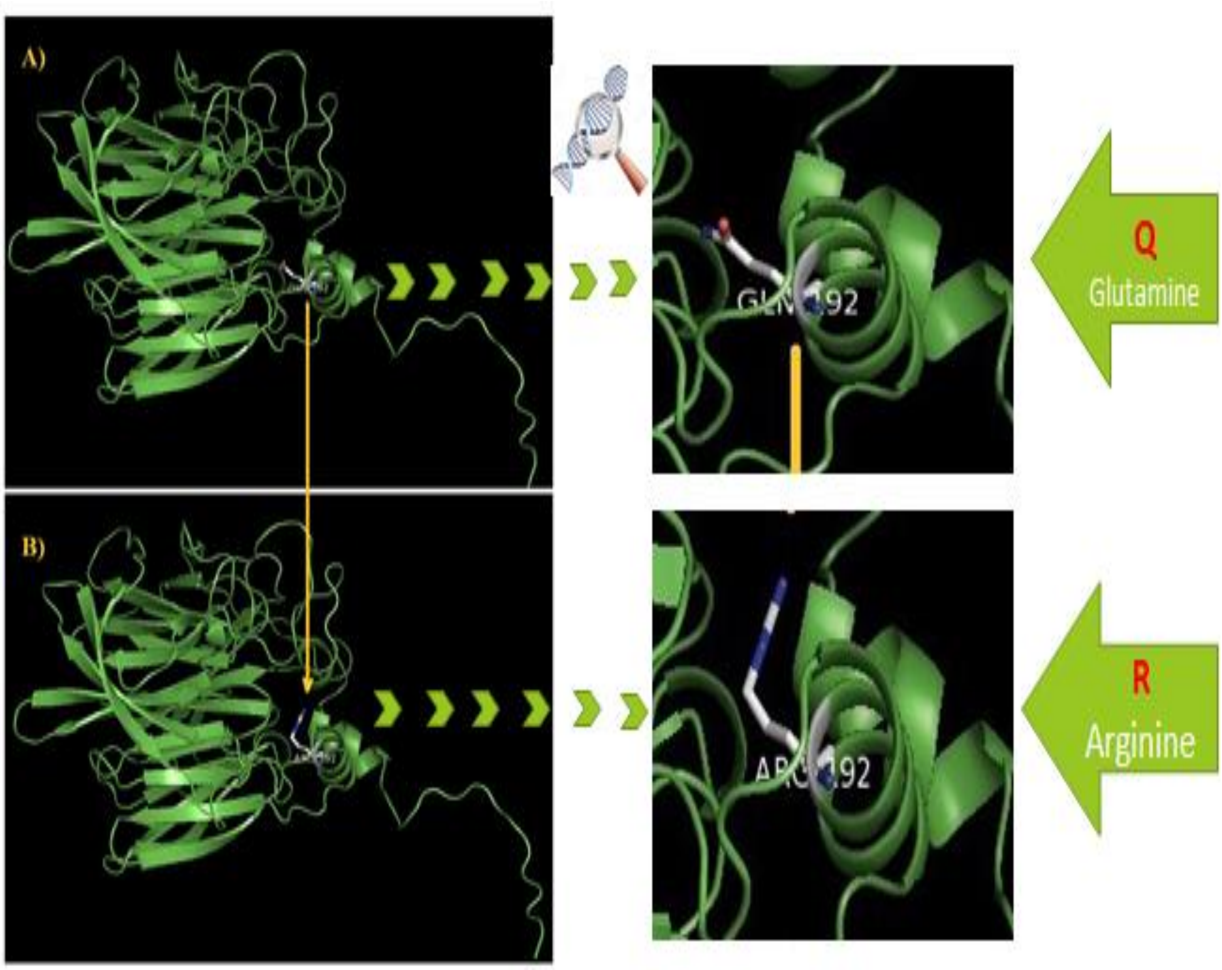


Figure (**3-5**): 3D Structure of human Paraoxonase, showing the effect of the genotype polymorphisms to the structure of the enzyme and the linking position with the HDL lipoprotein

3.6.3. Genotyping of PON1

PON1 (EC 3.1.8.1) is a calcium-dependent esterase found in plasma associated with high-density lipoprotein (HDL) and contributes to the lipoprotein's anti-oxidant impact [173]. PON1's antioxidant role has been broadened by some authors to include the protection of peroxidative damage to cell membranes in general [173]. The large variance in serum PON1 activity (40-fold) seen among people, however, makes clinical interpretation difficult. Both genetic and environmental factors can account for this variation [174]. The paraoxonase gene's promoter and coding regions are the primary determinants of its expression and enzymatic activity [175,176]. However, various environmental factors can influence serum paraoxonase activity. Reduced paraoxonase activity has been linked to pathologic conditions such as renal illness, diabetes mellitus, cardiovascular disease, and liver cirrhosis, and numerous dietary and lifestyle factors have been linked to serum paraoxonase activity. Tobacco smoking has been linked to lower PON1 activity in patients with coronary artery disease [177], and cigarette smoke extracts have been shown to suppress PON1 activity in vitro [178]. Simvastatin [179] and hormone replacement treatment [180] are two examples of pharmacologic therapy. On the other hand, has been shown to boost PON1 activity in the blood. Individual differences in paraoxonase activity are explained in part by genetic variation in the paraoxonase gene's coding region, which results in arginine substitution for glutamine at position 192 (Q192R) and methionine substitution for leucine at position 55 (M/L55). The M/L55 polymorphism is also a strong predictor of paraoxonase serum levels [181]. Several case–control studies have found a positive link between the PON1 R192 allele and coronary heart disease, while others have found no link [182]. In smokers, previous studies have shown a decrease in PON1 activity and HDLc concentration, as well as an increase in TC, TG, LDLc, Lp (a), and the ApoB/ApoA1 ratio. Smoking has long been linked to coronary artery disease and other vascular problems. A change in plasma lipid profile has been linked to the development of cardiovascular disease in smokers. The processes for the altered lipid profile in smokers are recalled in this context [183]. First, nicotine promotes adrenaline release from the adrenal cortex, resulting in an increase in blood free fatty acid concentrations, which stimulates hepatic cholesterol synthesis and secretion, as well as hepatic secretion of very low-density lipoprotein (VLDL) and hence increased TG [181]. Second, smoking lowers estrogen levels, which leads to a reduction in HDLc levels [181]. In addition, HDL levels in the blood were inversely associated to VLDL levels in the blood. Finally, smoking produces hyperinsulinemia by increasing insulin resistance. Because lipoprotein lipase activity is reduced in hyperinsulinemic circumstances, LDL and TG levels are raised. Furthermore, human serum paraoxonase has been proven to be a polymorph enzyme [181]. One of the diseases that is caused by a mix of hereditary and environmental variables is cancer [184]. The PON1 gene is an antioxidant enzyme with significant lipophilic antioxidant characteristics that helps maintain the antioxidant-oxidant equilibrium [185]. It is found on the long arm of chromosome 7q21.3. PON1 is also an esterase that binds to high-density lipoprotein and helps to scavenge reactive oxygen species (HDL). According to research, oxidative stress can contribute to cell proliferation and malignant transformation, as well as DNA damage and other biological components, resulting in tumor formation [186]. Researchers can predict PON1 gene polymorphism in cancer susceptibility because of PON1's ability to detoxify carcinogenic oxidative stress metabolites. PON1-Q192R and PON1-L55M, the two most prevalent functional genetic polymorphisms in PON1, have been identified at positions 192 and 55, respectively, as a result of the deep development of genetic investigations of PON1 [187]. The glutamine (Q genotype) was swapped for the arginine (R genotype) 192 of the gene 6 exon of the PON 1 gene, resulting in the PON1-Q192R polymorphism (rs662A > G). PON1-L55M (rs854560) was created when 55 leucine's (L genotype) were replaced with methionine (M genotype) at third exon 55 [188]. Furthermore, the two functional SNPs Q192R and L55M have been linked to the risk of a variety of malignancies, including mouth cancer, lung cancer, and embryonal tumors [188]. Given the importance of PON1 in tumor development and the link between genotype and phenotype, we hypothesize that the PON1 gene variants Q192 R and L55M are linked to tumor susceptibility. However, many research' findings are now inconsistent and ambiguous. As a result, a comprehensive meta-analysis of the link between the Q192R and L55M polymorphisms and cancer risk should be done [188]. Human paraoxonase 1 (PON1) is a gene that has been linked to a genetic vulnerability to coronary artery disease (CAD). In the PON1 gene, there are two frequent DNA polymorphisms. The distance between these polymorphisms is only 8.6 kb (GenBank accession number AC004022). Because of a leucine (L allele) and methionine (M allele) swap, one is found at amino acid position 55. The second is owing to a glutamine (Q allele) and arginine (R allele) exchange at amino acid position 192. In certain but not all populations, the 192R allele has been linked to a higher risk of coronary artery disease. The issue with the PON1 L55M polymorphism and cardiovascular risk is perplexing [189]. It is anesterase enzyme that metabolizes a variety of substrates, including pharmaceuticals and organophosphorus (OPs) chemicals [190]. At locations Gln192g Arg (Q192R) and Leu55g Met in the PON1 coding area, there are two polymorphisms (L55M). Because the two alloenzymes have varied affinities and catalytic activity toward a variety of substrates, Q192R has received more attention. Polymorphisms in the PON1 gene have been shown to alter PON1 activity in studies. Eckerson et al [191] conducted a study. PON1 activity was shown to be lower in PON1 192 Q allele carriers than in R allele carriers. PON1 activity has been found to be reduced in several cancers, such as gastric and pancreatic carcinoma. Although the link between PON1 polymorphisms and serum activity has been discovered in a variety of malignant tumors, including cancers of the ovary, lung, and liver [192]. The interaction between PON1 and ARE activity and CRC has not been well researched, and just a few published articles have looked into the link between PON1 Q192R and L55M and CRC [193]. The activity of paraoxonase (PON), which has the ability to breakdown lipid peroxides, is primarily responsible for HDL's antioxidant function. The human PON1 gene is found on the long arm of chromosome 7 between q21.3 and q22.1 [194]. PON1, a calcium-dependent antioxidant glycoprotein with a molecular weight of 43 kDa and one of the HDL structures, is one of the HDL structures. There are two types of polymorphisms in the PON1 coding region: L55M (163T > A) and Q192R (575A > G). Codon 55 leucine (L) was substituted by methionine (M) in L55M. (M). Glutamate (G) was substituted by arginine (R) at codon 192 in Q192R [195]. The 192 polymorphism is the most important predictor of PON1 activity, however the 55 polymorphism can also influence it. Although several case–control studies have been conducted to investigate the link between PON1 55M or 192R alleles and cardiovascular disease, the results have been inconsistent. Some researchers have discovered a link between the PON1 gene polymorphism and heart disease, whereas others have not [196]. Similarly, replacing leucine (L genotype) with methionine (M genotype) in position 55 results in the second polymorphism rs854560 (L55M). Homozygous QQ is the most frequent genotype, followed by heterozygous QR, and homozygous RR is the least common. The protein expressed by R has an 8-fold higher hydrolyzing activity than the protein encoded by Q. The serum protein concentration is also affected by this mutation. Individuals with homozygous RR alleles have higher enzyme concentrations than those with homozygous QQ alleles [197]. In addition to the polymorphisms at these locations, the PON1 promoter region has five polymorphisms. PON1 activity can vary by up to 40-fold between individuals and populations due to these genetic variations [198]. The A allele encodes a low-activity version of PON1 at the PON1 rs662 (also known as c.575A N G, Q192R, or p. Gln192Arg) locus (PON1 Arg192). The AG or G allele, on the other hand, is linked to the high-activity PON1 isoform (PON1 Gln192) [199]. PON1 Gln192 accelerates the hydrolysis of paraoxon, whereas PON1 Arg192 accelerates the hydrolysis of diazoxon, sarin, and soman (L55M) SNVs have no effect on the interaction of PON1 with substrates [200].

