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Effect of Mesencephalic Astrocyte-Derived Neurotrophic Factor and Oxidative Imbalance in Coronary Artery Diseases with/without Non-Alcoholic Fatty Liver in Iraqi Patients

A Thesis

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

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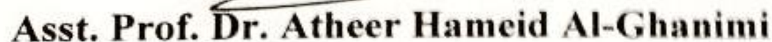

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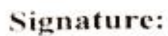

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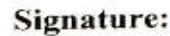

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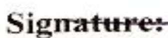

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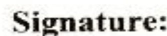

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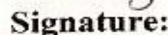

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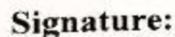

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To deliver the message and fulfill the trust and advise the nation to the prophet of mercy and light of the worlds, our master

Muhammad and his good and pure family

May God's peace and blessings be upon him and his family

To whom God has crowned with prestige and dignity...

To the one who taught me to give without waiting...

To whom I carry his name with pride…

My father

To the meaning of love and the meaning of tenderness and dedication….

To whom her supplication was the secret of my success…

To the most precious beloveds…

My mother

May Allah have mercy on her!

To my dear

Husband and Children

To my support and my strength my brothers and sisters

And to my big family and to my loved ones and friends

And to my distinguished teachers

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Summary

Coronary artery obstructive disease is a leading cause of death worldwide in low- and middle-income countries. It occurs at younger and older ages. Over time, this disease doubles in adults over 30 years of age. Coronary artery disease (CAD), also known as ischemic heart disease (IHD), refers to a group of closely associated syndromes caused by an imbalance between the myocardial oxygen demand and blood supply.

Non-alcoholic fatty liver disease (NAFLD) is a public health problem, affecting up to a third of the world's adult population. Several cohort studies have consistently documented that NAFLD (especially in it's more advanced forms) is associated with a higher risk of all-cause mortality and that the leading causes of death among patients with NAFLD are cardiovascular diseases (CVDs).

Oxidative Stress is an imbalance between the production of oxidants and antioxidant defenses that may result in damage to biological systems. The oxidative stress plays an essential role in the pathogenesis of chronic diseases such as cardiovascular diseases, diabetes, neurodegenerative diseases, and cancer.

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a novel evolutionarily conserved protein present in both vertebrate and invertebrate species. The liver is an important metabolic organ that modulates lipid homeostasis and multiple studies have revealed the critical role of MANF in regulating hepatic lipid metabolism. In fact, MANF is highly abundant in the liver. The pathogenesis of cardiovascular diseases (CVDs) is complex, with ER stress and inflammation playing important roles. In line with other tissues or cell types, intracellular MANF also exhibited protective functions against ER stress and inflammation in the cardiac context, including in ischemia/reperfusion.

The objectives of the present projects are to know the extent to which non-alcoholic fatty liver disease affects and its relationship to coronary artery obstructive disease, and assessment of MANF levels and what is their impact on both NAFLD and CAD.

The study was a case-control that included 120 samples (male and female) and their serum samples were collected from Kerbala Heart Center/ Kerbala Health Directorate l Kerbala - Iraq with ages ranging between 40 to 73 years. Time and duration of study from November 2022 to May 2023. The number of patients with coronary arteries was 60 and the number of healthy people was 60 the number of patients with non-alcoholic fatty liver disease was 37 and 23 without NAFD. The serum samples were withdrawn and kept at -20 °C. The Mesencephalic Astrocyte-Derived Neurotrophic Factor, liver function test, lipid profile, and albumin were determined at optimized conditions in the laboratory of the Department of Chemistry and Biochemistry, College of Medicine, University of Kerbala by using the Automated Biochemistry analyzer to measure liver function lipids profile and examine albumin, while MANF biomarker was determined by ELISA Sandwich technique.

The observed levels of MANF decreased in non-alcoholic fatty liver disease, steatosis, and Coronary artery disease in compare with control group Results indicated a significant difference in MANF levels among groups, and increased levels of MDA and decreased levels in antioxidant group GPX, TAC, and Se in CAD and NAFLD pateints. The mean levels of MANF in control was (348.62 ± 143.50) which was significantly higher than for the patient group (287.58 ± 76.71), ($p \leq 0.001$). The Distribution of serum level of MANAF, (348.62 ± 143.50), The level of MANF protects against fatty liver and reduces chronic coronary artery disease.

In Conclusions, the results presented here contribute to the determination of the functions of the physiological MANF protein in protecting against non-alcoholic fatty liver and chronic coronary artery obstructive disease, as decrease the level MANF protein leads to accumulation of liver fat and also causes increased cell death due to lack of oxygen.

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List of Abbreviations

•OH	Hydroxyl radical
4-HNE	4-hydroxynonenal
AA	Arachidonic acid
AAR	AST/ALT ratio
ACD	Atherosclerotic cardiovascular disease
ACS	Acute coronary syndrome
AF	Atrial fibrillation
AI	Artificial intelligence
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUC	Area under curve
BMI	Body mass index
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CAG	coronary angiography
CAT	Catalase
cCAD	Chronic coronary artery disease
CCTA	Coronary computed tomography angiography
cDNA	Cloning and sequencing of the MANF
CHD	Coronary heart disease
C-index	Cholesterol index
CMR	Cardiac magnetic resonance
COPD	Chronic obstructive pulmonary
CoQ	Coenzyme Q
CRP	C-reactive protein
CT	Computed tomography
CVD	Cardiovascular disease
DM	Diabetes mellitus
DTAC	Dietary total antioxidant capacity
ECG	Electrocardiogram
ELISA	Enzyme-linked immune sorbent assay
ER	Endoplasmatic Reticulum
EVINCI	evaluation of integrated cardiac imaging
FFAs	Free fatty acids
GDMT	Guideline-medical therapy
GGT	Gamma-glutamyl transferase
GPX	Glutathione Peroxidase

GSH	Glutathione
HCC	Hepatocellular carcinoma
HDL-C	High-density lipoprotein cholesterol
ICA	Invasive coronary angiography
IHD	Ischemic heart disease
INR	International normalized ratio
IR	Insulin resistance
LA	Lipoic acide
LAD	Lift anterior descending
LCX	Lift circumflex
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein cholesterol receptor
LFTs	Liver function test
LSEC	Liver sinusoidal endothelial cell
MAFLD	Metabolic dysfunction-associated fatty liver disease
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MDA	Malondialdehyde
MetS	Metabolic syndrome
MI	Myocardial infraction
ML	Machine learning
MRI	Magnetic resonance imaging
NADH	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver
NASH	Non-alcoholic steatohepatitis
NMR	Nuclear magnetic resonance
NOS	Nitric oxide synthases
Nox	NADPH oxidase
NSTEMI	Non-ST-elevation myocardial infarction
NTF	Neurotrophic factors
$O_2^{\bullet-}$	superoxide
OS	Oxidative stress
ox-LDL	The oxidized LDL
p38 MAPK	p38 mitogen-activated protein kinase
PCI	Percutaneous coronary intervention
PCSK9	proprotein convertase subtilisin/kexin type 9 gene
PET	positron emission tomography
PR	Positive remodeling
PRDXs	Peroxiredoxins
PT	Prothrombin time
RCA	Right coronary arteries

RNS	Reactive nitrogen species
ROC	Receiver operating characteristic
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
SD	Standard deviation
Se	Selenium
SIHD	Stable ischemic heart disease
SMC	Smooth muscle cell
SOD	Superoxide dismutase
SPECT	Single photon computed emission tomography
STEMI	ST-elevation myocardial infarction
T2D	Type 2 diabetes
TAC	Total antioxidant capacity
TB	Total bilirubin
TC	Total cholesterol
TG	Triglycerides
VLDL-C	Very low-density lipoprotein cholesterol

Chapter One

Introduction

and

Literature Review

1. Introduction

1.1 Heart disease

The term "heart disease" refers to several types of heart conditions. The most common type of heart disease is coronary artery disease (CAD), which affects the blood flow to the heart **(Riyaz, 2022)**. Decreased blood flow can cause a heart attack. Cardiovascular disease is the leading and most important cause of death in many countries. It is reported that the main cause of cardiovascular disease is coronary artery disease. Where 2 out of 10 deaths attributable to coronary artery disease **(Salehi *et al*., 2021).** Coronary artery disease is the most common and prevalent serious disease in men and women. As the primary cause of CVDs, atherosclerosis is usually caused by the deposition of lipid-containing plaques on the inner arterial wall, most of which was induced by hypercholesterolemia. Recent studies have revealed several important environmental factors (especially nutritional) associated with atherosclerosis. There is no doubt that high fat/high cholesterol diet is the leading cause of hyperlipidemia, especially hypercholesterolemia **(Liu *et al*., 2020).**

1.1.1. Coronary artery disease

Coronary artery obstructive disease is a leading cause of death worldwide, both in low- to middle-income countries. It occurs at younger and older ages. Over time, this disease doubles in adults over 30 years of age **(Hosseini, *et al*., 2021)**. Coronary artery disease (CAD), also known as Ischemic heart disease (IHD), refers to a group of closely associated syndromes caused by an imbalance between the myocardial oxygen demand

and blood supply. Dependent on the graded severity of coronary artery narrowing and the myocardial response one of four syndromes may progress to angina pectoris (chest pain), acute myocardial infarction, sudden cardiac death and chronic Ischemic heart disease with congestive heart failure **(Hosseini *et al.*, 2021).** when plaque builds up in the arteries, it is called atherosclerosis. Plaque in the arteries can rupture from blockages and cause blood flow to stop, which can lead to a heart attack **(Al-Tu'ma, & Al-Mayali, 2019)**. Coronary artery disease (CAD) is the most common type of CVD. CAD occurs when at least one of the left anterior descending (LAD), left circumflex (LCX) and right coronary (RCA) arteries are stenotic **(Tabas, 1999).**

1.1.1.1. Chronic coronary artery disease

Stable ischemic heart disease SIHD presents as stable angina. Stable angina typically presents as substernal chest pain or pressure that worsens with exertion or emotional stress and gets relieved with rest or nitroglycerin and is of 2 months duration. It is important to know that classic anginal symptoms could be absent and it could present differently with atypical symptoms and exertional dyspnea instead in certain demographic groups including women, the elderly age, and diabetics **(Bahit, et al., 2018**).

The patient has heart disease Grade I: daily activities are not limited, and general activities do not cause fatigue, palpitations, dyspnoea, or angina pectoris. Grade II: the physical activity of patients with heart disease is slightly restricted, and there are no symptoms at rest, but fatigue, palpitations, dyspnoea, or angina pectoris occur during normal activities. Grade III: the physical activity of patients with heart disease is significantly limited and less

than the usual general activities can cause the above symptoms. Grade IV: patients with heart disease cannot engage in any physical activity, and symptoms of heart failure also occur at rest, which is aggravated by physical activity **(Tian *et al*., 2022).** Chronic stable angina, the initial manifestation of CAD in approximately 50% of all patients,6 is usually caused by the obstruction of at least 1 large epicardial coronary artery by atheromatous plaque. Angina is due to the mismatch between myocardial oxygen demand and supply, resulting in myocardial ischemia. Angina pectoris is characterized by substernal discomfort, heaviness, or a pressure-like feeling, which may radiate to the jaw, shoulder, back, or arm and which typically lasts several minutes. These symptoms are usually brought on by exertion, emotional stress, a cold, or a heavy meal and are relieved by rest or nitroglycerin within minutes. Symptoms can be classified as characteristics of typical angina **(Allai *et al*., 2016).**

1.1.1.2. Pathogenesis of Coronary Artery Disease

Atherosclerosis is the main etiopathogenic process that causes CAD, and its progression is related to an interplay between environmental and genetic factors, In CAD, the extracellular matrix in the inner lining of the coronary arterial wall combines with lipoproteins, exposing them to more lipoprotein modification and inflammation, forming vulnerable atherosclerotic plaques **(Sayols-Baixeras *et al*., 2014).** As inflammation progresses, there is cell death, accumulation of extracellular lipids in the lesion's artery wall, and calcium deposition **(Libby & Theroux, 2005).** The atherosclerotic plaque thickens, causing stenosis of the coronary lumen **(Buja & Willerson, 1987).** which results in the restriction of blood flow and

delivery of oxygenated blood to the heart muscles, causing ischemia. Atherosclerotic lesions with thick fibrous caps and calcification but with relatively smaller lipid cores can slowly induce ischemia due to progressive plaque volume increase that encroaches the coronary lumen diameter. In contrast, some atherosclerotic lesions with larger lipid cores and thinner fibrous caps are vulnerable to rupture, in which the contents are suddenly spilled into the coronary lumen, triggering the thrombus formation which can occlude the lumen and completely disrupt myocardial blood flow **(Libby & Theroux, 2005)**. This leads to acute myocardial infarction (MI) in which heart muscles die due to a lack of oxygen for an extended time duration **(Jahmunah *et al*., 2021)**.

1.1.1.3. Risk factors for Coronary artery disease

has been found to play an important role in the development of such cardiovascular diseases

1.1.1.3.1. Modifiable Risk Factors

- **Cigarette smoking**

Smoking is considered a strong risk factor for myocardial infarction, premature atherosclerosis, and sudden cardiac death. Smoking results in early STEMI especially in otherwise healthier patients. Smoking causes an average of 7 years earlier and is more likely twice the chances of infarction than non-smokers **(Zhang, 2010).**

- **Dyslipidemia (high LDL or high TG low HDL)**

Elevated triglyceride levels and dense, small LDL particles act as predisposing risk factors for MI. Non-fasting triglyceride levels appear to be a strong and independent predictor of future risk of MI, particularly

when the total cholesterol level is also elevated. The reason behind it is that decreased HDL-C levels and increased triglyceride levels cause metabolic perturbations thus causing adverse consequences. To identify high-risk individuals, elevated triglyceride levels may become markers (**Stampfer *et al*., 1996).**

- **Hypertension**

Hypertension is a strong and independent risk factor for MI. It is a major risk factor for causing atherosclerosis in coronary blood vessels, resulting in a heart attack or MI. Hypertension and MI are closely linked **(Huma *et al*., 2012).**

- **Diabetes Mellitus**

Significant differences in measured parameters were noted when all diabetic and non-diabetic patients were compared to the control group. It was found that in men with myocardial infarction, there are significant differences between diabetic and nondiabetic patients concerning certain risk factors such as age, hypertension, and hypertriglyceridemia in diabetic patients, while smoking and family history are predominant factors in non-diabetic patients. However, newly diagnosed diabetic men have similar risk profiles to their known diabetic counterparts **(Sewdarsen *et al*., 1991).**

- **Obesity**

Increased BMI is directly related to the incidence of MI. Infarction is greatly enhanced by extreme obesity because it is a recognized risk

factor for MI. To reduce the population burden of MI in the US, strategies are devised to promote optimal body weight **(Kenchaiah *et al*., 2002)**.

- **Sedentary lifestyle**

Inactive people with multiple cardiac risk factors are more likely to develop MI. To get benefits, these individuals should start with modest exercise training. There should be aggressive risk factor modification before the performance of the vigorous activity **(Giri *et al*., 1999)**.

1.1.3.2. Non Modifiable Risk Factors

- **Family history**

The heritability of CAD risk has been reported to increase with the increase in the number of affected relatives and onset at a young age. Mendelian disorders are associated with CAD, which includes familial hypercholesterolemia. Hypercholesterolemia is a single-gene disorder caused by mutations in the LDL receptor genes (LDLR), in the lipid-binding domain of apolipoprotein B100 (APOB) and proprotein convertase subtilisin/kexin type 9 gene proprotein convertase subtilisin/kexin type 9 gene (PCSK9). Understanding the molecular basis of such disorders led to the discovery of pathways of LDL cholesterol metabolism which is associated with the pathogenesis of CAD. Variations in the genes associated with such disorders could help determine the disease **(Malakar *et al*., 2019).**

- **Age**

Older adults are more likely to die of heart disease. About 80% of heart disease deaths occur in people aged 65 or older (**Cox *et al*., 2022**).

- **Gender**

Men tend to have heart attacks earlier in life than women. Women's rate of heart attack increases after menopause but does not equal men's rate. Even so, heart disease is the leading cause of death for both men and women (**Czajkowski *et al*., 1997**).

1.1.1.4. Diagnosis of Coronary artery disease

The diagnosis of coronary artery disease is classified as follow:

1.1.1.4.1. Clinical Diagnosis of coronary artery diseases

The coronary artery diseases sign symptoms include:

- **Chest pain (angina).**

the majority of studies describe chest pain as the most frequent symptom in both genders (**Arslanian-Engoren *et al*., 2006; Araújo *et al*., 2018**). Angina is also the most frequently reported symptom in stable IHD in both men and women, but women more often present with atypical angina. While typical angina is characterized by retrosternal pain that is provoked by exertion and relieved by rest or nitroglycerine, atypical angina represents a more diverse symptom presentation with pain or discomfort not only in the chest but also in the arms, jaw, neck, and interscapular area (**Wenger, 2016**). These symptoms do not necessarily occur at exertion but can arise after exertion or be triggered by mental stress or even occur at rest. Symptoms may last intermittently over several hours, and atypical presentations include morevague symptoms such as fatigue, anxiety, dyspnoea, dyspepsia, and nausea (**Mehta *et al*., 2019**).

- **Shortness of breath.**

  The heart can't pump enough blood to meet the body's needs, this may develop shortness of breath or extreme fatigue with exertion **(McHorney *et al*., 2021).**

CAD is life-threatening and in the stage of CAD initiation experts may miss diagnosis for the absence of typical symptoms (**Kwok *et al*., 2021**).

1.1.1.4.2. Diagnostic Test

1.1.14.2.1.The Electrocardiography (ECG)

The ECG is the electrical activity of the heart which gets altered due to CAD, MI, and CHF (**Birnbaum *et al*., 2014**). An electrocardiogram (ECG) can record a patient's heart electrical signal activities over a long period **(Adler *et al*., 2015)** by measuring voltages from electrodes attached to the patient's chest, arms, and legs. ECGs are a quick, safe, and painless way to check for heart rate, heart rhythm, and signs of potential heart disease. A twelve-lead ECG is today's standard tool and is used by cardiologists for detecting various cardiovascular abnormalities. However, heart problems may not always be observed on a standard 10-second recording from the 12-lead ECG measurements performed in hospitals or clinics. Therefore, long-term ECG monitoring that tracks the patient's heart condition at all times and under any circumstance has become possible with the development of new sensing technologies. Portable ECG recording devices such as the Apple Watch, Omron Heart Scan, Qardio MD, and, more recently, the Astroskin Smart Shirt are revolutionizing cardiac diagnostics by measuring a patient's 24/7 cardiac activities and transmitting this

information to a cloud service to be stored and processed remotely **(Li, & Boulanger, 2020).**

1.1.1.4.3. Echocardiography

Echocardiography is an ultrasound of the heart. It is a useful and non-invasive mode of testing that is performed in both acute and chronic and inpatient and outpatient settings. In acute settings, it could tell about wall motion, valvular regurgitation and stenosis, infective or autoimmune lesions, and chamber sizes. It also is useful in the diagnosis of acute pulmonary pathologies like pulmonary embolism. It also evaluates the pericardial cavity. In chronic settings, it can be done to see the same information mentioned above and also a response to the therapy. It is also used in an outpatient setting as part of stress testing. In addition to diagnostics, it has also a role in therapeutics, for example, pericardiocentesis could be performed with the needle guided by echocardiography (**Shahjehan, and Bhutta, 2022**).

1.1.1.4.4. Coronary Angiography

Coronary angiography (CAG) is the current gold standard for diagnosing CHD. It is characterized by obtaining the most intuitive imaging results in a relatively short period **(Cobo *et al*., 2021; de la Torre Hernandez *et al*., 2021).** coronary angiography is considered the gold-standard method for the identification and characterization of coronary artery stenosis. Yet, ICA is limited by the evaluation of a 3D vascular structure as a two-dimensional imaging, and by the inability to accurately identify the hemodynamic significance of the intermediate coronary stenosis **(Tonino et al., 2010)**. The invasive physiologic assessment aims to overcome these limitations, assessing the ischemic impact of coronary stenoses **(Pijls *et al*., 1996).** Coronary

computed tomography angiography is recommended as a first-choice option for diagnosing CAD where there is clinical suspicion with a low probability according to stable CAD guidelines and should be considered in HF patients with a low-intermediate likelihood of CAD according to HF guidelines **(Knuuti *et al*., 2020; McDonagh *et al*., 2021)**, This is a rather challenging task considering symptom variability and clinical cofounders in the HF population. However, CCTA presents a high likelihood to "rule out" and a low likelihood to "rule in" CAD **(Pontone *et al*., 2020)**. Despite its ionizing radiation and possible contrast toxicity with the development of novel prospective gating techniques that require much lower dose radiation, and because of a shorter scan time, CCTA is an attractive option to CMR. It can accurately evaluate coronary anatomy, which is very helpful in HF patients when ruling out CAD as a possible cause. In the evaluation of integrated cardiac imaging for the detection and characterization of ischaemic heart disease (EVINCI) study, CCTA had better diagnostic accuracy than stress CMR, PET, SPECT, or stress echocardiography for diagnosing ischemic heart disease **(Neglia *et al*., 2015).** While coronary angiography is the gold standard diagnostic technique for CAD, it is invasive and costly. Therefore, combinations of more biomarkers need to be integrated using various methods for creating predictive, diagnostic, or prognostic tools for CAD. Machine learning (ML) has undergone an expansion in its application as a component of artificial intelligence (AI) and has enhanced the efficiency of the healthcare system **(Fernández-Ruiz, 2019)**. A previous study has shown that ML algorithms are effective for risk prediction, diagnosis, and imaging analysis of CVD **(Bertsimas *et al*., 2021).**

1.2.3. Mechanisms Linking NAFLD to CVD and other Cardiac Complications

The pathophysiology behind the association of NAFLD with CVD and other cardiac complications is incompletely understood and may involve other pathways besides insulin resistance, for example, low-grade inflammation, oxidative stress, and the effects of perturbations in the gut microbiota **(Stahl et al., 2019).** Low- grade systemic inflammation is a key feature of many metabolic diseases, such as T2DM, obesity, and related disorders including NAFLD. NAFLD is not only linked to CVD and T2DM but also chronic kidney disease (Adams et al., 2017). Over the past few years, growing evidence supports a strong correlation between NAFLD and increased cardiovascular disease (CVD) risk, independent of the presence of diabetes, hypertension, and obesity. This implies that NAFLD may also be directly involved in the pathogenesis of CVD. Notably, liver sinusoidal endothelial cell (LSEC) dysfunction appears to be implicated in the progression of NAFLD via numerous mechanisms, including the regulation of the inflammatory process, hepatic stellate activation, augmented vascular resistance, and the distortion of microcirculation, resulting in the progression of NAFLD. Vice versa, the liver secretes inflammatory molecules that are considered pro-atherogenic and may contribute to vascular endothelial dysfunction, resulting in atherosclerosis and CVD **(Nasiri-Ansari *et al.,* 2021)**. Importantly, these associations are especially relevant in patients with NASH, suggesting that liver inflammation may directly contribute to the development of these extrahepatic diseases. Multiple sources of cytokines drive liver inflammation and extrahepatic complications whereas it is recognized that liver fibrosis determines long-term liver prognosis in NAFLD, it is generally accepted that liver inflammation

precedes fibrosis in most instances. However, hepatic fat accumulation may also lead to liver damage, that is, fibrosis, independent from inflammation **(Don Giovanni *et al*., 2018)**. The mechanisms underlying excess cardiovascular risk in NAFLD patients are much more complex and involve both genetic mechanisms associated with the MetS and other species associated with NAFLD **(Lonardo, *et al*., 2018)(Targher, *et al*., 2018)** The studies from the past decade have projected the increasing morbidity and mortality of patients with NAFLD, not due to liver-related complications but primarily because of cardiovascular disease **(Mantovani, *et al*., 2016).** As shown in Figure **(1)**