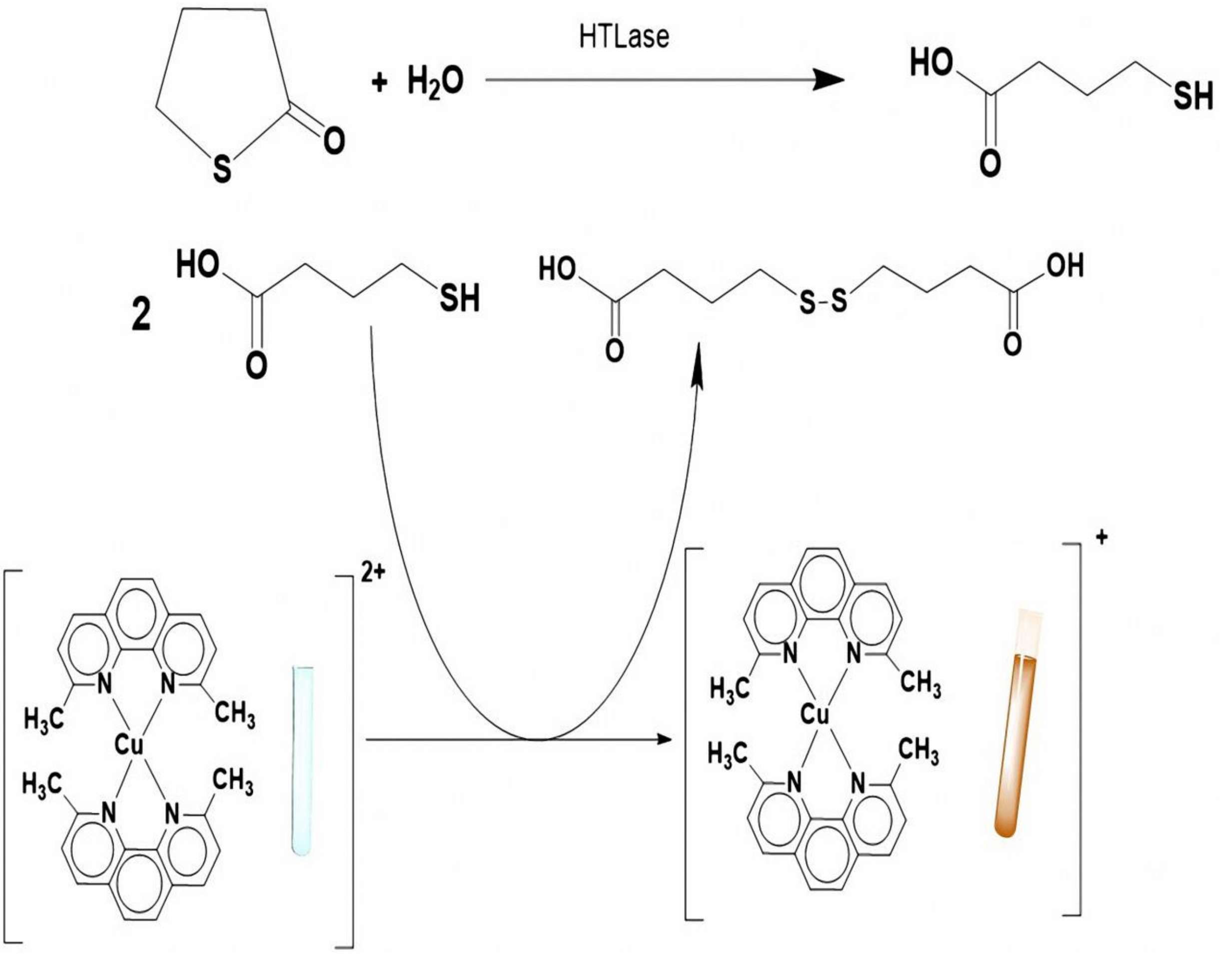
3.7. A quantitative method for assessment of homocysteine thiolactonase enzyme activity

3.7.1. Results and discussion

3.7.2. CUPRAC method as a suitable probe to assess HTase activity

The current study elucidates a simple protocol to assay HTLase activity in biological samples using the cupric neocuproine complex ($Cu(Nc)_2^{2+}$) as a chromogenic oxidizing reagent (CUPRAC method). The CUPRAC method was developed by Apace *et al.* [201] to measure antioxidant capacity. HTLase hydrolyzes substrates (homocysteine thiolactone (HTL) or γ-thiobutyrolactone (TBL)) back to homocysteine or 4-mercaptobutyric acid, respectively. The present protocol was dependent upon the reduction of ($Cu(Nc)_2^{2+}$) to highly colored Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$) by the produced 4-mercaptobutyric acid, and the result was quantified spectrophotometrically at 450 nm. The increment of absorbance being associated with the HTLase activity. The current assay is based on γ-thiobutyrolactone (TBL) substrate to estimate the HTLase activity, as shown in Scheme (1).

The formation of Cu(I)-neocuproine complex ($Cu(Nc)_2^+$) produced a single peak at 450 nm, and the absorbance was associated directly with the produced 4-mercaptobutyric acid levels after the completion of the HTLase enzyme reaction (Figure 3-6). The Units definition is the number of micromoles of homocysteine produced (i.e., substrate hydrolyzed) per unit time.



Scheme (1): Scheme of γ-thiobutyrolactone (TBL) hydrolases by homocysteine thiolactonase (HTLase). The reaction of Cu(II)-neocuproine complex with 4-mercaptobutyric acid, producing the yellow-orange colored Cu(I)-neocuproine chelate (λmax = 450 nm).

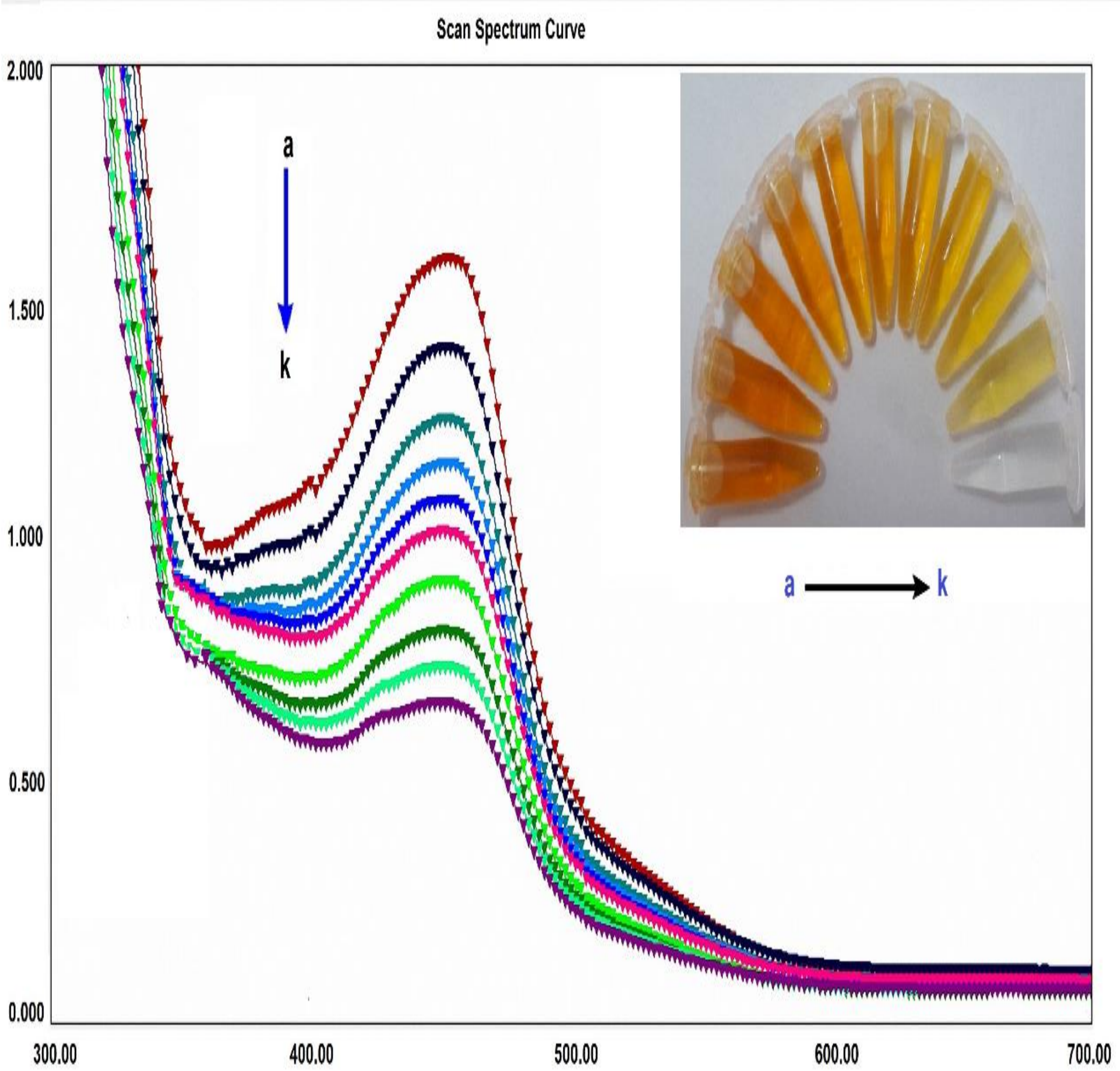


Figure (3-6). Homocysteine thiolactonase (HTLase) enzyme activity correlated with the spectrophotometric properties of the Cu(I)-neocuproine complex ($(Cu(Nc)_2^+)$). Absorption spectra achieved by the reduction of ($Cu(Nc)_2^{2+}$) to highly colored Cu(I)-neocuproine complex ($Cu(Nc)_2^+$) as a result to the libration 4-mercaptobutyric acid from the hydrolysis of γ-thiobutyrolactone by HTLase activity, and the result was measurement spectrophotometrically at 450 nm: (a to k) represent (800, 700, 600, 550, 500, 450, 400, 350, 300, 250) μM 4-mercaptobutyric acid.

3.7.3. Accuracy and selectivity

The current protocols used to assess HTase activity are subject to interference by various biomolecules such as sugars, amino acids, and proteins. Thus, to determine the interference of such organic compounds, we used 4-mercaptobutyric acid concentrations in four volumetric flasks according to the method described above (see 2.7). The assay recovery results for each possible interfering biochemical mixture are shown in Table 3-12, demonstrating that the current protocol is appropriate for assessing HTase enzyme activity in the presence of these interfering biomolecules.

The present assay uses a correction factor (control-test) to exclude the interference that arises from the presence of organic biomolecules in the sample that contains HTase enzyme activity. The absorbance of the test tube in the procedure is related to two types of compounds: produced 4-mercaptobutyric and interferences found in serum. The absorbance of the control test tube in the procedure relates to interference compounds found only in the serum. By subtracting the absorbance of the control test tube from the absorbance of the test tube, we eliminate the interference of any compound that may reduce Cu(II)-neocuproine complex ($Cu(Nc)_2^{2+}$) to highly colored Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$). That means the remaining absorbance belongs to produced 4-mercaptobutyric only.

Table 3-12. The recovery values obtained for each of the potential interfering biochemical mixtures during the assessment of HTase enzyme activity 4-mercaptobutyric acid using the current protocol.