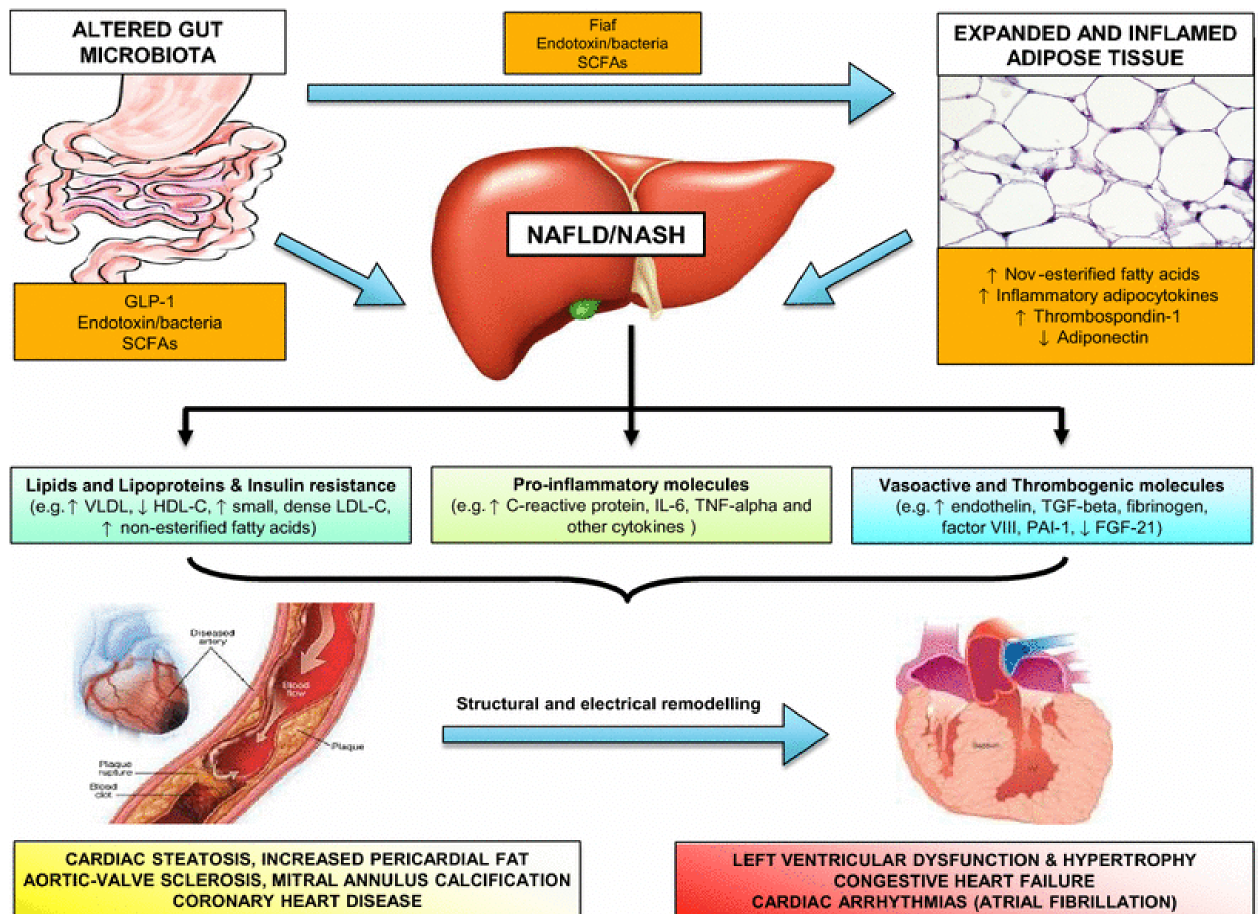


Figure 1: Putative mechanisms linking NAFLD to ischaemic heart disease and other cardiac complications. **(Targher *et al*., 2020).**

1.3. The Liver

The liver is one of the most important organs of the human body. Its role is fundamentally important, particularly in the transformation of toxic substances into elements that the body can eliminate (**Lorente *et al*., 2020**).

The liver is a critical hub for numerous physiological processes. These include macronutrient metabolism, blood volume regulation, immune system support, endocrine control of growth signaling pathways, lipid and cholesterol homeostasis, and the breakdown of xenobiotic compounds, including many current drugs. Processing, partitioning, and metabolism of macronutrients provide the energy needed to drive the aforementioned processes and are therefore among the liver's most critical functions. Moreover, the liver's capacities to store glucose in the form of glycogen, with feeding, and assemble glucose via the gluconeogenic pathway, in response to fasting, are critical. The liver oxidizes lipids, but can also package excess lipids for secretion to and storage in other tissues, such as adipose. Finally, the liver is a major handler of protein and amino acid metabolism as it is responsible for the majority of proteins secreted in the blood (whether based on mass or range of unique proteins), the processing of amino acids for energy, and disposal of nitrogenous waste from protein degradation in the form of urea metabolism. Over the course of evolution, this array of hepatic functions has been consolidated in a single organ, the liver**.** The goal of this primer is to concisely summarize hepatic functions concerning macronutrient metabolism. Introducing concepts critical to liver development, organization, and physiology sets the stage for these functions. It is important to emphasize that insight into hepatic pathologies and potential therapeutic avenues to treat these conditions requires an understanding

of the development and physiology of specialized hepatic functions **(Trefts *et al*., 2017).** The liver also plays a significant role in metabolism, regulation of red blood cells (RBCs), and glucose synthesis and storage. Typically when reviewing liver function tests, the discussion includes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), 5'nucleotidase, total bilirubin, conjugated (direct) bilirubin, unconjugated (indirect)bilirubin, prothrombin time (PT), the international normalized ratio (INR), lactate dehydrogenase, total protein, globulins, and albumin. These tests can help determine the area of hepatic injury, and the elevation pattern can help organize a differential diagnosis **(Ribeiro, *et al*., 2019).** The term "liver function tests "is a misnomer as many of the tests do not comment on the function of the liver but rather pinpoint the source of the damage. Elevations in ALT and AST in out of proportion to ALP, and bilirubin denote a hepatocellular disease. An elevation in ALP and bilirubin in disproportion to ALT and AST would characterize a cholestatic pattern. A mixed injury pattern is defined as an elevation of alkaline phosphatase and AST/ALT levels. Isolated hyperbilirubinemia is defined as an elevation of bilirubin with normal alkaline phosphatase and AST/ALT levels **(Vagvala, & O'Connor, 2018).**

1.3.1. Liver Cells

Liver tissue consists of a mass of cells tunneled through bile ducts and blood vessels. Hepatic cells make up about 60 percent of the tissue and perform more metabolic functions than any other group of cells in the body. A second group of cells, called Kupffer cells, line the smallest channels of the liver's vascular

system and play a role in blood formation, antibody production, and ingestion of foreign particles and cellular debris**(Britannica, 2020).**

1.3.2. Liver Function

The liver is located in the right upper quadrant of the abdomen and below the diaphragm, is responsible for several functions, including primary detoxification of various metabolites, synthesizing proteins, and producing digestive enzymes **(Lala *et al*., 2022).**

A. Synthesis function

Albumin is synthesized by the hepatic parenchymal cells at a rate dependent on colloidal osmotic pressure and dietary protein intake. The rate of albumin synthesis is also subject to feedback regulation determined by the plasma albumin concentration. Maintenance of plasma albumin concentrations can be achieved with only 10% of normal hepatocyte mass. The half-life of albumin is 21 days. Traces of albumin can be found in almost all extracellular body fluids. Little is lost from the body by excretion **(Rozga, *et al.,* 2013).** It is catabolized in various tissues, which are taken up by cells by pinocytosis. Its constituent amino acids are released by intracellular proteolysis and returned to the body pool. With any liver disease, there is a fall in serum albumin, reflecting decreased synthesis. If liver function is normal and serum albumin is low, this may reflect poor protein intake (malnutrition) or protein loss (nephrotic syndrome, malabsorption, or protein-losing enteropathy) **(Chen, *et al.,* 2021).**

B. Excretion of bilirubin, cholesterol, hormones, and drugs.

C. Metabolism of fats, proteins, and carbohydrates.

D. Enzyme activation.

E. Storage of glycogen, vitamins, and minerals.

(Buliarca et al., 2021).

1.3.3. Liver Enzymes

ALT is an enzyme that is found primarily in hepatocytes (lower concentrations in cardiac, renal, and muscle tissue) and thus is specific to hepatocellular injury. ALT levels often fluctuate throughout the day ALT facilitates the formation of glutamate and pyruvate in the hepatocyte which is important for energy production **(Aulbach, & Amuzie, 2017).** The normal range for ALT in males is between 29-33 IU/L and 19-25 IU/L for females. ALT levels have been a point of debate recently as newer studies are suggesting the need for a lower ALT cutoff to increase the sensitivity of the test. It's believed that the current ALT cutoffs were defined by using patients with possible underlying subclinical liver disease and hence decrease the sensitivity of the test **(Prati *et al*., 2002)**. It is important to note that the reference ranges for labs differ across countries and sometimes even between different centers in the same country.

AST is an enzyme that like ALT is also found in the liver however has other sites where its presence is not as minimal as ALT. These sites are primarily skeletal muscle, cardiac muscle, renal tissue, and brain. It occurs as 2 isoenzymes that are not differentiated on standard testing and hold little clinical value. AST facilitates amino acid metabolism (Sparling, et al., 2016). When it comes to AST, caution must be practiced when evaluating abnormal levels due to its presence in other tissues. The normal range for AST is < 35 IU/L **(Pagana et al., 2021).**

ALP is an enzyme that is primarily found in the hepatobiliary tract, bone, placenta, and to a smaller extent in intestinal tissue. ALP is involved in multiple dephosphorylating reactions. The normal range for ALP is between 30-120 IU/L. ALP is generally higher in children and adolescents due to the increased osteoblastic activity associated with bone growth **(Lowe *et al*., 2022).**

GGT is an enzyme that is found in multiple organs in the body including the pancreas, seminal vesicles, kidneys, biliary tract, and liver. Its elevation is usually considered significant for hepatobiliary disease when accompanied by an elevation in other liver biochemical tests. It is generally elevated in biliary disease, cytochrome-inducing medications, and alcohol abuse. GGT is involved in glutathione metabolism and production in multiple tissues in the body. Normal GGT levels range between 0-30 IU/L. GGT levels are generally 6-8 times higher in infants **(Cabrera-Abreu, & Green, 2002).**

1.3.4. Fatty Liver

Patients are usually asymptomatic, and "fatty liver" is usually an incidental finding on imaging done for other purposes. When patients do have symptoms, they are usually nonspecific, constitutional, or right upper quadrant discomfort **(Loria *et al*., 2010).**

1.3.4.1. Non-Alcoholic Fatty Liver

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide. It is strongly associated with obesity, type 2 diabetes (T2DM), and other metabolic syndrome features. **(Nasiri-Ansari *et al*., 2021).**

NAFLD is a spectrum of liver disease ranging from benign steatosis to cirrhosis requiring a liver transplant. It is one of the most common chronic liver conditions necessitating a liver transplant. There are a variety of causes of

NAFLD, ranging from metabolic syndrome, pregnancy, nutrition, drugs, toxins, and more. It is most commonly seen in diabetics and obese patients. It can also present in asymptomatic patients receiving workups for other reasons. It can sometimes present with right upper quadrant pain and/or discomfort. Liver enzymes can be elevated, classically with an elevated ALT: AST ratio **(Su *et al*., 2019; Adams *et al*., 2005).**

NAFLD or "Fatty Liver" corresponds to the presence of macrovesicular changes without inflammation (steatosis) and lobular inflammation in the absence of significant alcohol use. It can be divided into two subgroups: NAFL (Non-Alcoholic Fatty Liver) or simply Steatosis and NASH (Non-Alcoholic Steatohepatitis). NAFL is defined as the presence of hepatic steatosis with no evidence of hepatocellular injury in the form of ballooning of the hepatocytes. NASH is defined as the presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning), Malloryhyaline, and mixed lymphocytic and neutrophilic inflammatory infiltrate in perivenular areas with or without fibrosis **(Brunt *et al*., 1999; Antunes *et al*., 2022).** NAFL (Non-Alcoholic Fatty Liver) or simply Steatosis and NASH (Non-Alcoholic Steatohepatitis) could only be distinguished with histology and liver biopsy **(Wilkins *et al*., 2013).**

Table 1: Non-alcoholic fatty liver disease spectrum.

Non-alcoholic fatty liver disease spectrum		
NAFL	Steatosis changes. No cellular ballooning, hepatocyte inflammation, or fibrosis	Prevalence of 25% approximately. Reversible
NASH	Steatosis changes. Cellular ballooning and hepatocyte inflammation. No fibrosis	Prevalence of 1.5%-6.45% approximately. Generally irreversible is reversible in some patient
NASH-related liver cirrhosis	Hepatocyte destruction and fibrosis	Prevalence of 1%-2% approximately. Irreversible
Healthy liver ←→ NAFL → NASH → NASH-related cirrhosis		

NAFL: Non-alcoholic fatty liver; NASH: Non-alcoholic steatohepatitis (**Kalas *et al*., 2021**).

1.3.4.1.1. The pathophysiology of NAFLD

The pathophysiology of NAFLD is complex and multifactorial. In recent years, there has been a shift away from the two-hit hypothesis introduced in the 90s. This theory involved a first hit in the form of insulin resistance (IR), followed by a second hit characterized by oxidative stress with subsequent lipid peroxidation, the release of inflammatory cytokines and adipokines, and mitochondrial dysfunction, leading to the development of NASH **(Day, & James, 1998).** It is now evident that the underlying mechanism of NAFLD is more intricate, and it constitutes multiple parallel factors acting synergistically

in a genetically predisposed individual as part of an interorgan cross-talk between adipose tissue, liver, pancreas, and the gut **(Buzzetti *et al*., 2016).** That following an unhealthy diet and sedentary lifestyle has a key role in inducing hepatic de novo lipogenesis, with a subsequent influx of free fatty acids into hepatocytes, as well as promoting adipose tissue dysfunction with a release of adipokines and inflammatory cytokines. Fat deposition in the liver leads to increased lipotoxicity, followed by mitochondrial dysfunction and oxidative stress **(Marchesini *et al*., 1999; Cusi, 2009**; **Manne *et al*., 2018).** At the same time, an altered gut microbiome leads to increased bowel permeability and absorption of FFAs, raising their circulation levels and stimulating proinflammatory cytokine production. With the background of genetic factors and epigenetic changes, these events might affect hepatocyte fat content and liver inflammatory environment, causing a state of chronic inflammation with or without progression to hepatocyte death, activation of hepatic stellate cells, and deposition of a fibrous matrix **(Buzzetti *et al*., 2016).**

Lipid accumulation in nonadipose tissue is a key factor for the progression of insulin resistance, DM (Diabetes mellitus), and cardiovascular disease **(Farrell, 2009).**

Apart from the risk of liver fibrosis/cirrhosis, patients with NAFLD have an increased risk of all-cause mortality, especially due to cardiovascular disease (CVD). This risk is attributed to the common predisposing factors for both NAFLD and CVD, with the endothelium emerging as a key player. Recent data indicate that both vascular endothelium dysfunction and liver sinusoidal endothelial cell LSEC dysfunction play particularly significant roles not only in the pathogenesis and progression of NAFLD but also in the interplay between CVDs and NAFLD (**Nasiri-Ansari *et al*., 2022).**

1.3.4.1.2 Epidemiology of NAFLD

The estimated prevalence of non-alcoholic fatty liver disease (NAFLD) worldwide is approximately 25% **(Abdul-Rasool *et al*., 2022).** However, the real prevalence of NAFLD and the associated disorders is unknown mainly because reliable and applicable diagnostic tests are lacking. This is further complicated by the lack of consensus on the terminology of different entities such as NAFLD or nonalcoholic steatohepatitis (NASH). Although assessing fatty infiltration in the liver is simple by ultrasound, the gold standard for the assessment of fibrosis, the only marker of progression towards more severe liver disease is still liver biopsy. Although other non-invasive tests have been proposed, they must still be validated in large series **(Araújo et al., 2018).**

1.3.4.1.3. Risk Factors of NAFLD

The primary risk factors of NAFLD are obesity, type II diabetes, and metabolic syndrome including dyslipidemia and hypertension **(Angulo, 2007).** However, diseases other than metabolic syndrome can be associated with hepatic fat, and these might enter into the differential diagnosis of fatty liver disease of the usual type. Some of these have specific clinical and pathologic features that make their distinction from NAFLD straightforward **(Kneeman *et al*., 2012).**

- **Metabolic Syndrome**

MetS is a clinical entity that is defined as the presence of any 3 of the following 5 traits: (1) serum triglyceride (TG) level of 150 mg/dL or higher, (2) serum high-density lipoprotein (HDL) level of less than 40 mg/dL in men or less than 50 mg/dL in women, (3) systemic blood pressure of 130/85 mm Hg or higher, (4) fasting plasma glucose level of

100 mg/dL or higher, and (5) an increase in waist circumference (this differs based on national or regional cut points) **(Goyal, 2022).** A key role in NAFLD pathogenesis is played by metabolic dysregulation, as a consequence of insulin resistance and excessive accumulation of hepatic lipids, mainly in the form of triglycerides (TG) (**Bhalwar, 2020**).

NAFLD has been considered as a hepatic component of metabolic syndrome (MetS), **(Masella *et al*., 2005),** but recently an association between NAFLD and MetS in type 2 diabetes mellitus has been described but the phenomenon is very complex. Indeed NAFLD is mutually and bidirectionally linked with MetS of which it is both the cause and the consequences **(Villegas *et al*., 2011).**

- **Obesity**

Several studies involving severely obese adults have shown a high correlation between hypertriglyceridemia and NAFLD. Obesity is a significant independent risk factor for NAFLD development and progression. A study of 381,655 individuals reported that obesity increased the odds of NAFLD 3.5-fold **(Li *et al*., 2016).** This robust meta-analysis was controlled for confounding conditions including diabetes, hypertension, alcohol intake, and physical activity. In addition, each unit increase in BMI was positively correlated in a dose-dependent fashion to NAFLD risk **(Safari, & Gérard, 2019).** the reasoning for obesity-mediated NAFLD risk is based on increased IR and inflammation

- **Type 2 diabetes mellitus**

T2DM and insulin resistance have contributed to the development of NAFLD. Several studies have described a higher prevalence of NAFLD

among patients with T2DM compared with nondiabetics, with a prevalence estimated to be 40% to 69.5%. T2DM not only increases the prevalence of NAFLD but is also associated with more severe forms of NAFLD, including NASH and fibrosis **(Meroni *et al*., 2020).**

- **Genetic polymorphism**

Family studies and interethnic differences in susceptibility suggest that genetic factors play an important role in modulating the prevalence, severity, and progression of the disease. Polymorphisms in genes affecting lipid metabolism, oxidative stress, and inflammatory cytokines may be directly linked to the development and progression of steatohepatitis and fibrosis **(Riazi *et al*., 2022).**

- **Gender**

There are conflicting data on the influence of gender on NAFLD. Although early studies suggested that NAFLD was more common in women, recent studies have shown that NAFLD may be evenly distributed between women and men, or may even have a higher prevalence in men. In other studies, NAFLD was significantly more prevalent in men stratified cohorts into lean and overweight-obese groups and reported that lean NAFLD was more **(Lim, & Bernstein, 2018)**.

1.3.4.1.4. Stages of NAFLD

The different stages of non-alcoholic fatty liver disease (NAFLD). First, the healthy livers develop non-alcoholic fatty liver (NAFL) with hepatocellular steatosis as the main feature. If left untreated, NAFL may progress to a more severe form of non-alcoholic steatohepatitis (NASH), defined as inflammation and fibrosis in addition to hepatocellular steatosis. As the disease progresses,

NASH may progress to cirrhosis and even to hepatocellular carcinoma (HCC) **(Guo *et al.*, 2020)**.

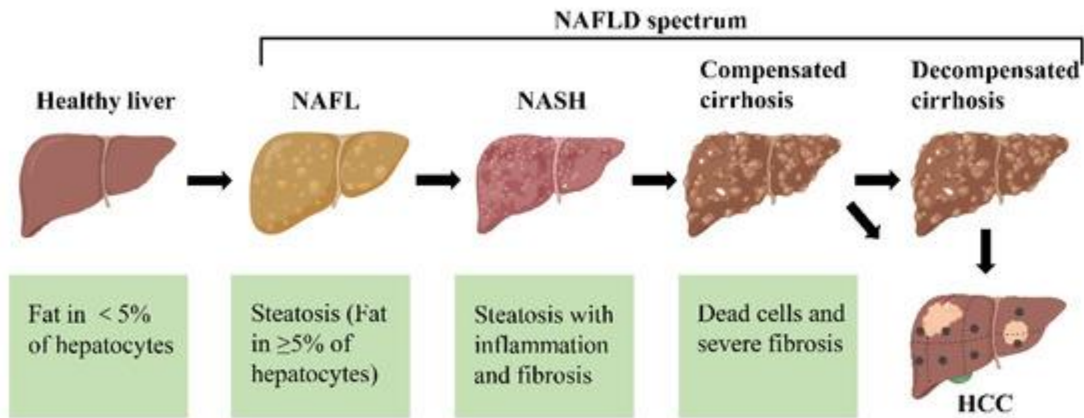


Figure 2: Illustrates the stages of progression of the non-alcoholic liver fat disease (Guo *et al.*, 2020).

1.3.4.1.5. Biochemical changes of NAFLD

Various changes in the biochemical profile can be observed in patients with the disease. Elevated serum transaminases level remains the most common or sometimes the only abnormal laboratory finding. Although the prime abnormality, liver enzymes may be normal in greater than 70% of the patients with NAFLD **(Obika, & Noguchi, 2011).** Serum levels of alkaline phosphatase (AL), γ-glutamyl transferase (GGT), or both are frequently elevated although the level is lower than in alcoholic hepatitis. There is also an increase in triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) levels posing a cardiovascular risk **(Siddiqui *et al.*, 2015).**

1.3.4.1.6. Diagnosis of NAFLD

Early diagnosis is very important in the timely management of NAFLD. But there is not a single biochemical marker for confirmation of NAFLD **(Pardhe *et al.*, 2018).**

Imaging studies play a key role in the diagnosis of NAFLD. The mainstay is ultrasonography; it is the least invasive, and relatively inexpensive. The sensitivity for an ultrasound to detect NAFLD is in the range of 60% to 90% with a specificity of around 90%. Unenhanced abdominal computed tomography and magnetic resonance are alternatives but are more costly and are not significantly superior to ultrasonography **(Kosmalski *et al*., 2018; Agganis *et al*., 2018; Ratziu *et al*., 2005).**

As proposed by the clinical care pathway from the American Gastroenterology Association (AGA), the first biochemical evidence alluding to a diagnosis of Non-Alcoholic Fatty Liver Disease (NAFLD) is the abnormal serum liver biochemistry Even though the majority of patients with NAFLD and NASH are asymptomatic, a mild elevation of liver function tests (usually less than five times the upper limit of normal) might be observed **(Kanwal *et al*., 2021).** It is important to highlight that the degree of aminotransferase rise does not reflect the degree of hepatocellular injury associated with NAFLD/NASH **(Giboney, 2005).** AST/ALT ratio (AAR) is a fundamental index used for the non-invasive staging of liver fibrosis and it is associated with advanced fibrosis and increased mortality in these patients **(Tahan *et al*., 2008).** GGT value is a component of various liver fibrosis diagnostic models, including FibroTest and Hepascore. The Prothrombin/International Normalised Ratio (INR) and albumin are also markers of hepatic synthetic function **(Kurokawa, & Ohkohchi, 2017**).