	Added 4-mercaptobutyric acid concentration µmol/L	Found 4-mercaptobutyric acid concentration µmol/L	Recovery%
Volumetric flask 1	400	400	100
Volumetric flask 2	400	398	99.5
Volumetric flask 3	400	405	98.7
Volumetric flask 4	400	404	99

3.7.4. Sensitivity and linearity

The CUPRAC method was linear ($y = 0.9546x + 14.331$) within the range of 5–1000 U. L^{-1} of HTase activity (Pearson's $r = 0.999$), as described in Fig (3-7). The high level of sensitivity of the HTase assay was confirmed by obtained values of LOQ (6U. L^{-1}) and LOD (2 U. L^{-1}). Linearity of the present protocol was similar to the H^+ ions liberating method.

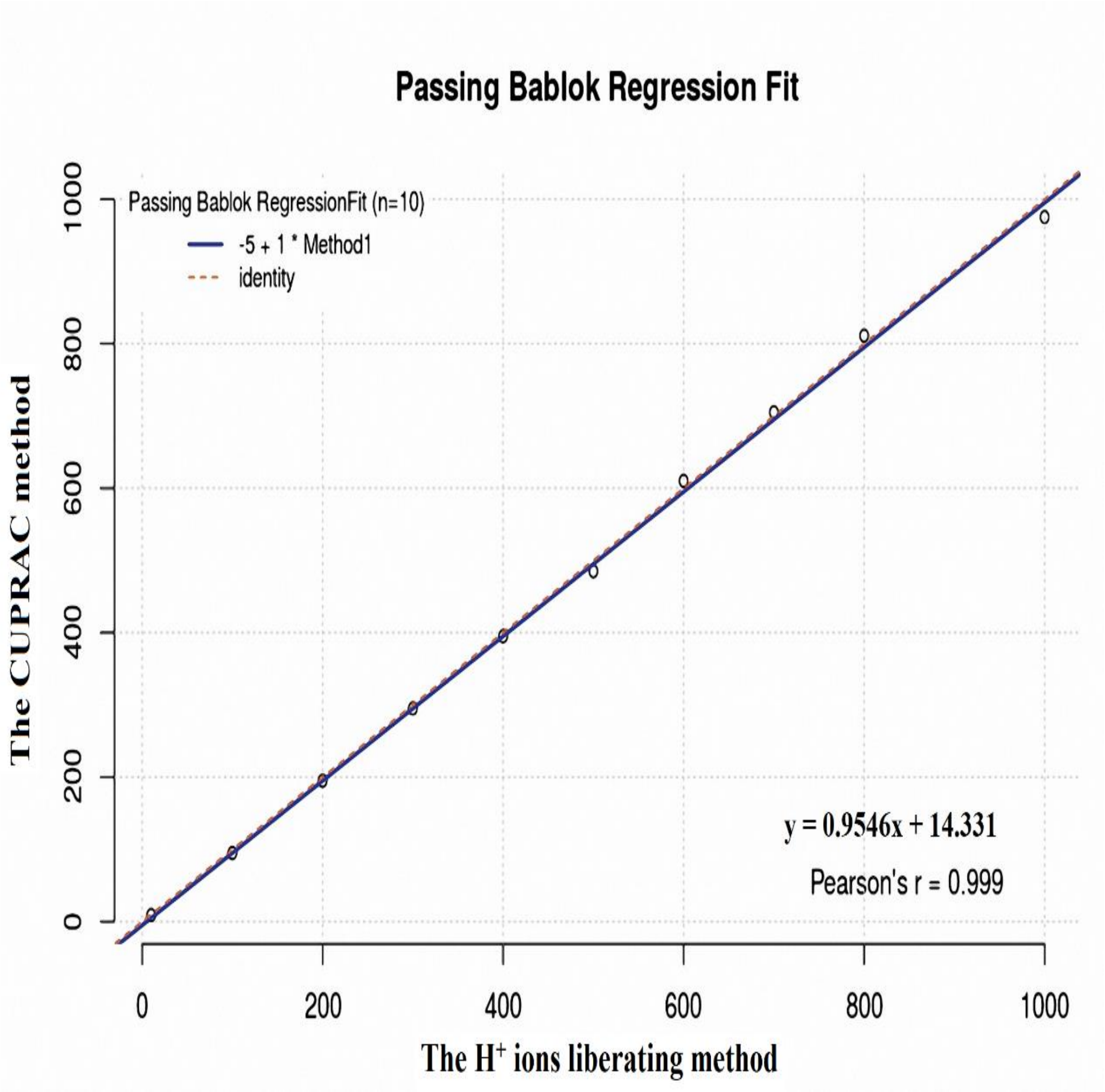


Fig (3-7). Linearity of the current protocol for a series of dilutions of HTLase activity achieved by plotting a straight line between the H^+ ions liberating method and CUPRAC method.

Using the Passing-Bablok correlation analysis, a strong correlation was found between the H^+ ions liberating and CUPRAC methods (Fig. 3-7). The Pearson correlation between the H^+ ions liberating and the CUPRAC methods for the various samples was established, with a Pearson $r > 0.99$.

3.7.5. Validation and reproducibility

Bland-Altman plot analyses were used to verify the current CUPRAC protocol by comparing HTase enzyme activity assessed by H^+ ions liberating method as documented by Billecke *et al*. [129] using matched enzymatic samples. The Bland-Altman plot was used to determine the limits of agreement between the two quantitative methods [202] and was also used to verify the characteristics of the methods and the assumptions of normality of differences between the investigated methods using a graphical approach. The resulting graph is an XY scatter diagram, with the x-axis representing the mean of the two methods' results ([A - B]/2) and the y-axis representing the absolute difference between them ([B - A]). According to Bland and Altman, 95% of the result points could equal ± 1.96 SD of the mean difference. The correlation coefficient between the two methods was 0.9996, showing that the current method is comparable to the reference method (as shown in Fig. 3-8).

Bland-Altman plot (Fig. 3-9) proves the relative difference between the H^+ ions liberating and CUPRAC methods, with the mean relative bias.

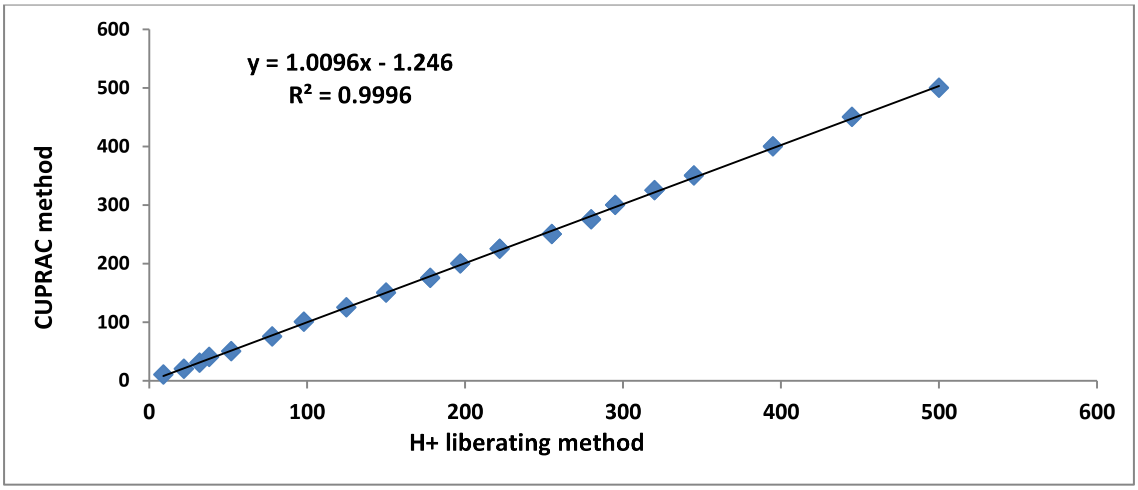


Fig (3-8). HTase enzyme activity was measured over a range of HTase enzyme dilutions, using the H^+ ions liberating and CUPRAC methods.

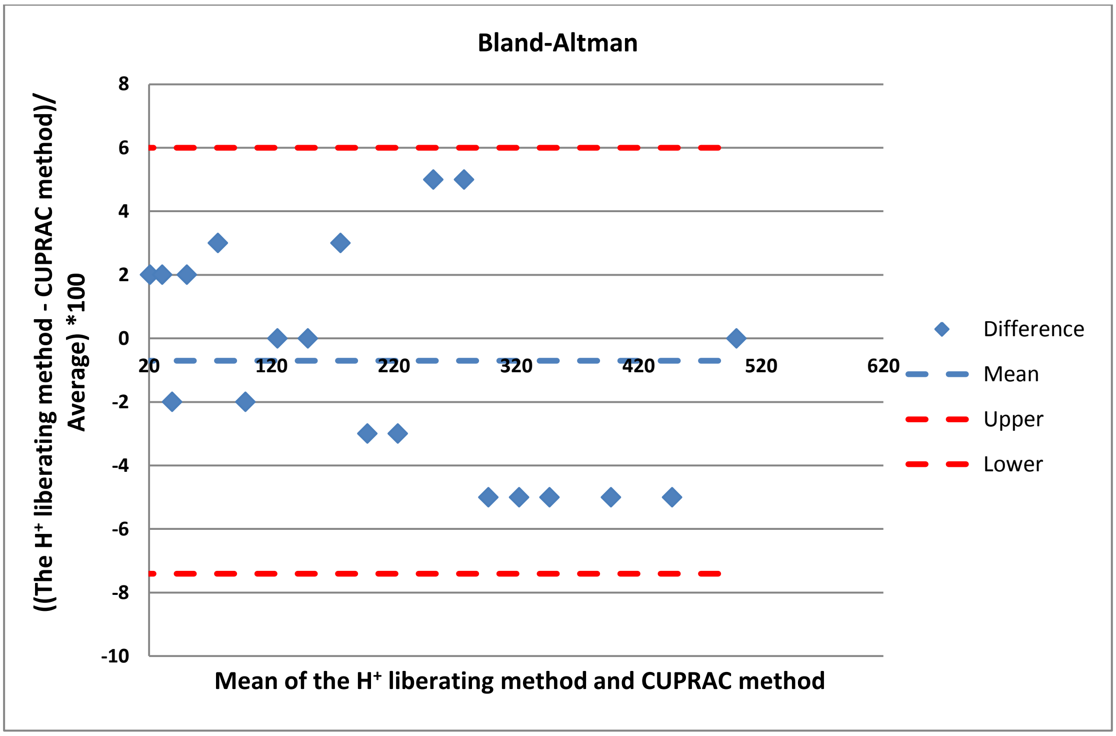


Fig (3-9). Bland-Altman plot indicating the relative difference between the H^+ ions liberating and CUPRAC methods, with the mean relative bias.

There are many publications on measuring HTase activity. Most articles assessed HTLase activity by using the method of liberating H^+ ions during thiolactone hydrolysis to homocysteine as a function to the homocysteine thiolactonase activity [129]. There are two disadvantages to this approach. The first is its reliance on phenol red to determine the H^+ ions liberated from thiolactone hydrolysis. Phenol red is a pH indicator that is very sensitive to the change in H^+ ion concentration [203], so it is necessary to prepare an appropriate buffer solution to be used in the assessment of enzymatic activity and to obtain reliable results. The second disadvantage relates to preparation of a precise standard HCl titration curve to calculate the H^+ ions liberated from thiolactone hydrolysis. This is because hydrochloric acid is not suitable for use as a primary standard solution [204].