1.4. Oxidants and Antioxidants

Oxidative stress plays an essential role in the pathogenesis of chronic diseases such as cardiovascular diseases, diabetes, neurodegenerative diseases, and cancer. Long-term exposure to increased levels of pro-oxidant factors can

cause structural defects at a mitochondrial DNA level, as well as functional alteration of several enzymes and cellular structures leading to aberrations in gene expression. The modern lifestyle associated with processed food, exposure to a wide range of chemicals, and lack of exercise play an important role in oxidative stress induction **(Zuo *et al*., 2015).** Free radical oxidants such as reactive oxygen species, reactive nitrogen species, and reactive sulfur species are produced inside cells through various metabolic processes. The body is equipped with an antioxidant defense system that guards against oxidative damage caused by these reactive oxidants and plays a major role in protecting cells from oxidative stress and damage. Antioxidants such as glutathione (GSH), thioredoxin, ascorbic acid, and enzymes, for example, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) counter oxidative stress, and protect lipids, proteins, and DNA. Antioxidants such as tocopherols, ascorbic acid, carotenoids, flavonoids, and amino acids are also natural antioxidants present in foods. There is increasing demand and availability of designer foods fortified with antioxidants and probiotics that may be important in human health **(Ali *et al*., 2020).**

1.4.1. Oxidative Stress

The term ‘oxidative stress’ was first coined by Helmut Sies1 as an imbalance between the production of oxidants and antioxidant defenses that may result in damage to biological systems. Since then, the field of redox biology has evolved from concepts of oxidative stress in pathology to redox signaling in physiology **(Sies *et al*., 2017; Valko *et al*., 2007).** Oxidative stress has been shown to participate in a wide range of diseases including atherosclerosis, chronic obstructive pulmonary disease (COPD), Alzheimer

disease and cancer, which has revealed the multiple mechanisms by which oxidants contribute to cellular damage **(Forman *et al*., 2014)**. However, the extent to which oxidative stress participates in the pathology of diseases is quite variable, such that the effectiveness of increasing antioxidant defense may be limited in some diseases. Oxidative stress involves the chemistry of reactions of so-called reactive species derived from oxygen and nitrogen **(Lonardo *et al*., 2018).**

1.4.1.1. Reactive oxygen and Nitrogen species

Reactive oxygen species (ROS) are involved in many important cellular activities including gene transcription, signaling transduction, and immune response. Common ROS include hydroxyl radical (•OH), superoxide ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) **(Gilgun-Sherki *et al*., 2001).** Overproduction of ROS can result in oxidative damage to biomolecules such as lipids, proteins, and DNA, which has been implicated in the development of aging as well as various ailments including cancer, respiratory, cardiovascular, neurodegenerative, and digestive diseases. It is reported that the deleterious effects of excess ROS, or oxidative stress (OS), eventually lead to cell death **(Liu *et al*., 2018).** Reactive oxygen and nitrogen species (RONS) include two classes of chemically-reactive molecules containing oxygen (reactive oxygen species, ROS) and nitrogen (reactive nitrogen species, RNS). Both classes are referred to as RONS. The majority of RONS carry unpaired electrons and are called free radicals. In mammalians, a major function of specialized enzymes, such as NADPH-oxidase, myeloperoxidase, and nitric oxide synthase (NOS), is the generation of RONS. The controlled generation of RONS in the extracellular space by these enzymes was developed evolutionarily as part of the innate immune system to kill bacteria. However, an overwhelming release

of RONS may also induce deleterious effects, causing damage to host biological structures. Another group of enzymes releases RONS intracellularly as a byproduct of metabolic processes. For instance, superoxide ($O_2^{\bullet-}$) is released as a byproduct of mitochondrial respiration and monooxygenase activity of cytochrome p450. Intracellular RONS, as well as excessive release of extracellular RONS, were thought to induce deleterious effects, causing oxidative damage to different kinds of biomolecules. These processes are referred to as “oxidative stress” **(Alkadi et al., 2020).**

1.4.1.2. Free Radicals

Free radicals are the products of normal cellular metabolism. A free radical can be defined as an atom or molecule containing one or more unpaired electrons in a valency shell or outer orbit and is capable of independent existence. The odd number of electron(s) of a free radical makes it unstable, short-lived, and highly reactive. Because of their high reactivity, they can abstract electrons from other compounds to attain stability. Thus the attacked molecule loses its electron and becomes a free radical itself, beginning a chain reaction cascade that finally damages the living cell **(Pham-Huy *et al*., (2008); Valko *et al*., (2007).** Both ROS and RNS collectively constitute free radicals and other nonradical reactive species. The ROS/RNS play a twofold job as both beneficial and toxic compounds to the living system. At moderate or low levels ROS/RNS have beneficial effects and involve various physiological functions such as immune function (i.e. defense against pathogenic microorganisms), several cellular signaling pathways, mitogenic response, and in redox regulation **(Nordberg, & Arnér, (2001); YlÄ-Herttuala, (1999).** But at higher concentrations, both ROS as well as RNS generate oxidative stress and nitrosative stress, respectively, causing potential damage to the biomolecules.

Oxidative stress and nitrosative stress are developed when there is an excess production of ROS/RNS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other side. Most importantly, the excess ROS can damage the integrity of various biomolecules including lipids **(Stadtman, & Levine, 2000)**, proteins (Marnett, **(2000**), and DNA **(Phaniendra *et al*., 2015),** leading to increased oxidative stress in various human diseases such as diabetes mellitus, neurodegenerative diseases, rheumatoid arthritis, cataracts, cardiovascular diseases, respiratory diseases as well as in aging process **(Erickson, (1997).**

Reactive oxygen species (ROS) are generally small, short-lived, and highly reactive molecules that are formed by incomplete one-electron reduction of oxygen. ROS are generated by multiple cellular organelles, including mitochondria, peroxisomes, and endoplasmic reticulum **(Dubois-Deruy *et al*., 2020); Chen, & Zweier, (2014).** ROS can also be produced in Fenton and Haber-Weiss reactions, thymidine catabolism, and polyamine catabolism. Mitochondria are the major source of ROS generation, as a by-product of respiration (**Merksamer *et al*., 2008)** As shown in the figure **(3).**

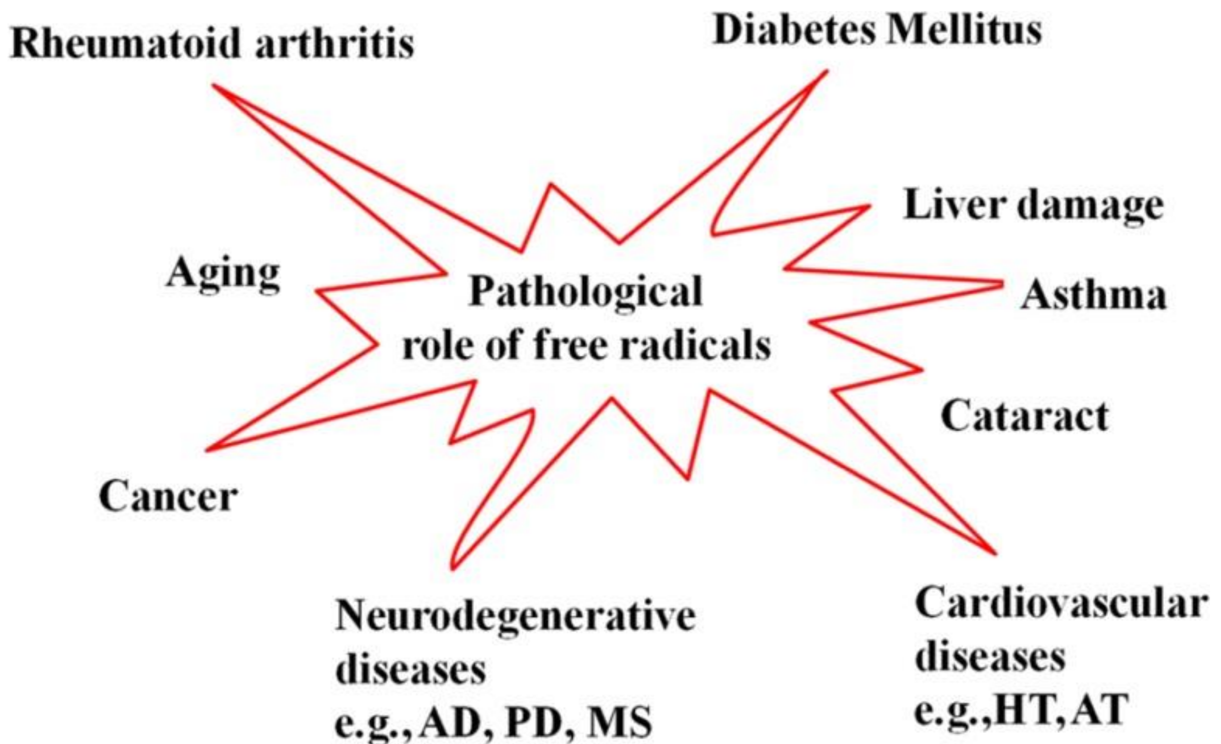


figure 3: Pathological role of free radicals (Erickson, (1997).

Free radicals are generated in our bodies by several systems. A balance between free radicals and antioxidants is an important matter for appropriate physiological function. If free radicals become greater than the ability of the body to control them, a case known as oxidative stress appears, and as a result of that, several human diseases spread in the body. Antioxidants can contribute to facing this oxidative stress **(Suroowan et al., 2022).**

1.4.1.3. Lipid peroxidation

Lipids have an important role in human homeostasis as they perform three primary biological functions within the organism: they serve as structural components of cell membranes, function as energy storehouses, and act as signaling molecules. There is a wide diversity of lipid composition described in cell membranes, organelles and tissues **(van Meer, (2005).** and in both the

current classification systems, lipids are grouped into eight categories. The cellular lipid profile comprises more than 1000 different molecular species **(Pamplona *et al*., 2019).** Polyunsaturated fatty acids (PUFAs) are the most abundant constituents, mainly located at the sn-2 position in glycerophospholipids within cellular membranes. It is known that PUFA residues of lipids are very sensitive to oxidation, and this sensitivity increases so does the number of double bonds per fatty acid molecule **(Sies, & Jones, 2020).** Lipid peroxidation often occurs in response to oxidative stress, where reactive oxygen species (ROS) cause the oxidation of lipids containing carbon-carbon double bonds in membrane lipid bilayers. ROS may be defined as "derivatives of molecular oxygen that occur as a normal attribute of aerobic life" **(Esterbauer *et al*., 1999).** Lipid peroxidation occurs in three major phases: initiation, chain propagation, and termination. A single initiation reaction will result in 200–400 propagation cycles, giving rise to unsaturated aldehydes 4-hydroxy-2-nominall and acrolein), dialdehydes (malondialdehyde and glyoxal), and ketoaldehydes (4-oxo-2-nominal and isoketals) (**Sies, & Jones, D. P. (2020).** Some of them are highly reactive and are considered second toxic messengers, which disseminate and magnify oxidative damage **(Barrera *et al*., 2018)**. The most commonly determined aldehydes so far are 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). The former is endowed with the highest biological activity and the latter, highly produced during lipid peroxidation, is commonly used as a measure of oxidative stress **(Ayala et al., 2014).** Arachidonic acid (AA) is the main precursor of bicyclic endoperoxide **(Puchau *et al*., 2009).**

1.4.1.3.1. Malondialdehyde

MDA originates from polyunsaturated fatty acids when a carbon-carbon double bond is attacked by a free radical, resulting in the formation of unsaturated lipid radicals with H_2O release. Further O_2 capture will lead to the formation of peroxyl radicals and lipid hydroperoxides. On the one hand, the peroxyl radical can develop cyclization thanks to their *cis*-double bond homoallylic to the peroxyl group. The intermediate free radicals formed after cyclization can cyclize again to form bicycle endoperoxides, structurally related to prostaglandins, and undergo cleavage to produce MDA. As shown in (**Figure 4**).

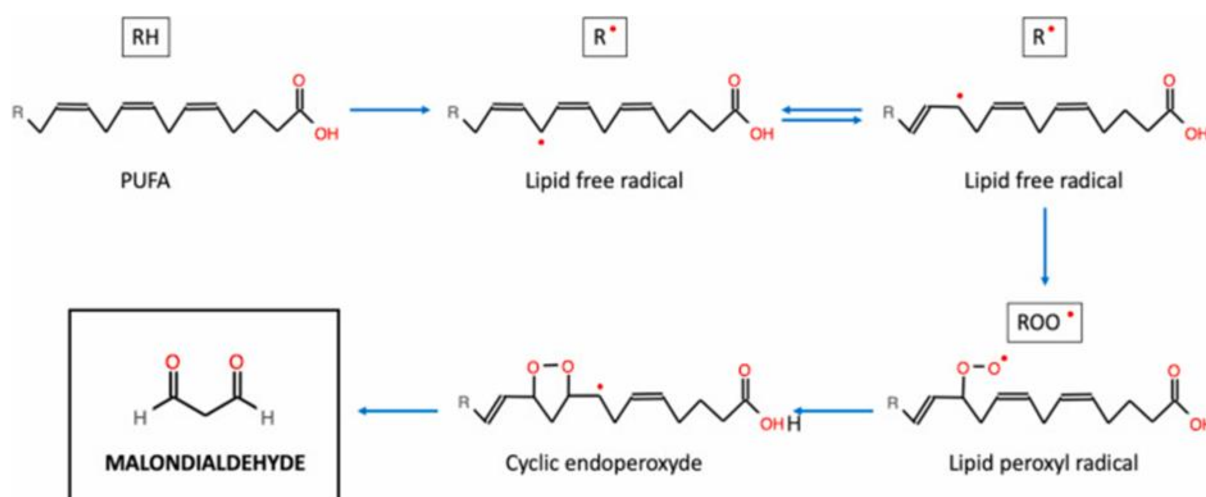


Fig. 4. MDA formation through lipid peroxidation (Nilsson, *et al*., 2019)