The second useful method for assessing HTase activity depends upon Ellman's reagent to monitor thiolactone hydrolysis spectrophotometrically. The method is based on the increase of absorbance at 405nm when DTNB is reduced to a yellow thionitrophenylate anion (5-mercapto-2-nitrobenzoic acid) by a thiol-generated group at neutral and alkaline pH [130]. The first disadvantage of the spectrophotometric method is linked to the ideal pH of the reaction. Yellow thionitrophenylate anion has minimum stability at pH7, whereas almost 100% stability occurred at alkaline pH [205]. Although alkaline pH promotes paraoxonase/HTase activity, Hcy-thiolactone is unstable in alkaline solutions, so the HTLase assay was performed at pH 7.0. [206]. Also, a number of studies indicated that Ellman's reagent is relatively unstable [207, 208]. Finally, DTNB interacts with free sulfhydryl groups in the sample [208] and thus may affect HTLase activity.

3.8. Conclusions

This paper describes a simple method for assessing the activity of the HTase enzyme activity, which can be completed in only a few steps. This method allows the measurement of HTase enzyme activity in biological samples that contain high concentrations of vitamins, amino acids, proteins, antioxidants, or other interfering substances, as well as low concentrations of substrate. Utilizing the reduction of Cu(II)-neocuproine complex ($Cu(Nc)_2^{2+}$) to highly colored Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$) by the produced 4-mercaptobutyric acid provided an ideal approach to assaying HTLase activity, as the increment of absorbance correlated with HTLase activity.

Conclusion

It is concluded that: -

1- This describesa simplemethod forassessing the activity ofthe HTase enzymes activity which canbe completed inonly a fewsteps. This method allowsthe measurement ofHTase enzyme activityin biological samplesthat containhigh concentrationsof vitamins, amino acids, proteins,antioxidants, or other interfering substances, as well as lowconcentrations substrate.Utilizing the reduction ofCu(II)-neocuproine complex (Cu(Nc)22+) tohighly colored Cu(I)-neocuproine complex (Cu(Nc)2+)by the produced 4-mercaptobutyric acid provided an ideal approach to assaying HTLase activity, as the increment of absorbance correlatedwith HTLase activity.

2- Smoking complications are associated with increment reactive oxygen species concentration and decrement total antioxidant levels. The individuals' TOS and TAS levels were assessed. While cigarette smokers' serum total antioxidant capacity was considerably lower than that of non-smokers. These findings point to oxidative stress and a smoker's oxidant defense mechanism being compromised.

3- The complications of smoking are associated with an increase in lipid peroxide concentration in smokers compared to non-smokers, and consequently an increase in lipid peroxide concentration, which leads to high oxidative stress due to the formation of free radicals and the emergence of more pathological conditions in smokers.

4- The results of the current clinic study observed low levels of paraoxonase activities PON1 and Lactonase (LAC) in smokers and non-smokers. A decrease in the concentration of paraoxonase activity was observed for smokers who are more susceptible to oxidative stress. Low-density lipoprotein (LDL) smokers are more susceptible to oxidation and have higher levels of oxidized LDL, according to. Given the many negative effects of oxidized LDL, this would be an important link between smoking and vascular disease. Smoking may increase oxidative stress by reducing antioxidant defense mechanisms as well as the production of reactive oxygen radicals in smoke. These findings suggest a possible adaptive response that may be attributed to the elevated formation of ROS. This mechanism may require higher levels of antioxidant enzymes to protect tissues from the increased level of oxidative stress in relation to metabolic conditions.

5- The present genetic study observed non-significant differences in the distribution of the PON1 192 genotype with allele frequencies [Q/R variation of the PON1 gene (rs662) between smokers and non-smokers. obtained, the Q to R substitution at position 192 is activity-limiting since the R allele codons for the protein with paraoxon hydrolases activity several times greater than in QR genotypes and least activities in QQ genotypes.

Recommendations

The study recommends the following: -

1- Studies related to the enzyme Paraoxonase in general are few and because it is one of the most important enzymes that fight free radicals, we recommend intensifying the study to know the important roles of this enzyme more broadly.

2- In the current study, the focus was on the relationship of the enzyme homocysteine thiolactonase with smoking, so it is important to focus on studying the effect of other sources of free radicals on the work of the enzyme.

3- Study the key role of paraoxonase gene expression in smokers and non-smokers.

4- Establishing programs to encourage students to quit smoking.

References

References:

1. Skalis G, Archontakis S, Thomopoulos C, Andrianopoulou I, Papazachou O, Vamvakou G, Aznaouridis K, Katsi V, Makris T. A single-center, prospective, observational study on maternal smoking during pregnancy in Greece: The HELENA study. Tobacco Prevention & Cessation. 2021;7.
2. St Claire S, Gouda H, Schotte K, Fayokun R, Fu D, Varghese C, Prasad VM. Lung health, tobacco, and related products: gaps, challenges, new threats, and suggested research. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2020 May 1;318(5):L1004-7.
3. Harutyunyan A, Movsisyan N, Petrosyan V, Petrosyan D, Stillman F. Reducing children’s exposure to secondhand smoke at home: a randomized trial. Pediatrics. 2013 Dec 1;132(6):1071-80.
4. Turner MC, Andersen ZJ, Baccarelli A, Diver WR, Gapstur SM, Pope III CA, Prada D, Samet J, Thurston G, Cohen A. Outdoor air pollution and cancer: An overview of the current evidence and public health recommendations. CA: a cancer journal for clinicians. 2020 Nov;70(6):460-79.
5. Goniewicz ML, Smith DM, Edwards KC, Blount BC, Caldwell KL, Feng J, Wang L, Christensen C, Ambrose B, Borek N, van Bemmel D. Comparison of nicotine and toxicant exposure in users of electronic cigarettes and combustible cigarettes. JAMA network open. 2018 Dec 7;1(8): e185937-.
6. Rahman Z, Singh VP. The relative impact of toxic heavy metals (THMs) (arsenic (As), cadmium (Cd), chromium (Cr)(VI), mercury (Hg), and lead (Pb)) on the total environment: an overview. Environmental monitoring and assessment. 2019 Jul;191(7):1-21.
7. Ali H, Khan E. Trophic transfer, bioaccumulation, and biomagnification of non-essential hazardous heavy metals and metalloids in food chains/webs—Concepts and implications for wildlife and human health. Human and Ecological Risk Assessment: An International Journal. 2019 Aug 18;25(6):1353-76.
8. Anyanwu BO, Orisakwe OE. Current mechanistic perspectives on male reproductive toxicity induced by heavy metals. Journal of Environmental Science and Health, Part C. 2020 Jul 2;38(3):204-44.

9. El-Mahdy MA, Mahgoup EM, Ewees MG, Eid MS, Abdelghany TM, Zweier JL. Long-term electronic cigarette exposure induces cardiovascular dysfunction similar to tobacco cigarettes: role of nicotine and exposure duration. American Journal of Physiology-Heart and Circulatory Physiology. 2021 May 1;320(5):H2112-29.
10. Lama D, Prajapati B, Shrestha N, Kunwar S, Jaiswal S, Thakur RK. Hemoglobin and C-reactive protein level among smoker and non-smoker individuals of Kathmandu valley, Nepal. World Journal of Biology Pharmacy and Health Sciences. 2020;3(1):017-23.
11. Thirumalai T. Cigarette Smoke-induced Oxidative Stress: A Systematic Review. BR Nahata Smriti Sansthan International Journal of Phramaceutical Sciences & Clinical Research. 2021 Aug 12;1(2).
12. Grzybowski A, Och M, Kanclerz P, Leffler C, Moraes CG. Primary open angle glaucoma and vascular risk factors: a review of population based studies from 1990 to 2019. Journal of clinical medicine. 2020 Mar;9(3):761.
13. Antoni S, Ferlay J, Soerjomataram I, Znaor A, Jemal A, Bray F. Bladder cancer incidence and mortality: a global overview and recent trends. European urology. 2017 Jan 1;71(1):96-108.
14. Olson S, Anderson KM. Immigration as a social determinant of health. National Academy of Sciences. 2018.
15. Myers PL, editor. Tobacco use and ethnicity. Routledge; 2018 Oct 24.
16. Routledge K. The Mystery of Easter Island. Lulu. com; 2020 Apr 12.
17. World Health Organization. Tobacco and its environmental impact: an overview. World Health Organization; 2017.
18. Daenen K, Andries A, Mekahli D, Van Schepdael A, Jouret F, Bammens B. Oxidative stress in chronic kidney disease. Pediatric nephrology. 2019 Jun;34(6):975-91.
19. Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. Toxicology. 2011 May 10;283(2-3):65-87.
20. Alrouji M, Manouchehrinia A, Gran B, Constantinescu CS. Effects of cigarette smoke on immunity, neuroinflammation and multiple sclerosis. Journal of neuroimmunology. 2019 Apr 15;329:24-34.
21. Checa J, Aran JM. Airway redox homeostasis and inflammation gone awry: from molecular pathogenesis to emerging therapeutics in respiratory pathology. International Journal of Molecular Sciences. 2020 Jan;21(23):9317.

22. Mittler R. ROS are good. Trends in plant science. 2017 Jan 1;22(1):11-9.
23. Gruhlke MC, Slusarenko AJ. The biology of reactive sulfur species (RSS). Plant Physiology and Biochemistry. 2012 Oct 1;59:98-107.
24. Mohanlal SM. Phytochemical Investigations on 'Black Glumed'njavara (Oryza sativa l.), the Medicinal Rice, as Compared to Staple Varieties and Evaluation of their Antioxidant, Anti-inflammatory and Anticancer Effects. Pennsylvania State University: Citeseer. 2011 Dec 19.
25. Choudhury FK, Rivero RM, Blumwald E, Mittler R. Reactive oxygen species, abiotic stress and stress combination. The Plant Journal. 2017 Jun;90(5):856-67.
26. Desai N. Challenges in development of nanoparticle-based therapeutics. The AAPS journal. 2012 Jun;14(2):282-95.
27. Kehrer JP, Klotz LO. Free radicals and related reactive species as mediators of tissue injury and disease: implications for health. Critical reviews in toxicology. 2015 Oct 21;45(9):765-98.
28. Foyer CH, Shigeoka S. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant physiology. 2011 Jan 1;155(1):93-100.
29. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Current biology. 2014 May 19;24(10):R453-62.
30. Maqsoudlou A, Assadpour E, Mohebodini H, Jafari SM. Improving the efficiency of natural antioxidant compounds via different nanocarriers. Advances in colloid and interface science. 2020 Apr 1;278:102122.
31. Alkadi H. A review on free radicals and antioxidants. Infectious Disorders-Drug Targets (Formerly Current Drug Targets-Infectious Disorders). 2020 Feb 1;20(1):16-26.
32. Maia LB, Moura JJ. How biology handles nitrite. Chemical reviews. 2014 May 28;114(10):97.
33. da Costa Silva T, Justino AB, Prado DG, Koch GA, Martins MM, de Souza Santos P, de Morais SA, Goulart LR, Cunha LC, de Sousa RM, Espindola FS. Chemical composition, antioxidant activity and inhibitory capacity of α-amylase, α-glucosidase, lipase and non-enzymatic glycation, in vitro, of the leaves of Cassia bakeriana Craib. Industrial Crops and Products. 2019 Nov 15;140:111641.