1.4.2. Diseases Associated with Oxidants

The overproduction of reactive oxygen species (ROS) has been implicated in the development of various chronic and degenerative diseases such as cancer, respiratory, neurodegenerative, and digestive diseases. Under physiological conditions, the concentrations of ROS are subtly regulated by antioxidants, which can be either generated endogenously or externally supplemented. A

combination of antioxidant deficiency and malnutrition may render individuals more vulnerable to oxidative stress, thereby increasing the risk of cancer ccurrence. In addition, the antioxidant defense can be overwhelmed during sustained inflammation such as in chronic obstructive pulmonary diseases, inflammatory bowel disease, neurodegenerative disorders, cardiovascular diseases, and aging **(Madamanchi et al., 2005).**

The majority of CVD is correlated with atherosclerosis development, in which OS plays a causal role **(Azumi *et al*., 2002).** Excessive ROS can be generated in vascular cells from NAD(P)H oxidase (Nox), nitric oxide synthases (NOS) uncoupling, and mitochondria, which cause oxidative modifications of low-density lipoprotein (LDL) **(Cachofeiro, *et al*., 2008).** The oxidized LDL (ox-LDL) transported through the arterial lumens induces apoptosis of endothelial cells and smooth muscle cells (SMCs). By taking up ox-LDL, macrophages may transform into foam cells, which secrete growth mediators to attract SMCs into the intima. SMCs can secret an extracellular matrix that forms a thin fibrous cap surrounding the fatty streak **(Heitzer *et al*., 2001)** With the continuous propagation of SMCs, monocytes, and macrophages, fatty streaks are ultimately converted into more advanced fibrous plaque **(Cachofeiro *et al*., 2008),** potentially leading to vessel occlusion **(Heitzer *et al*., 2001).** Further, OS has also been implicated in the development of cardiac hypertrophy, ischemic reperfusion injury, and myocyte apoptosis, all of which may contribute to heart failure **(Fukai, & Ushio-Fukai,** (**2020**).

1.4.3. Antioxidants

Antioxidant defense is an important part of organisms' adaptation to environmental stresses. Cells have developed different antioxidant responses to maintain redox homeostasis including endogenous antioxidant and redox-

dependent transcriptional regulation pathways. Antioxidant molecules are nucleophilic and react with oxidants, which are generally electrophiles **(Cao et al., 2019).**

Antioxidants are generally classified as primary antioxidants, which react directly with free radicals and thereby inhibit the propagation step, or secondary antioxidants which inhibit the initiation and propagation reactions **(Valencia-Perez *et al*., 2015).** The main function of primary antioxidants is to donate hydrogens to the lipid-free radical, which turns itself into a free radical. The antioxidant free radical then can react with other lipid peroxide radicals or other antioxidant free radicals to finish the reaction. Several primary antioxidants are endogenous in food systems, such as tocopherols, ascorbic acid, flavonoids, carnosine, and glutathione **(Buettner, 1993).** The effectiveness of primary antioxidants depends on their chemical structure, including their ability to donate electrons, and on their antioxidant radical stability **(Bjerkeng, & Johnsen,** (**1995**).

Secondary antioxidants are oxygen liberators and chelators. Some examples of these types of compounds are superoxide dismutase, catalase, glutathione peroxidase, and carotenoids, which act by decreasing the active oxygen levels. Chelating agents include citrates, phosphates, ceruloplasmin, and some free amino acids. **(Kinoshita *et al*., 1986).** Antioxidants are added directly to food; however, sanitary regulations restrict their direct use in some products, so there is special interest in incorporating them into the product packaging **(Guerra-Araiza *et al*., 2013).**

1.4.3.1. Exogenous Antioxidant

Non-enzymatic antioxidants, include carotenoids, vitamins, flavonoids, non-flavonoids, organosulfur compounds, mitochondria-targeted antioxidants, and minerals **(Park *et al*., 2020).** Carotenoids are colorful pigments that are found in fruits, vegetables, and seaweeds, with important anti-inflammatory, antioxidant, and anti-apoptotic activity **(Young, & Lowe, 2018; Nishiumi *et al*., 2011)** Flavonoids, a class of polyphenolic compounds produced by plants, are abundant in fruits and vegetables, as well as in tea **(Shahidi, & Ambigaipalan, 2015).** These compounds possess many biological properties, including antioxidants, and anti-inflammatory and minerals such as selenium, zinc, iron, and copper **(Cho *et al*., 2020; Pradas et al., 2018**).

Under conditions such as aging and many diseases, endogenous antioxidant defenses seem to be inadequate to prevent free radicals damage to DNA, lipids, proteins, and other biomolecules. So, the development and the use of different exogenous antioxidants that might ameliorate neural injury by oxidative stress had been increased during the last years. In this context, natural antioxidants like flavonoids (quercetin, curcumin, luteolin, and catechins), as well as magnolol and honokiol, are shown to be efficient inhibitors of the oxidative process and they have been considered as a better therapeutic option with the traditional antioxidants like vitamin C, vitamin E, and β -carotene. Nevertheless, as flavonoids, magnolol, honokiol, and traditional antioxidants have some different targets and mechanisms of action as well as different functions, if they will be used in combination, better results could be obtained on different processes and pathologies **(Halliwell, 2007).**

Vitamins regulate various key enzymatic processes in the liver, and alterations in vitamin metabolism are reported to play a crucial role in NAFLD progression.

Vitamins A, C, and E are well-studied against NAFLD due to their antioxidant activities. Similarly, modulation of vitamins D and B12 and folate levels in serum also had a strong correlation with NAFLD severity **(Bouayed, & Bohn, (2010).**

1.4.3.1.1. Selenium

Selenium deficiency has been associated with incidences of CVD for example Keshan disease which is characterized by cardiomyopathy, a disease that was endemic in some parts of China with low selenium soils. Experiments that focused on selenium deficiency in the development of CVD indicated that cardiac pathologies may be due to increased oxidative stress and Reactive oxygen species (ROS) generated during ischemia appeared to damage the myocardium and its blood vessels leading to poor post-ischemic recovery. In animal experiments, selenium supplementation was used to reduce the production of ROS during ischemia and reperfusion injury by increasing the activity of GPx-1 Selenium supplementation has also been suggested to reduce mortality among people with very low selenium intake, as observed after supplementation for a period of four. Although the above observations indicate a relationship between suboptimal selenium intake and the incidence of CVD, evidence of selenium having protective effects on CVD is still inconclusive. While some studies indicate that suboptimal selenium is an independent risk factor for myocardial infarction, others have not found this to be the case. A study by Lubos *et al.* found an association of low selenium levels with increased risk of the acute coronary syndrome but not with stable angina pectoris **(Lubos *et al.*, 2010**), while another study by Vinceti *et al* suggests that chronic overexposure to environmental selenium may increase blood pressure **(Vinceti *et al*. 2019**). Evidence on the association between low selenium levels and

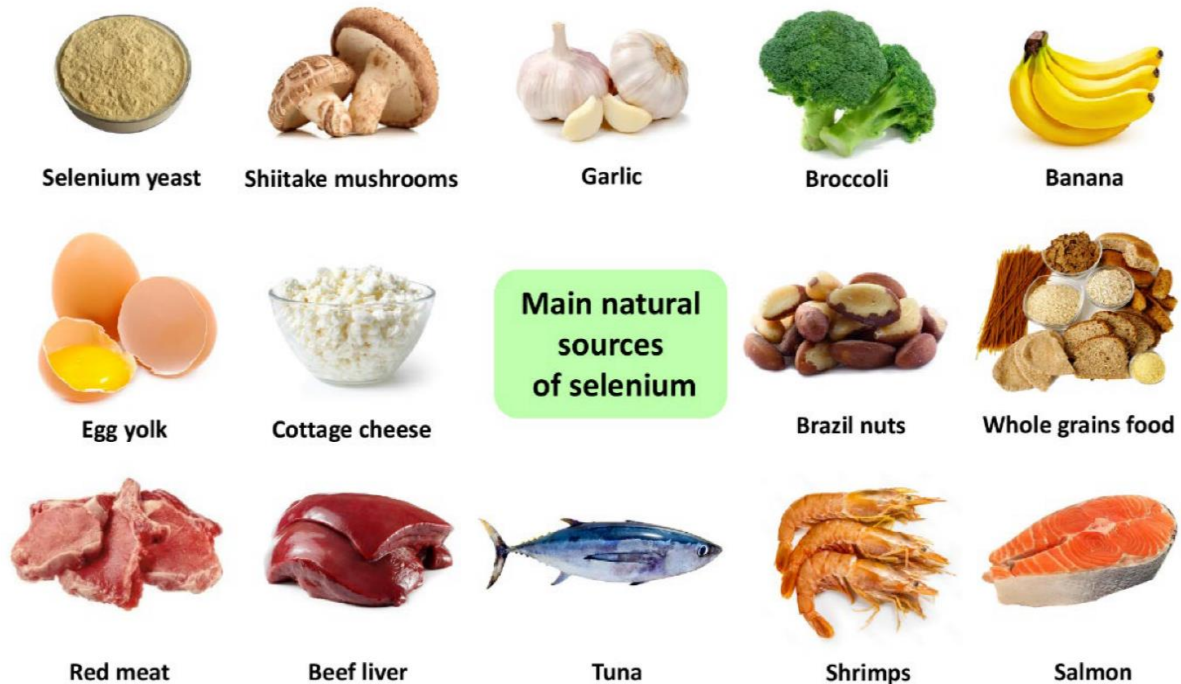
increased mortality is also conflicting. While some studies indicate that selenium can reduce mortality, others have found no association or even indicate that selenium in certain levels could increase mortality. The daily selenium intake should be within the recommended daily allowance (50–300 μg/day) to prevent the harmful effects of selenium that may occur at levels beyond 300 μg/day. There is a need for further studies on specific types of CVD and selenium as some seem to be more associated with selenium than others **(Kuria *et al*., 2021).** Numerous scientific findings linking low selenium levels with increased cardiovascular risk have raised high expectations for the beneficial effects of its supplementation. Unfortunately, the results of clinical trials conducted so far are truly diverse, and the meta-analyses consistently report the lack of impact of supplementation on reducing the risk of incidence and mortality due to cardiovascular diseases.Despite the trace amount of selenium and zinc in the body, these elements are necessary for its proper functioning. Their impact on maintaining the body's oxidoreductive balance and protecting cells from free radicals is particularly important. Both their deficiency and excess are unfavorable for health. Keshan disease has a confirmed cause and effect relationship with selenium deficiency. Findings on the relationship between selenium and other cardiovascular diseases such as atherosclerosis **(Yang *et al*., 2022)**

The presence of high Selenium as antioxidant selenoenzymes and selenoproteins may help to reduce the production of oxidized LDL and, therefore, would reduce the incidence of heart diseases **(Rocca *et al*., 2019)**.

- **Sources of Se in the Human Diet**

Organic Se from food is considered a safe and efficient source of supporting human health. Among the sources of organic Se for humans, It found in foods

of animal, vegetable, and mushroom origin. The main animal sources of Se are red meats, poultry, beef or sheep liver, seafood, eggs, and dairy products. The main kinds of food containing Se in comparatively high amounts are shown in **Figure 5.**



Figur 5. Main natural sources of selenium (Thiry, *et al*., 2012)

1.4.3.2. Endogenous Antioxidants

Antioxidants can be described as a system that protects biomolecules and the organism against the harmful effects of free radicals and reduces or repairs the damage done by ROS to the target molecule, and this is called antioxidant defense such as superoxide dismutase, catalase, glutathione peroxidase **(Martín-Sierra et al., 2019; Varesi *et al*., 2023).**

A. Enzymatic

the enzymatic antioxidants are the first line of defense and act by breaking down and removing free radicals. Some examples are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and peroxiredoxins (PRDXs) **(O'Flaherty, 2014).** These antioxidant enzymes have different expression profiles, subcellular locations, and substrates, which show the complex nature of redox control. In general, SOD, CAT, and GPX act synergistically to convert superoxide anion (O2 -•) to hydrogen peroxide and then to water, using cofactors such as copper, zinc, manganese, and iron **(Bouayed, & Bohn, 2010).**

B. Non-Enzymatic

Non-enzymatic direct antioxidants are typically small molecule scavengers that bind ROS such as endogenous uric acid, glutathione (GSH), LA, NADPH, coenzyme Q, albumin, and bilirubin **(Malekmohammad, Sewell, & Rafieian-Kopaei, 2019).**

Lipoic acid LA, an endogenous antioxidant with multiple biological functions, also termed α-lipoic acid, pyruvate oxidation factor, thioctic acid, and lipoate, is a fatty acid containing 8 carbons and 2 sulfur molecules in a dithiolane ring. Lipoic acid is synthesized by mitochondria. It is a cofactor for mitochondrial α-ketoacid dehydrogenases (e.g., the pyruvate dehydrogenase complex), and it inhibits atherosclerotic lesion development **(Waslo *et al*., 2019).**

Coenzyme Q is present in cellular membranes. It is a lipophilic antioxidant with anti-inflammatory properties, and coenzyme Q10 is the main form found in humans. CoQ inhibits lipid and protein oxidation and reduces the conversion of α-tocopherol radical to α-tocopherol. It is capable of scavenging peroxyl radicals, thereby improving endothelial function **(Waslo *et al*., 2019).**

Bilirubin the key actions of bilirubin as an endogenous non-enzymatic antioxidant *via* scavenging oxidants, inhibiting protein oxidation, inhibiting in *vivo* leukocyte adhesion to endothelial cells, and the phosphorylation of retinoblastoma tumor suppressor protein in addition to p38 mitogen-activated protein kinase (p38 MAPK) **(Malekmohammad, & Rafieian-Kopaei, 2019**).

Uric acid is the end-product of purine catabolism. There are two immediate sequential precursors-hypoxanthine followed by xanthine—the conversions of which are both catalyzed by xanthine oxidase. Mechanistically, uric acid increases cytokine production, scavenges OH as well as HOCl, and incites inflammatory responses, endothelial dysfunction, and plaque instability **(Ames et al., 1981).**

1.4.3.2.1. **Glutathione**

Glutathione (GSH), a ubiquitous low molecular weight thiol, is considered the most abundant endogenous antioxidant molecule **(Cao *et al*., 2019**). GSH is a reduced peptide consisting of three residues (*γ*-l-glutamyl-l-cysteinyl glycine), which can donate an electron to form oxidized GSSG. Alterations in the ratio of the redox pair 2GSH/GSSG towards a more oxidized status form the biochemical basis of targeting redox-sensitive cysteine residues in proteins. As an antioxidant, GSH removes ROS directly or indirectly and limits the lifetime

of the oxidative signal **(Diaz-Vivancos *et al*., 2015)**. GSH is also a substrate of several antioxidant enzymes. The indirect ROS-scavenging functions of GSH by revitalizing other antioxidant enzymes are also very important Glutathione is an important small water-soluble tripeptide antioxidant present in cells **(Generation, 2019).**

1.4.3.3. Total Antioxidant Capacity

an individual antioxidant or food item may not accurately reflect the total antioxidant capacity of a diet, therefore, dietary total antioxidant capacity (DTAC) has been developed and attracted considerable attention **(Puchau *et al*., 2010).** DTAC has been proposed as a method for studying the potential beneficial impacts of whole dietary antioxidants and has been found to have a strong correlation with plasma antioxidant capacity **(Psaltopoulou *et al*., 2011)**. A wide range of studies indicated that plasma TAC levels are improved by consuming antioxidant-rich food sources such as fruits and vegetables. According to several studies, higher DTAC might beneficially affect various metabolic disturbances. Furthermore, diets high in antioxidants could negatively be associated with plasma C-reactive protein (CRP), a major biomarker for systemic inflammation, and in turn, triggering metabolic disorders, such as central obesity and glucose intolerance. In addition to glucose abnormality, a cross-sectional study showed that higher DTAC was associated with lower odds of impaired lipid profiles **(Mohammadi, *et al*., 2022).** Total antioxidant intake was also linked to lower body mass index (BMI) and systolic blood pressure. In addition, decreased TAC of plasma has been associated with metabolic syndrome in adults **(Psaltopoulou *et al*., 2011).**

1.5. Metabolic Syndrome and Coronary Artery Disease

The metabolic syndrome predicts cardiovascular disease and T2D. The risk associated with the metabolic syndrome does not exceed its components, whereof elevated blood pressure is the most frequent **(Nilsson, Tuomilehto, & Rydén, 2019).**

1.6. Mesencephalic astrocyte-derived neurotrophic factor (MANF)

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a novel evolutionarily conserved protein present in both vertebrate and invertebrate species. MANF shows distinct structural and functional properties than the traditional neurotrophic factors (NTF). MANF is composed of an N-terminal saposin-like lipid-binding domain and a C-terminal SAF-A/B, Acinus, and PIAS (SAP) domain connected by a short linker. The two well-described activities of MANF include:

- its role as a neurotrophic factor that plays direct neuroprotective effects in the nervous system
- cell protective effects of non-neuronal diseases, include retinal damage, diabetes mellitus, liver injury, myocardial infarction, nephrotic syndrome **(Wu, *et al*., 2021).**

MANF is also known as arginine-rich mutated in early tumors **(Ezhilarasan, & Lakshmi, 2022).** Independent *in vitro* studies have demonstrated that MANF can influence the biological function of different cell types that participate in the regenerative response:

- cytoprotection of damaged cells
- regulation of immune cells

- regulation of stem cell function (although this has only been demonstrated for NSCs and may be associated with its neurotrophic function **(Tseng et al., 2018; Sousa-Victor, Jasper, & Neves, 2018).**

A lot of previous studies have shown that MANF protects normal cells against the apoptosis induced by various stimuli and promotes the survival and proliferation of normal cells **(Yang *et al*., 2023).** Proposed model for the function of MANF in tissue repair through a synergistic activity as an inhibitor of apoptosis and inflammation As shown in the **figure (6).**

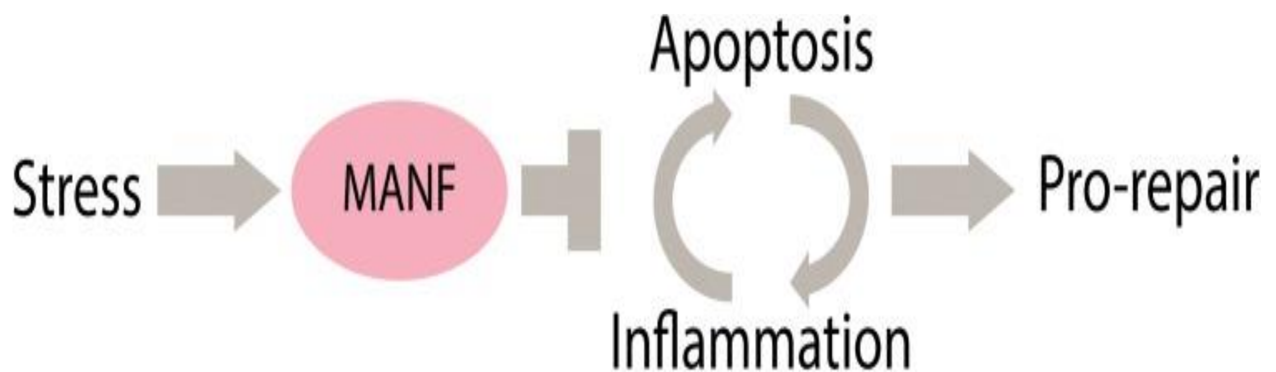


FIGURE: 6. Function of MANF in tissue repair through a synergistic activity as an inhibitor of apoptosis and inflammation (Tadimalla *et al*., 2008).

1.6.1. Chemistry of MANF

Cloning and sequencing of the MANF cDNA verified that MANF encoded by a 4.3 kb gene with 4 exons is located on human chromosome 3 **(Mizobuchi *et al*., 207).** The MANF primary transcript encompasses 1109 bp, which codes for a predicted 179 amino acid protein. The N-terminal 21 amino acids serve as the signal peptide, targeting nascent MANF to the ER. The secreted form of MANF is the full-length protein without the signal sequence, i.e. 158 amino acids with a molecular weight of 18 kDa **(Hellman *et al*., 2011).**

Nuclear magnetic resonance (NMR) spectroscopy of the three-dimensional solution structure of human MANF reveals that MANF is composed of an N-terminal saposin-like domain (residues 1–95) and a C-terminal SAP (SAF-A/B, Acinus, and PIAS) domain (residues 104–158), which are connected with a short linker (residues 96–103) **(Kim, Park, & Chen, 2017).**

1.6.2. Biochemical Roles for MANF

Although MANF was initially discovered by their neurotrophic activities, further studies revealed that MANF is highly expressed in non-neural tissues and that their cytoprotective activity extends beyond the dopaminergic system **(Mätlik *et al*., 2018; Neves *et al*., 2016).** Moreover, MANF is found in circulation in the blood and it has been associated with several non-neuronal diseases in humans, including inflammatory diseases **(Yavarna, et al., 2015; Galli *et al*., 2016).** suggesting a role beyond neuroprotection. Consistently, recent data suggest that cytoprotection accounts only partially for MANF biological activity. The study found that MANF can also act directly on immune cells and modulate their inflammatory phenotype by reducing pro-inflammatory signaling and promoting pro-reparative activation of macrophages and that this function is required for the protective function observed in *vivo* **(Petrova *et al*., 2003).** MANF is an ER stress-inducible protein originally identified as a survival-promoting factor for brain dopaminergic neurons. MANF can be induced by several ER stress inducers in many cell types in *vivo* and in *vitro* and can exert a protective role against ER stress-induced damages. The pancreatic-specific MANF deficiency leads to ER stress, and apoptosis of pancreatic beta cells and diabetes, whereas recombinant MANF enhances pancreatic beta cell proliferation. In addition, MANF has also been shown to

protect human pancreatic beta cells against experimentally stress-induced cell death **(Tseng *et al*., 2017).**

1.6.3. MANF in metabolism regulation

- **Effect of MANF on glucose homeostasis**

Impaired glucose homeostasis is often caused by decreased insulin sensitivity in multiple tissues and/or insufficient insulin secretion in dysfunctional pancreatic β cells. Others found a correlation between circulating MANF levels and insulin resistance in patients with prediabetes and T2DM **(Tang, & He, 2022).**

- **Effect of MANF on lipid metabolism**

Recent studies revealed the emerging importance of MANF in lipid metabolism. Clinical studies found upregulated serum MANF levels in patients with hyperlipidemia and MANF levels were negatively correlated with total cholesterol and low-density lipoprotein cholesterol levels **(Fu et al., 2021).**

- **Effect of MANF on energy balance**

As compared with the canonical neurotrophic factors, MANF involved in energy homeostasis is only beginning to emerge and its regulatory roles in the central nervous system and peripheral tissues seem inconsistent. MANF is abundantly expressed in the brain.

The high level of MANF in the hypothalamus persists into adulthood, although its expression in other brain regions decreases as the brain matures **(Wang *et al*., 2014).**

- **Effect of MANF on Inflammation**

Chronic low-grade inflammation plays a key role in the initiation, propagation, and development of metabolic diseases.