34. Wen C, Zhang J, Zhang H, Duan Y, Ma H. Plant protein-derived antioxidant peptides: Isolation, identification, mechanism of action and application in food systems: A review. Trends in Food Science & Technology. 2020 Sep 23.
35. Mirończuk-Chodakowska I, Witkowska AM, Zujko ME. Endogenous non-enzymatic antioxidants in the human body. Advances in medical sciences. 2018 Mar 1;63(1):68-78.
36. Maes M, Bonifacio KL, Morelli NR, Vargas HO, Moreira EG, Stoyanov DS, Barbosa DS, Carvalho AF, Nunes SO. Generalized anxiety disorder (GAD) and comorbid major depression with GAD are characterized by enhanced nitro-oxidative stress, increased lipid peroxidation, and lowered lipid-associated antioxidant defenses. Neurotoxicity research. 2018 Oct;34(3):489-510.
37. Islam MR, Ali S, Karmoker JR, Kadir MF, Ahmed MU, Nahar Z, Islam SM, Islam MS, Hasnat A, Islam MS. Evaluation of serum amino acids and non-enzymatic antioxidants in drug-naïve first-episode major depressive disorder. BMC psychiatry. 2020 Dec;20(1):1-8.
38. Kumari S, Badana AK, Malla R. Reactive oxygen species: a key constituent in cancer survival. Biomarker insights. 2018 Feb 6;13:1177271918755391.
39. Ighodaro OM, Akinloye OA. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria journal of medicine. 2018;54(4):287-93.
40. Csiszár J, Horváth E, Bela K, Gallé Á. Glutathione-related enzyme system: glutathione reductase (GR), glutathione transferases (GSTs) and glutathione peroxidases (GPXs). InRedox State as a Central Regulator of Plant-Cell Stress Responses 2016 (pp. 137-158). Springer, Cham.
41. Tan BL, Norhaizan ME, Liew WP, Sulaiman Rahman H. Antioxidant and oxidative stress: a mutual interplay in age-related diseases. Frontiers in pharmacology. 2018 Oct 16;9:1162.
42. Mehta SK, Gowder SJ. Members of antioxidant machinery and their functions. Basic principles and clinical significance of oxidative stress. 2015 Nov 11:60-85.
43. Muztaba M, Khan MI, Khalid M, Tarique M, Akhtar J, Ahmad M. CoQ10: A miraculous and clinically vital coenzyme for normal body

functions, prevention and treatment of diseases. Journal of Pharmaceutical Sciences and Research. 2018 May 1;10(5):976-82.

44. Crotty GF, Ascherio A, Schwarzschild MA. Targeting urate to reduce oxidative stress in Parkinson disease. Experimental neurology. 2017 Dec 1;298:210-24.
45. Ali SS, Ahsan H, Zia MK, Siddiqui T, Khan FH. Understanding oxidants and antioxidants: Classical team with new players. Journal of food biochemistry. 2020 Mar;44(3):e13145.
46. Carocho M, Ferreira IC. A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food and chemical toxicology. 2013 Jan 1;51:15-25.
47. Pisoschi AM, Pop A. The role of antioxidants in the chemistry of oxidative stress: A review. European journal of medicinal chemistry. 2015 Jun 5;97:55-74.
48. Jeszka-Skowron M, Zgoła-Grześkowiak A, Grześkowiak T. Phenolic Compounds in Coffee and Tea Beverages. InAnalytical Methods in the Determination of Bioactive Compounds and Elements in Food 2021 (pp. 31-81). Springer, Cham.
49. Luna-Guevara ML, Luna-Guevara JJ, Hernández-Carranza P, Ruíz-Espinosa H, Ochoa-Velasco CE. Phenolic compounds: A good choice against chronic degenerative diseases. InStudies in natural products chemistry 2018 Jan 1 (Vol. 59, pp. 79-108). Elsevier.
50. Jain A, Sirisha VL. Algal Carotenoids: Understanding Their Structure, Distribution and Potential Applications in Human Health. Encyclopedia of Marine Biotechnology. 2020 Aug 11;1:33-64.
51. Nile SH, Park SW. Edible berries: Bioactive components and their effect on human health. Nutrition. 2014 Feb 1;30(2):134-44.
52. Alva V, Lupas AN. The TULIP superfamily of eukaryotic lipid-binding proteins as a mediator of lipid sensing and transport. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2016 Aug 1;1861(8):913-23.
53. Solnica B, Sygitowicz G, Sitkiewicz D, Cybulska B, Jóźwiak J, Odrowąż-Sypniewska G, Banach M. 2020 Guidelines of the Polish Society of Laboratory Diagnostics (PSLD) and the Polish Lipid

Association (PoLA) on laboratory diagnostics of lipid metabolism disorders. Archives of medical science: AMS. 2020;16(2):237.

54. Kotur- Stevuljević J, Vekić J, Stefanović A, Zeljković A, Ninić A, Ivanišević J, Miljković M, Sopić M, Munjas J, Mihajlović M, Spasić S. Paraoxonase 1 and atherosclerosis- related diseases. Biofactors. 2020 Mar;46(2):193-205.
55. Ali SS, Ahmad WA, Budin SB, Zainalabidin S. Implication of dietary phenolic acids on inflammation in cardiovascular disease. Reviews in cardiovascular medicine. 2020 Jun 30;21(2):225-40.
56. Stadler JT, Marsche G. Obesity-related changes in high-density lipoprotein metabolism and function. International Journal of Molecular Sciences. 2020 Jan;21(23):8985.
57. Rochu D, Chabrière E, Masson P. Paraoxonase-1 and its interactions with HDL: molecular structures of PON1 and HDL. InThe HDL Handbook 2010 Jan 1 (pp. 99-131). Academic Press.
58. Vekic J, Zeljkovic A, Stefanovic A, Jelic-Ivanovic Z, Spasojevic-Kalimanovska V. Obesity and dyslipidemia. Metabolism. 2019 Mar 1;92:71-81.
59. Soppert J, Lehrke M, Marx N, Jankowski J, Noels H. Lipoproteins and lipids in cardiovascular disease: from mechanistic insights to therapeutic targeting. Advanced Drug Delivery Reviews. 2020 Jul 27.
60. Ferri N, Corsini A, Sirtori CR, Ruscica M. Present therapeutic role of cholesteryl ester transfer protein inhibitors. Pharmacological research. 2018 Feb 1;128:29-41.
61. Dijk W, Kersten S. Regulation of lipoprotein lipase by Angptl4. Trends in Endocrinology & Metabolism. 2014 Mar 1;25(3):146-55.
62. Levinson SS, Wagner SG. Implications of reverse cholesterol transport: recent studies. Clinica Chimica Acta. 2015 Jan 15;439:154-61.
63. Kontush A. HDL and Reverse Remnant-Cholesterol Transport (RRT): relevance to cardiovascular disease. Trends in Molecular Medicine. 2020 Aug 26.
64. Hafiane A, Gasbarrino K, Daskalopoulou SS. The role of adiponectin in cholesterol efflux and HDL biogenesis and metabolism. Metabolism. 2019 Nov 1;100:153953.

65. Brites F, Martin M, Guillas I, Kontush A. Antioxidative activity of high-density lipoprotein (HDL): Mechanistic insights into potential clinical benefit. BBA clinical. 2017 Dec 1;8:66-77.
66. Taler-Verčič A, Goličnik M, Bavec A. The structure and function of paraoxonase-1 and its comparison to paraoxonase-2 and-3. Molecules. 2020 Jan;25(24):5980.
67. Khalil A, Fulop T, Berrougui H. Role of paraoxonase1 in the regulation of high-density lipoprotein functionality and in cardiovascular protection. Antioxidants & redox signaling. 2021 Jan 20;34(3):191-200.
68. Shokri Y, Variji A, Nosrati M, Khonakdar-Tarsi A, Kianmehr A, Kashi Z, Bahar A, Bagheri A, Mahrooz A. Importance of paraoxonase 1 (PON1) as an antioxidant and antiatherogenic enzyme in the cardiovascular complications of type 2 diabetes: Genotypic and phenotypic evaluation. Diabetes research and clinical practice. 2020 Mar 1;161:108067.
69. Wysocka A, Zwolak A. The Relevance of Noncoding DNA Variations of Paraoxonase Gene Cluster in Atherosclerosis-Related Diseases. International journal of molecular sciences. 2021 Jan;22(4):2137.
70. Bosak A, Bavec A, Konte T, Šinko G, Kovarik Z, Goličnik M. Interactions of paraoxonase-1 with pharmacologically relevant carbamates. Molecules. 2020 Jan;25(1):211.
71. Dzoyem JP, Kuete V, Eloff JN. Biochemical parameters in toxicological studies in Africa: significance, principle of methods, data interpretation, and use in plant screenings. InToxicological survey of African medicinal plants 2014 Jan 1 (pp. 659-715). Elsevier.
72. Taler-Verčič A, Goličnik M, Bavec A. The structure and function of paraoxonase-1 and its comparison to paraoxonase-2 and-3. Molecules. 2020 Jan;25(24):5980.
73. Manco G, Porzio E, Suzumoto Y. Enzymatic detoxification: a sustainable means of degrading toxic organophosphate pesticides and chemical warfare nerve agents. Journal of Chemical Technology & Biotechnology. 2018 Aug;93(8):2064-82.
74. Santos CR, Costa PA, Vieira PS, Gonzalez SE, Correa TL, Lima EA, Mandelli F, Pirolla RA, Domingues MN, Cabral L, Martins MP. Structural insights into β-1, 3-glucan cleavage by a glycoside hydrolase family. Nature Chemical Biology. 2020 Aug;16(8):920-9.

75. Blaha-Nelson D, Krüger DM, Szeler K, Ben-David M, Kamerlin SC. Active site hydrophobicity and the convergent evolution of paraoxonase activity in structurally divergent enzymes: the case of serum paraoxonase 1. Journal of the American Chemical Society. 2017 Jan 25;139(3):1155-67.
76. Cui T, Lahiri A, Carstens T, Borisenko N, Pulletikurthi G, Kuhl C, Endres F. Influence of water on the electrified ionic liquid/solid interface: a direct observation of the transition from a multilayered structure to a double-layer structure. The Journal of Physical Chemistry C. 2016 May 5;120(17):9341-9.
77. Mortazavi H, Omidi-Ardali H, Amini SA, Saffari-Chaleshtori J, Samani KG. In vivo/in silico insight into the effect of titanium dioxide nanoparticle on serum paraoxonase 1 activity in rat. Journal of Biomolecular Structure and Dynamics. 2020 Dec 28:1-1.
78. Fathima Nasreen, J. A Study of Paraoxonase Gene Polymorphisms in Coronary Artery Disease-Understanding the Biochemical and Genetic Basis of Coronary Artery Disease. Diss. PSG Institute of Medical Sciences and Research, Coimbatore, 2020.
79. Ben-David M, Soskine M, Dubovetskyi A, Cherukuri KP, Dym O, Sussman JL, Liao Q, Szeler K, Kamerlin SC, Tawfik DS. Enzyme evolution: an epistatic ratchet versus a smooth reversible transition. Molecular biology and evolution. 2020 Apr 1;37(4):1133-47.
80. She ZG, Chen HZ, Yan Y, Li H, Liu DP. The human paraoxonase gene cluster as a target in the treatment of atherosclerosis. Antioxidants & redox signaling. 2012 Mar 15;16(6):597-632.
81. Salnikov ES, Aisenbrey C, Anantharamaiah GM, Bechinger B. Solid-state NMR structural investigations of peptide-based nanodiscs and of transmembrane helices in bicellar arrangements. Chemistry and physics of lipids. 2019 Mar 1;219:58-71.
82. Ben-David M, Soskine M, Dubovetskyi A, Cherukuri KP, Dym O, Sussman JL, Liao Q, Szeler K, Kamerlin SC, Tawfik DS. Enzyme evolution: an epistatic ratchet versus a smooth reversible transition. Molecular biology and evolution. 2020 Apr 1;37(4):1133-47.
83. Ben-David M, Sussman JL, Maxwell CI, Szeler K, Kamerlin SC, Tawfik DS. Catalytic stimulation by restrained active-site floppiness—the case of high density lipoprotein-bound serum

paraoxonase-1. Journal of molecular biology. 2015 Mar 27;427(6):1359-74.

84. Yang G, Anderson DW, Baier F, Dohmen E, Hong N, Carr PD, Kamerlin SC, Jackson CJ, Bornberg-Bauer E, Tokuriki N. Higher-order epistasis shapes the fitness landscape of a xenobiotic-degrading enzyme. Nature chemical biology. 2019 Nov;15(11):1120-8.
85. Masi S, Ambrosini S, Mohammed SA, Sciarretta S, Lüscher TF, Paneni F, Costantino S. Epigenetic remodeling in obesity-related vascular disease. Antioxidants & Redox Signaling. 2021 May 20;34(15):1165-99.
86. Behl T, Kumar S, Sehgal A, Singh S, Kumari S, Brisc MC, Munteanu MA, Brisc C, Buhas CL, Judea-Pusta C, Nistor-Cseppento DC. Rice bran, an off-shoot to newer therapeutics in neurological disorders. Biomedicine & Pharmacotherapy. 2021 Aug 1;140:111796.
87. Kotur- Stevuljević J, Vekić J, Stefanović A, Zeljković A, Ninić A, Ivanišević J, Miljković M, Sopić M, Munjas J, Mihajlović M, Spasić S. Paraoxonase 1 and atherosclerosis- related diseases. Biofactors. 2020 Mar;46(2):193-205.
88. Manco G, Porzio E, Carusone TM. Human Paraoxonase-2 (PON2): Protein Functions and Modulation. Antioxidants. 2021 Feb;10(2):256.
89. Bianchi C, Miccoli R, Del Prato S. Hyperglycemia and vascular metabolic memory: truth or fiction?. Current diabetes reports. 2013 Jun 1;13(3):403-10.
90. Rysz J, Gluba-Brzózka A, Rysz-Górzyńska M, Franczyk B. The role and function of HDL in patients with chronic kidney disease and the risk of cardiovascular disease. International journal of molecular sciences. 2020 Jan;21(2):601.
91. Reiss AB, Voloshyna I, De Leon J, Miyawaki N, Mattana J. Cholesterol metabolism in CKD. American Journal of Kidney Diseases. 2015 Dec 1;66(6):1071-82.
92. Grunenwald A, Roumenina LT, Frimat M. Heme Oxygenase 1: A Defensive Mediator in Kidney Diseases. International Journal of Molecular Sciences. 2021 Jan;22(4):2009.
93. Losappio V, Franzin R, Infante B, Godeas G, Gesualdo L, Fersini A, Castellano G, Stallone G. Molecular mechanisms of premature aging in hemodialysis: The complex interplay between innate and adaptive

immune dysfunction. International journal of molecular sciences. 2020 Jan;21(10):3422.

94. Miljkovic M, Stefanovic A, Vekic J, Zeljkovic A, Gojkovic T, Simic-Ogrizovic S, Bogavac-Stanojevic N, Cerne D, Ilic J, Stefanovic I, Jelic-Ivanovic Z. Activity of paraoxonase 1 (PON1) on HDL2 and HDL3 subclasses in renal disease. Clinical biochemistry. 2018 Sep 1;60:52-8.
95. Jakubowski H. Homocysteine in protein structure/function and human disease. Chemical biology of homocysteine-containing proteins. 2013.
96. George AK, Majumder A, Ice H, Homme RP, Eyob W, Tyagi SC, Singh M. Genes and genetics in hyperhomocysteinemia and the “1-carbon metabolism”: implications for retinal structure and eye functions. Canadian journal of physiology and pharmacology. 2020;98(2):51-60.
97. Škovierová H, Vidomanová E, Mahmood S, Sopková J, Drgová A, Červeňová T, Halašová E, Lehotský J. The molecular and cellular effect of homocysteine metabolism imbalance on human health. International journal of molecular sciences. 2016 Oct;17(10):1733.
98. Chen J, Li J, Wang J, Zeng D, Chen J, Zhang Y, Wu M, Zhang D, Hong X. Association of serum VLDL level with hyperhomocysteinemia in hypertensive patients: A cross-sectional study. Clinical and Experimental Hypertension. 2021 Jan 2;43(1):26-33.
99. Kumar A, Palfrey HA, Pathak R, Kadowitz PJ, Gettys TW, Murthy SN. The metabolism and significance of homocysteine in nutrition and health. Nutrition & metabolism. 2017 Dec;14(1):1-2.
100. Youssef-Saliba S, Vallée Y. Sulfur amino acids: from prebiotic chemistry to biology and vice versa. Synthesis. 2021 Apr 1.
101. Khodadadi S, Riazi GH, Ahmadian S, Hoveizi E, Karima O, Aryapour H. Effect of N-homocysteinylation on physicochemical and cytotoxic properties of amyloid β-peptide. FEBS letters. 2012 Jan 20;586(2):127-31.
102. Perła-Kaján J, Jakubowski H. Paraoxonase 1 and homocysteine metabolism. Amino acids. 2012 Oct;43(4):1405-17.
103. Kowalska K, Socha E, Milnerowicz H. The role of paraoxonase in cardiovascular diseases. Annals of Clinical & Laboratory Science. 2015 Mar 1;45(2):226-33.

104. Jakubowski H. Homocysteine editing, thioester chemistry, coenzyme A, and the origin of coded peptide synthesis. Life. 2017 Mar;7(1):6.

105. Palfrey H. Effects of Sitagliptin on Cardiovascular Structural and Biochemical Changes in Rats Fed Atherogenic Diets (Doctoral dissertation, Southern University and Agricultural and Mechanical College).

106. Kamps JJ, Hopkinson RJ, Schofield CJ, Claridge TD. How formaldehyde reacts with amino acids. Communications Chemistry. 2019 Nov 7;2(1):1-4.

107. Jakubowski H. Homocysteine modification in protein structure/function and human disease. Physiological reviews. 2019 Jan 1;99(1):555-604.

108. Gupta N, Gill KD, Singh S. Paraoxonase 1 (PON1) activity, polymorphisms and coronary artery disease. future. 2018;4:5.

109. Ginsberg G, Neafsey P, Hattis D, Guyton KZ, Johns DO, Sonawane B. Genetic polymorphism in paraoxonase 1 (PON1): Population distribution of PON1 activity. Journal of Toxicology and Environmental Health, Part B. 2009 Oct 8;12(5-6):473-507.

110. Juliana P, Singh RP, Singh PK, Poland JA, Bergstrom GC, Huerta-Espino J, Bhavani S, Crossa J, Sorrells ME. Genome-wide association mapping for resistance to leaf rust, stripe rust and tan spot in wheat reveals potential candidate genes. Theoretical and applied genetics. 2018 Jul;131(7):1405-22.

111. Cornelli U, Lu C, Sun X, Wang J, Perricone R, Ballanti E, Gao YF. Humoral factors in the skin. InPractical Immunodermatology 2017 (pp. 115-142). Springer, Dordrecht.

112. Lockridge O. Review of human butyrylcholinesterase structure, function, genetic variants, history of use in the clinic, and potential therapeutic uses. Pharmacology & therapeutics. 2015 Apr 1;148:34-46.

113. Ergen A, Kılıcoglu O, Ozger H, Agachan B, Isbir T. Paraoxonase 1 192 and 55 polymorphisms in osteosarcoma. Molecular biology reports. 2011 Aug 1;38(6):4181-4.

114. Hassan MA, Al-Attas OS, Hussain T, Al-Daghri NM, Alokail MS, Mohammed AK, Vinodson B. The Q192R polymorphism of the paraoxonase 1 gene is a risk factor for coronary artery disease in Saudi

subjects. Molecular and cellular biochemistry. 2013 Aug;380(1):121-8.

115. Shokri Y, Variji A, Nosrati M, Khonakdar-Tarsi A, Kianmehr A, Kashi Z, Bahar A, Bagheri A, Mahrooz A. Importance of paraoxonase 1 (PON1) as an antioxidant and antiatherogenic enzyme in the cardiovascular complications of type 2 diabetes: Genotypic and phenotypic evaluation. Diabetes research and clinical practice. 2020 Mar 1;161:108067.

116. La Du BN, Aviram M, Billecke S, Navab M, Primo-Parmo S, Sorenson RC, Standiford TJ. On the physiological role (s) of the paraoxonases. Chemico-biological interactions. 1999 May 14;119:379-88.

117. Draganov DI, La Du BN. Pharmacogenetics of paraoxonases: a brief review. Naunyn-Schmiedeberg's archives of pharmacology. 2004 Jan 1;369(1):78-88.

118. Moss DW. Isoenzymes. Springer Science & Business Media; 2012 Dec 6.

119. Annan DA. Paraoxonase 1 (PON 1) Q192R Genotype Distribution in Ghanaians with Type II Diabetes Mellitus (Doctoral dissertation, University of Ghana).

120. Darney K, Kasteel EE, Buratti FM, Turco L, Vichi S, Béchaux C, Roudot AC, Kramer NI, Testai E, Dorne JL, Di Consiglio E. Bayesian meta-analysis of inter-phenotypic differences in human serum paraoxonase-1 activity for chemical risk assessment. Environment international. 2020 May 1;138:105609.

121. Souza-Nogueira AD, Camargo AE, Remondi FA, Paoliello MM, Richter RJ, Furlong CE, Barbosa DS, Maes M, Moreira EG. Paraoxonase 1 (PON1) Q192R genotypes and their interaction with smoking strongly increase atherogenicity and the Framingham risk score. Archives of endocrinology and metabolism. 2016 Aug 25;60:426-35.

122. Ayub A, Mackness MI, Arrol S, Mackness B, Patel J, Durrington PN. Serum paraoxonase after myocardial infarction. Arteriosclerosis, thrombosis, and vascular biology. 1999 Feb;19(2):330-5.

123. Jakubowski H, Ambrosius WT, Pratt JH. Genetic determinants of homocysteine thiolactonase activity in humans: implications for atherosclerosis. FEBS letters. 2001 Feb 23;491(1-2):35-9.

124. Jakubowski H. Calcium-dependent Human Serum Homocysteine Thiolactone Hydrolase: A protective mechanism against protein N-homocysteinylation. Journal of Biological Chemistry. 2000 Feb 11;275(6):3957-62.

125. Lakshman MR, Gottipati CS, Narasimhan SJ, Munoz J, Marmillot P, Nylen ES. Inverse correlation of serum paraoxonase and homocysteine thiolactonase activities and antioxidant capacity of high-density lipoprotein with the severity of cardiovascular disease in persons with type 2 diabetes mellitus. Metabolism. 2006 Sep 1;55(9):1201-6.

126. Domagała TB, Łacinski M, Trzeciak WH, Mackness B, Mackness MI, Jakubowski H. The correlation of homocysteine-thiolactonase activity of the paraoxonase (PON1) protein with coronary heart disease status. Cell Mol Biol (Noisy-le-grand). 2006 Dec 31;52(5):4-10.

127. Yang X, Li Y, Li Y, Ren X, Zhang X, Hu D, Gao Y, Xing Y, Shang H. Oxidative stress-mediated atherosclerosis: mechanisms and therapies. Frontiers in Physiology. 2017 Aug 23;8:600.

128. Khalil A, Fulop T, Berrougui H. Role of Paraoxonase1 in the Regulation of High-Density Lipoprotein Functionality and in Cardiovascular Protection. Antioxidants & Redox Signaling. 2020 Feb 24.

129. Billecke S, Draganov D, Counsell R, Stetson P, Watson C, Hsu C, La Du BN. Human serum paraoxonase (PON1) isozymes Q and R hydrolyze lactones and cyclic carbonate esters. Drug Metabolism and Disposition. 2000 Nov 1;28(11):1335-42.

130. Kosaka T, Yamaguchi M, Motomura T, Mizuno K. Investigation of the relationship between atherosclerosis and paraoxonase or homocysteine thiolactonase activity in patients with type 2 diabetes mellitus using a commercially available assay. Clinica Chimica Acta. 2005 Sep 1;359(1-2):156-62.

131. Erel O. A new automated colorimetric method for measuring total oxidant status. Clinical biochemistry. 2005 Dec 1;38(12):1103-11.

132. Apak R, Güçlü K, Özyürek M, Karademir SE. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of

neocuproine: CUPRAC method. Journal of agricultural and food chemistry. 2004 Dec 29;52(26):7970-81.

133. Apak R, Güclü K, Özyürek M, Celik SE. Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay. Microchimica Acta. 2008 Apr 1;160(4):413-9.

134. Hodges DM, DeLong JM, Forney CF, Prange RK. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta. 1999 Feb;207(4):604-11.

135. Shimasaki H. [36] Assay of fluorescent lipid peroxidation products. Methods in enzymology. 1994 Jan 1;233:338-46.

136. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clinical chemistry. 1972 Jun 1;18(6):499-502.

137. Camps J, Marsillach J, Joven J. The paraoxonases: role in human diseases and methodological difficulties in measurement. Critical reviews in clinical laboratory sciences. 2009 Mar 1;46(2):83-106.

138. Ramaseshan R, Sundarrajan S, Liu Y, Barhate RS, Lala NL, Ramakrishna S. Functionalized polymer nanofibre membranes for protection from chemical warfare stimulants. Nanotechnology. 2006 May 30;17(12):2947.

139. Dias, Clara Lúcia Gonçalves. HDL in HIV-1 infection: a quality perspective through paraoxonase-1 activities. Diss. Faculdade de Ciências e Tecnologia, 2013.

140. Kumar A. Effect of simvastatin on paraoxonase 1 (PON1) activity and oxidative stress. Asian Pacific Journal of Tropical Medicine. 2010 Apr 1;3(4):310-4.

141. Zaggout FR. Entrapment of phenol red pH indicator into a sol–gel matrix. Materials Letters. 2006 Apr 1;60(8):1026-30

142. Hashim HO, Al-Shuhaib MB. A Novel DNA Extraction Protocol from Frozen Blood of Normal Individuals and Patients Who Received Systemic Chemotherapy.

143. De Vrieze J, Ijaz UZ, Saunders AM, Theuerl S. Terminal restriction fragment length polymorphism is an “old school” reliable

technique for swift microbial community screening in anaerobic digestion. Scientific reports. 2018 Nov 14;8(1):1-2.

144. Jaber HT, Hailu A, Pratlong F, Lami P, Bastien P, Jaffe CL. Analysis of genetic polymorphisms and tropism in East African Leishmania donovani by Amplified Fragment Length Polymorphism and kDNA minicircle sequencing. Infection, Genetics and Evolution. 2018 Nov 1;65:80-90.

145. Sriphairoj K, Na-Nakorn U, Klinbunga S. Species identification of non-hybrid and hybrid Pangasiid catfish using polymerase chain reaction-restriction fragment length polymorphism. Agriculture and Natural Resources. 2018 Feb 1;52(1):99-105.

146. Tyagi AK, Khoshbeen MB, Curtis PH, Uppugunduri CR, Ansari M. Development and validation of an allele-specific PCR assay for genotyping a promoter and exonic single nucleotide polymorphisms of MGMT gene. Journal of biological methods. 2018;5(2).

147. Ohashi M, Liu F, Hai Y, Chen M, Tang MC, Yang Z, Sato M, Watanabe K, Houk KN, Tang Y. SAM-dependent enzyme-catalysed pericyclic reactions in natural product biosynthesis. Nature. 2017 Sep;549(7673):502-6.

148. Bahar B, Tuncel AF, Holmes EW, Holmes DT. An interactive website for analytical method comparison and bias estimation. Clinical biochemistry. 2017 Dec 1;50(18):1025-9.

149. Shrivastava A, Gupta V. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chronicles of young scientists. 2011 Jan 1;2(1):21.

150. Di Meo S, Venditti P. Evolution of the knowledge of free radicals and other oxidants. Oxidative medicine and cellular longevity. 2020 Apr 23;2020.

151. Gulcin İ. Antioxidants and antioxidant methods: An updated overview. Archives of toxicology. 2020 Mar;94(3):651-715.

152. Altan N, Dincel AS, Koca C. Diabetes mellitus and oxidative stress. Turk J Biol Biochem 2006;31(2):51–6.

153. Mahmood I, Abdulla KS, Othman SH. The total antioxidant status in cigarette smoking individuals. MJBU 2007;25(1):46–50.

154. Baser H, Can U, Baser S, Yerlikaya FH, Aslan U, Hidayetoglu BT. Assesment of oxidative status and its association with thyroid

autoantibodies in patients with euthyroid autoimmune thyroiditis. Endocrine. 2015 Apr;48(3):916-23.

155. Roy J, Galano JM, Durand T, Le Guennec JY, Chung- Yung Lee J. Physiological role of reactive oxygen species as promoters of natural defenses. The FASEB Journal. 2017 Sep;31(9):3729-45.

156. Valko M, Jomova K, Rhodes CJ, Kuča K, Musílek K. Redox-and non-redox-metal-induced formation of free radicals and their role in human disease. Archives of toxicology. 2016 Jan 1;90(1):1-37.

157. Karademirci M, Kutlu R, Kilinc I. Relationship between smoking and total antioxidant status, total oxidant status, oxidative stress index, vit C, vit E. The clinical respiratory journal. 2018 Jun;12(6):2006-12.

158. Di Meo S, Venditti P. Evolution of the knowledge of free radicals and other oxidants. Oxidative medicine and cellular longevity. 2020 Apr 23;2020.

159. Di Meo S, Reed TT, Venditti P, Victor VM. Role of ROS and RNS sources in physiological and pathological conditions. Oxidative medicine and cellular longevity. 2016 Oct;2016.

160. Tsikas D. Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. Analytical biochemistry. 2017 May 1;524:13-30.

161. Reactive Dicarbonyl Scavenging Effectively Reduces MPO-Mediated Oxidation of HDL and Restores PON1 Activity - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/Mechanism-of-malondialdehyde-MDA-formation-from-peroxidation-of-high-density_fig1_342577135 [accessed 20 Jul, 2021]

162. Mahadesh Prasad AJ, Kemparaju K, Elizabeth AF, Jaichander P, Arun A, Cletus JM. Lack of correlation of paraoxonase (PON1) activity with smoking among the south Indians and risk of cardiovascular disease. World Appl Sci J. 2010;9:194-8.

163. Marek G, Ściskalska M, Grzebieniak Z, Milnerowicz H. Decreases in paraoxonase-1 activities promote a pro-inflammatory effect of lipids peroxidation products in non-smoking and smoking patients with acute pancreatitis. International Journal of Medical Sciences. 2018;15(14):1619.

164. Camps J, Marsillach J, Joven J. The paraoxonases: role in human diseases and methodological difficulties in measurement. Critical reviews in clinical laboratory sciences. 2009 Mar 1;46(2):83-106.

165. Ayan D, Şeneş M, Çaycı AB, Söylemez S, Eren N, Altuntaş Y, Öztürk FY. Evaluation of paraoxonase, arylesterase, and homocysteine thiolactonase activities in patients with diabetes and incipient diabetes nephropathy. Journal of medical biochemistry. 2019 Oct;38(4):481.

166. Bizoń A, Milnerowicz H. The effect of divalent metal chelators and cadmium on serum phosphotriesterase, lactonase and arylesterase activities of paraoxonase 1. Environmental toxicology and pharmacology. 2018 Mar 1;58:77-83.

167. Esse R, Barroso M, Tavares de Almeida I, Castro R. The contribution of homocysteine metabolism disruption to endothelial dysfunction: state-of-the-art. International journal of molecular sciences. 2019 Jan;20(4):867.

168. Perła-Kaján J, Jakubowski H. Paraoxonase 1 and homocysteine metabolism. Amino acids. 2012 Oct;43(4):1405-17.

169. Sugamura K, Keaney Jr JF. Reactive oxygen species in cardiovascular disease. Free radical biology and medicine. 2011 Sep 1;51(5):978-92.

170. Koubaa N, Nakbi A, Hammami S, Attia N, Mehri S, Hamda KB, Farhat MB, Miled A, Hammami M. Association of homocysteine thiolactonase activity and PON1 polymorphisms with the severity of acute coronary syndrome. Clinical biochemistry. 2009 Jun 1;42(9):771-6.

171. Ergueder IB, Ucar A, ARITÜRK I, Ergueder T, Avci A, Hasipek S, SUNGUROĞLU K. The effects of cigarette smoking on serum oxidant status, and cholesterol, homocysteine, folic acid, copper, and zinc levels in university students. Turkish Journal of Medical Sciences. 2009 Sep 23;39(4):513-7.

172. Daly C, Fitzgerald AP, O'Callaghan P, Collins P, Cooney MT, Graham IM, COMAC Group. Homocysteine increases the risk associated with hyperlipidaemia. European journal of preventive cardiology. 2009 Apr 1;16(2):150-5.

173. Mahadesh Prasad AJ, Kemparaju K, Elizabeth AF, Jaichander P, Arun A, Cletus JM. Lack of correlation of paraoxonase (PON1)

activity with smoking among the south Indians and risk of cardiovascular disease. World Appl Sci J. 2010;9:194-8.

174. Ferré N, Camps J, Fernandez-Ballart J, Arija V, Murphy MM, Ceruelo S, Biarnés E, Vilella E, Tous M, Joven J. Regulation of serum paraoxonase activity by genetic, nutritional, and lifestyle factors in the general population. Clinical chemistry. 2003 Sep 1;49(9):1491-7.

175. Mackness B, Durrington PN. Polymorphisms of paraoxonase genes and low-density lipoprotein lipid peroxidation. The Lancet. 1999;353(9151):468-9.

176. Leviev I, James RW. Promoter polymorphisms of human paraoxonase PON1 gene and serum paraoxonase activities and concentrations. Arteriosclerosis, thrombosis, and vascular biology. 2000 Feb;20(2):516-21.

177. James RW, Leviev I, Righetti A. Smoking is associated with reduced serum paraoxonase activity and concentration in patients with coronary artery disease. Circulation. 2000 May 16;101(19):2252-7.

178. Nishio E, Watanabe Y. Cigarette smoke extract inhibits plasma paraoxonase activity by modification of the enzyme's free thiols. Biochemical and biophysical research communications. 1997 Jul 18;236(2):289-93.

179. Tomás M, Sentí M, García-Faria F, Vila J, Torrents A, Covas M, Marrugat J. Effect of simvastatin therapy on paraoxonase activity and related lipoproteins in familial hypercholesterolemic patients. Arteriosclerosis, thrombosis, and vascular biology. 2000 Sep;20(9):2113-9.

180. Sutherland WH, Manning PJ, de Jong SA, Allum AR, Jones SD, Williams SM. Hormone-replacement therapy increases serum paraoxonase arylesterase activity in diabetic postmenopausal women. Metabolism-Clinical and Experimental. 2001 Mar 1;50(3):319-24.

181. Mouhamed DH, Ezzaher A, Mechri A, Neffati F, Omezzine A, Bouslama A, Gaha L, Douki W, Najjar MF. Effect of cigarette smoking on paraoxonase 1 activity according to PON1 L55M and PON1 Q192R gene polymorphisms. Environmental health and preventive medicine. 2012 Jul;17(4):316-21.

182. Wang M, Lang X, Zou L, Huang S, Xu Z. Four genetic polymorphisms of paraoxonase gene and risk of coronary heart

disease: a meta-analysis based on 88 case–control studies. Atherosclerosis. 2011 Feb 1;214(2):377-85.

183. Costa, Cibelle Neiva Cavalcanti Mariano da. "Biochemical and molecular characterisation of the dyslipidaemia in Portugal." (2018).

184. Chou CH, Chou YE, Chuang CY, Yang SF, Lin CW. Combined effect of genetic polymorphisms of AURKA and environmental factors on oral cancer development in Taiwan. PloS one. 2017 Feb 2;12(2):e0171583.

185. Hassan M, A Sehgal S, Rashid S. Regulatory cascade of neuronal loss and glucose metabolism. CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders). 2014 Sep 1;13(7):1232-45.

186. Linton MF, Yancey PG, Davies SS, Jerome WG, Linton EF, Song WL, Doran AC, Vickers KC. The role of lipids and lipoproteins in atherosclerosis. Endotext [Internet]. 2019 Jan 3.

187. Wigner P, Grębowski R, Bijak M, Saluk-Bijak J, Szemraj J. The Interplay between Oxidative Stress, Inflammation and Angiogenesis in Bladder Cancer Development. International Journal of Molecular Sciences. 2021 Jan;22(9):4483.

188. De Benedetti, S. T. E. F. A. N. O. "Sporadic Amyotrophic Lateral Sclerosis in patients with common geographical origin: a multidisciplinary study." (2017).

189. Boes E, Coassin S, Kollerits B, Heid IM, Kronenberg F. Genetic-epidemiological evidence on genes associated with HDL cholesterol levels: a systematic in-depth review. Experimental gerontology. 2009 Mar 1;44(3):136-60.

190. Tang JU, ROSE RL, Chambers JE. Metabolism of organophosphorus and carbamate pesticides. InToxicology of organophosphate & carbamate compounds 2006 Jan 1 (pp. 127-143). Academic Press.

191. Eckerson HW, Wyte CM, La Du BN. The human serum paraoxonase/arylesterase polymorphism. American journal of human genetics. 1983 Nov;35(6):1126.

192. Fang DH, Fan CH, Ji Q, Qi BX, Li J, Wang L. Differential effects of paraoxonase 1 (PON1) polymorphisms on cancer risk: evidence from 25 published studies. Molecular biology reports. 2012 Jun 1;39(6):6801-9.

193. Yigittürk O, Turgay F, Kızıldağ S, Özsoylu D, Balcı GA. Do PON1–Q192R and PON1–L55M polymorphisms modify the effects of hypoxic training on paraoxonase and arylesterase activity?. Journal of Sport and Health Science. 2020 Nov 11.
194. Ahmed SH, Kharroubi W, Kaoubaa N, Zarrouk A, Batbout F, Gamra H, Najjar MF, Lizard G, Hininger-Favier I, Hammami M. Correlation of trans fatty acids with the severity of coronary artery disease lesions. Lipids in health and disease. 2018 Dec;17(1):1-3.
195. Chen H, Ding S, Zhou M, Wu X, Liu X, Liu J, Wu Y, Liu D. PON1 L55M and Q192R gene polymorphisms and CAD risks in patients with hyperlipidemia. Herz. 2018 Nov;43(7):642-8.
196. Dardiotis E, Aloizou AM, Siokas V, Tsouris Z, Rikos D, Marogianni C, Aschner M, Kovatsi L, Bogdanos DP, Tsatsakis A. Paraoxonase-1 genetic polymorphisms in organophosphate metabolism. Toxicology. 2019 Jan 1;411:24-31.

197. Tomatir AG, Pehlivan S, Sahin HH, Balci SO, Budeyri S, Pehlivan M. Q192R and L55M polymorphisms of paraoxonase 1 gene in chronic myelogenous leukemia and chronic lymphocytic leukemia. Anticancer research. 2015 Sep 1;35(9):4807-12.
198. Costa LG, Giordano G, Cole TB, Marsillach J, Furlong CE. Paraoxonase 1 (PON1) as a genetic determinant of susceptibility to organophosphate toxicity. Toxicology. 2013 May 10;307:115-22.
199. Vickers NJ. Animal communication: when i'm calling you, will you answer too?. Current biology. 2017 Jul 24;27(14):R713-5.
200. Grzegorzewska AE, Ostromecka K, Adamska P, Mostowska A, Warchoł W, Jagodziński PP. Paraoxonase 1 gene polymorphisms concerning non-insulin-dependent diabetes mellitus nephropathy in hemodialysis patients. Journal of Diabetes and its Complications. 2020 Nov 1;34(11):107687.

201. Apak R, Güclü K, Özyürek M, Celik SE. Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay. Microchimica Acta. 2008 Apr 1;160(4):413-9.

202. Bland JM, Altman DG. Measuring agreement in method comparison studies. Statistical methods in medical research. 1999 Apr;8(2):135-60.

203. Zaggout FR. Entrapment of phenol red pH indicator into a sol–gel matrix. Materials Letters. 2006 Apr 1;60(8):1026-30.

204. Harvey D. Modern analytical chemistry. New York: McGraw-Hill; 2000 Jan.

205. Kalab M. Factors affecting the Ellman determination of sulfhydryl groups in skimmilk powder and gels. Journal of Dairy Science. 1970 Jun 1;53(6):711-8.

206. Beltowski J, Wójcicka G, Jakubowski H. Modulation of paraoxonase 1 and protein N-homocysteinylation by leptin and the synthetic liver X receptor agonist T0901317 in the rat. Journal of endocrinology. 2010 Feb 1;204(2):191.

207. Piggott AM, Karuso P. Fluorometric assay for the determination of glutathione reductase activity. Analytical chemistry. 2007 Nov 15;79(22):8769-73.

208. Dingova D, Leroy J, Check A, Garaj V, Krejci E, Hrabovska A. Optimal detection of cholinesterase activity in biological samples: Modifications to the standard Ellman's assay. Analytical biochemistry. 2014 Oct 1;462:67-75.

الخلاصة

يرتبط دخان السجائر بعدد من المشكلات الصحية الرئيسية، بما في ذلك سرطان الفم وسرطان الحلق والسكتة الدماغية وسرطان الكبد وسرطان المثانة. تدخين السجائر عبارة عن مزيج معقد من حوالي 7000 مادة كيميائية. يحتوي أي وعاء سجائر يحتوي على دخان على جزيئات دخان تفاعلي (ROS)مثل الجذور الفائقة (O^{2-})، وبيروكسيد الهيدروجين (H_2O_2)والهيدروكسيل (OH)، وجذور البيرو كسيل. (ROO •) تم العثور على أي عضو يلعب دورًا في نمو السرطان ، مثل الرئتين والرقبة والمريء والبنكرياس والبروستاتا والمثانة. الجذور الحرة المتمحورة حول النيتروجين وكذلك المؤكسدات غير الراديكالية. يمكن أن تؤدي هذه الأنواع من الأكسجين، بدورها، إلى تحفيز الضرر التأكسدي وتعزيزه في شكل بيروكسيد الدهون.

- في عام 2021 أجريت تجربة سريرية في جامعة بابل. جمعت عينات الدم من طلاب جامعة بابل. تم تسجيل ما مجموعه(150) من غير المدخنين و (150)من المدخنين في الدراسة. تمت دراسة المجموعتين التاليتين في الدراسة الحالية:

1- المجموعة الأولى: 150 موضوعاً لغير المدخنين.
2- المجموعة الثانية: 150 مدخناً سيجارة.

- تتناول هذه الدراسة خمسة أجزاء: -

1- تم تقييم مستويات TOS، TAS للأفراد. تحتوي السجائر على تراكيز مصل أعلى من أنواع المؤكسدات الكلية مقارنة بغير المدخنين. بينما كانت السعة الكلية لمضادات الأكسدة لدى مدخني السجائر أقل بكثير من قدرة غير المدخنين. تشير هذه النتائج إلى تعرض الإجهاد التأكسدي للخطر وآلية الدفاع المؤكسد للمدخن.

2- تم فحص تركيز بيروكسيد الدهون في مصل غير المدخنين ومدخني السجائر. تظهر نتائج الدراسة الحالية ارتفاعًا كبيرًا ((p 0.05في تركيز Malondialdehyde في جميع مدخني السجائر الذين شملتهم الدراسة، مقارنة بمجموعة التحكم غير المدخنين في تركيز homocysteine thiolactonase في جميع مجموعة مدخني السجائر في الدراسة ومقارنة بمجموعة التحكم لغير المدخنين [1.1091 ng / ml]في مجموعة مدخني السجائر بشكل أكثر وضوحًا هي [1.2304 نانو غرام / مل]. تم العثور على العديد من الجذور الحرة والمركبات شديدة التفاعل الأخرى في دخان السجائر. قد تؤدي التركيزات المتزايدة لهذه الجزيئات التفاعلية في الأنسجة إلى أكسدة الدهون، مما يؤدي إلى إطلاق مركبات مثل MDA، والتي تم اكتشافها كأحد منتجات أكسدة الدهون.

3- تصف الدراسة الحالية بروتوكول قياس طيفي بسيط لتقييم مادة الهوموسيستين ثيولاكتوناز وتوضح إمكانية استنساخه ودقته. تشير نتيجة هذه الدراسة إلى زيادة معنوية ($p < 0.05$) في تركيز مادة الهوموسيستين ثيولاكتوناز في جميع مجموعات مدخني السجائر التي شملتها الدراسة ومقارنة مع مجموعة غير المدخنين [146.393 نانو غرام / مل] في مجموعة مدخني السجائر انخفضت بشكل ملحوظ إلى [104.636 نانو غرام / مل].

4- تمت دراسة مستوى نشاط باراوكسوناز، وأظهرت نتائج الدراسة انخفاض معنوي ($p < 0.05$) في تركيز نشاط باراوكسوناز في كل مجموعة مدخني سجائر الدراسة ومقارنة مع مجموعة غير المدخنين (145.33 نانو غرام / مل] في مجموعة مدخني السجائر. انخفض بشكل ملحوظ ليكون [128.56 نانو غرام / مل].

5- تهدف هذه الدراسة إلى معرفة تأثير تدخين السجائر على ثيولاكتوناز الهوموسيستين (HTLase) وفقًا لتعدد الأشكال الجينية PON1 Q192R والعلاقة بين هذه المعلمة وحالة الأكسدة / مضادات الأكسدة. دراسة المعلمات التالية. فحص نشاط homocysteine thiolactonase (HTLase) و paroxonase-1 (PON1). تقييم حالة مضادات الأكسدة الكلية وتركيز الأكسدة الكلي.

6- قياس مستوى الدهون وبيروكسيد الدهون. دراسة تعدد الأشكال الجيني homocysteine thiolactonase (HTLase) (تعدد الأشكال الجيني PON1 Q192R). حاول تطوير طريقة دقيقة جديدة لتقييم نشاط homocysteine thiolactonase (HTLase) ومقارنتها بالطرق العادية.

- تم الوصول إلى مدى تأثير التدخين على نشاط مادة homocysteine thiolactonase، وكذلك تطوير طريقة جديدة لقياس نشاط الإنزيم، ومقارنتها بالطرق المعتادة.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل / كلية العلوم

قسم الكيمياء

الدور الرئيسي لنشاط الهوموسيستين ثيولاكتوناز في تنظيم حالة مضادات الأكسدة لمدخني السجائر وفقًا لتعدد الأشكال الجيني PON1 Q192R

رسالة

مقدمة إلى مجلس كلية العلوم / جامعة بابل كجزء من متطلبات نيل درجة الماجستير في العلوم / الكيمياء

من قبل

نصر جبر عبيد شناوه

بكالوريوس علوم كيمياء –جامعة بابل

2007

بإشراف

أ. د. محمود حسين هدوان

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