Evidence has linked MANF to metabolism-related systemic inflammation, including its anti-inflammatory effects in pancreatic β cells, liver, and adipose tissues. A series of studies demonstrated that MANF is highly expressed in several immune cell types of sponges, flies, and mammals and is dynamically regulated in response to damage and inflammatory signals. The exact mechanisms remain largely unknown, but all evidence to date supports that increasing MANF expression leads to inhibiting proinflammatory signaling **(Neves, *et al*., 2016).**

1.6.4. Clinical Considerations of MANF in chronic metabolic disease

Metabolic dysfunction-associated fatty liver disease, Cardiovascular diseases, Diabetes, Obesity, and Kidney diseases **(Tang, & He, 2022).**

1.6.4.1. MANF in Liver Disease

The liver is an important metabolic organ that modulates lipid homeostasis and multiple studies have revealed the critical role of MANF in regulating hepatic lipid metabolism. In fact, MANF is highly abundant in the liver **(Wu *et al*., 2021).** Metabolic dysfunction-associated fatty liver disease (MAFLD) is a redefinition of the previous term non-alcoholic fatty liver disease **(Eslam *et al*., 2020).** Studies of human and animal models implicated MANF in NAFLD pathogenesis. Serum MANF level was significantly reduced in non-alcoholic steatohepatitis patients. In addition, both *in vitro* and *in vivo* studies have demonstrated the critical roles of MANF in suppressing lipogenesis **(He *et al*., 2020).** Also, decreased MANF expression led to increased liver inflammation and fibrosis, whereas MANF supplementation could reverse age-dependent inflammatory changes **(Sousa-Victor *et al*., 2019).**

1.6.4.2. MANF in Heart Disease

The pathogenesis of cardiovascular diseases (CVDs) is complex, with ER stress and inflammation playing important roles. In line with other tissues or cell types, intracellular MANF also exhibited protective functions against ER stress and inflammation in the cardiac context, including in ischemia/reperfusion **(Arrieta *et al*., 2020),** and myocardial infarction *in vivo*, as well as simulated ischemia in *vitro* (**Tadimalla *et al*., 2008**). More importantly, recombinant MANF showed a protective function when added to cultured cell medium *in vitro*. In fact, the expression of MANF in the heart is relatively low under basal conditions, whereas under ER stress stimuli, MANF expression and secretion are significantly upregulated in the cardiac context **(Danilova *et al*., 2019).** This induction may be a compensatory response repressing the chronic ER stress and inflammation in the heart, which supports the protective role of MANF. Clinical studies found a negative correlation between circulating MANF levels and atrial apoptosis in human chronic atrial fibrillation **(Wang *et al*., 2020).** Also, a significantly decreased circulating MANF level was found to be correlated with an increased risk of future CVD in adult patients **(Ren *et al*., 2021).**

Aim of Study:

1.To know the extent of the effect of non-alcoholic fatty liver disease on obstructive coronary artery disease.

2.and obstructive coronary artery disease. To know the effect of Mesencephalic Astrocyte-Derived Neurotrophic Factor in diagnosing non-alcoholic fatty liver disease

3. Studying the various markers included in this study in each disease as compared with control group.

4.Association with various oxidants; antioxidant Parameters and serum liver enzymes activity level

Chapter two

Material and Method

2. Materials and Methods

2.1. Subjects

2.1.1. Study Design

A case-control study was conducted in the Department of Biochemistry, College of Medicine, University of Karbala. This study was accomplished for the period of November 2022 to May 2023.

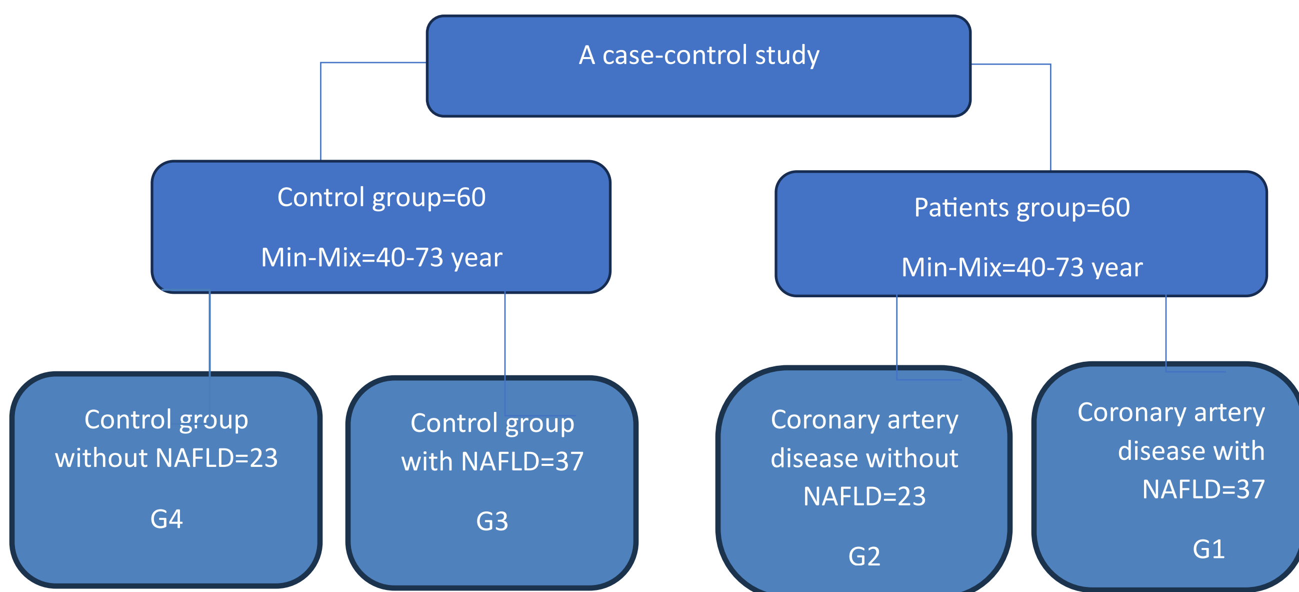


Figure: (2.1) Scheme of the study design

2.1.2. Patients

Patients with coronary artery disease were selected from Karbala heart center. The age range of the patients (40-70) years, and six 45 male and 15 female. The numbers patients with NAFLD 37 and patients without NAFLD 23.

A particular questionnaire form including descriptive information was designed and filled with each patient. The questionnaire included names, ages, sex, age groups, physical activity, smoking, family history of other diseases, BMI by examination and severity of coronary artery disease by Percutaneous coronary intervention PCI.

Inclusion Criteria

All patients were subjected to the full clinical history and clinical examination. The diagnosis of the chronic coronary arteries clinical conditions cases was identified based on signs and symptoms diagnostic by Consultant physician Dr. Ahmed Qasim Al-Haidari, Electrocardiogram (ECG), Echocardiogram (ECHO), Coronary computed tomography (CCT), Coronary Angiogram, and Percutaneous coronary intervention PCI, patients with NAFLD diagnostic by Consultant physician and diagnostic by used ultra sound.

Exclusion Criteria

Patients with the Presence of alcoholic Fatty Liver diseases, Acute Coronary Syndrome, and Acute Heart Failure were excluded from this study.

2.1.3. Control group

A control group of 60 subjects (15 female and 45 male) was chosen from well-known volunteer participants the age of control group (40-70) years.

2.1.4. Approval of the ethical committee

The protocols of the study were approved by Ethical Committee after a verbal written informed consent for participation and for taking the blood for investigations from everyone in rolled in this study.

2.1.5. Sample Collection

Five milliliters of fasting venous blood were drawn from all subjects by using a sterile disposable syringe. Blood was put into a gel tube and left at room temperature for nearly 50 minutes for clotting, then separated into five Eppendorf tubes and stored at -20 °C in the deep freezer. One Eppendorf was used to measure serum MANF by using the enzyme-linked immune sorbent assay (ELISA) technique, Two Eppendorf tube was used to measured serum MDA, serum Glutathione peroxidase, and serum Total antioxidant capacity by colorimetric methods by using Spectrophotometer and Three Eppendorf tube was used to measured serum lipid profile, and liver function test by using Auto analyzer SMART-120 and four Eppendorf tube were used to measured Selenium measured by Atomic absorption.

2.2. Chemicals and Kits

The Kits used in current study were summarized in **Table (2.1).**

Table (2.1): Chemicals and kits used in current study and their suppliers

No.	Chemicals and Kits	Company and Country
1	ELISA Mesencephalic astrocyte-derived neurotrophic factor(MANF) human serum (cat no YLA3587HU:)	Shanghai YL Biont/ China
2	Cholesterol Kit	GIESSE/Italy
3	Alanine Transaminase Kit	GIESSE/Italy
4	Aspartate Transaminase Kit	GIESSE/Italy
5	Alkaline phosphate Kit	GIESSE/Italy
6	HDL-Cholesterol Kit	GIESSE/Italy
7	Hydrogen peroxide	BDH/UK
8	Bilirubin Kit	GIESSE/Italy
9	Phosphate buffer	BDH/UK
10	Albumin Kit	GIESSE/Italy
11	Thiobarbituric acid (TBA)	BDH/UK

12	Triglyceride kit	GIESSE/Italy
13	Copper(II) chloride Solution $CuCl_2 \cdot 2. H_2O$	BDH/UK
14	{2,9-dimethyl-1,10-phenanthroline}	BDH/UK
15	[5,5'-Dithio-bis-(2-nitobenzoicacid) (DTNB)].	BDH/UK
16	Ammonium acetate (NH_4Ac)	BDH/UK
17	TDAC	BDH/UK
18	Glutathione	BDH/UK

2.3. Instruments and Lab Equipment

The instruments and laboratory tools used in this study were summarized in Table (2.2).

Table (2.2): Instruments and Lab Equipment used in this study.

No	Instruments and Lab Equipment	Company and Country
1	Atomic absorption spectrometry	SHIMADZU AA-6300/ Japan
2	Auto analyzer SMART-120	Geno Lab TEK/ USA
3	Centrifuge	Kokusan/ Germany
4	Deep freezer	Fisher Scientific/ USA
5	ELISA instrument system	Bio Tek/ USA
6	Gel tube (6ml)	Arth AL-Rafidin/ China
7	Hitachi cups	Arth AL-Rafidin/ China
8	Incubator, TPM- 900	Siroca crossline/Japan
9	Micropipette	Bioasic/ Canada
10	Refrigerator	Concord/ Lebanon
11	Sensitive balance	A&D/ Japan
12	Syringe (5ml)	Arth AL-Rafidin/ China
13	Vortex- mixture	Clay Adams/ Germany
14	Water path	Memmert/ Germany
15	Eppendorf tubes	ATACO/ China
16	Pipette tips	Arth AL-Rafidin/ China
17	Flame Atomic Absorption Spectrometry	SHIMADZU AA-6300/Japan

2.4. Methods

2.4.1. Body Mass Index measurement

Obesity was categorized using the body mass index (BMI) which was calculated from the following equation (**Keys, *et al.*, 1972**):

BMI (Kg/m^2) = Weight (kg) / Height (m^2).

WHO classification was used for BMI evaluation. Normal BMI levels ranged between (20-24.9) kg/m2 while overweight range between (25- 29.9) kg/m2 and when BMI $\geq$ 30 kg/m^2, the woman is considered obese (**Namjou, *et al.*, 2021**).

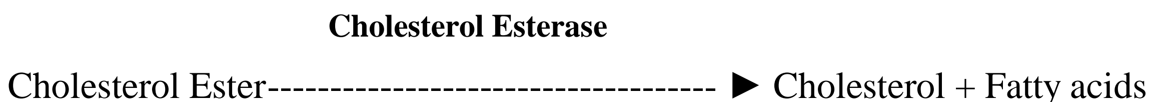
2.4.2. Measurement of Serum Lipid Profile

Total cholesterol, Triglyceride, and HDL were measured by Biochemistry Auto analyzer.

2.4.2.1. Measurement of Serum Total Cholesterol Concentration

Principle: Esterified cholesterol is hydrolyzed into free cholesterol and fatty acid by cholesterol esterase (CHE). Cholesterol oxidase (CHOD) oxidizes the free cholesterol into cholesta-4-ene-3-one with the formation of hydrogen peroxide. In the presence of peroxidase (POD), hydrogen peroxide reacts with a derivative of phenol and 4-amino antipyrine to produce a colored complex whose color intensity is directly proportional to the total cholesterol concentration in the sample (**Trinder, 1969; Bishop, 2020**).

A schematic representation of the reaction is shown in the following equations:



Cholesterol Oxidase

Peroxidase

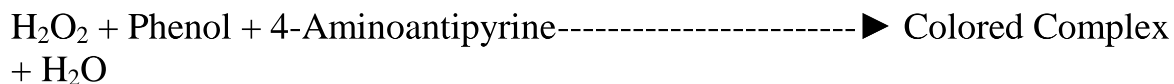


Table (2-3): Reagents used for total cholesterol assay.

Reagent (A) Vol = 50/100/250/1000 mL	Buffer4-AAP CHE CHOD POD Derivative of phenol	100 mmol/L 1 mmol/L 300 U/L 300 U/L 1500 U/L 1 mmol/L
Standard Vol = 5 mL	Cholesterol	200 mg/L

4-AAP, 4-aminoantipyrine; CHE, cholesterol esterase; CHOD, cholesterol oxidase; POD, peroxidase.

Reagent Preparation: liquid reagent, bring to room temperature (15-25 C before use. Mixed 6.250 ml from reagent A and 0.125 from reagent B.

Procedure:

Concentrations of total cholesterol were measured by using autoanalyzer chemistry and the procedure for blank, standard, and sample measurement is demonstrated in Table (2-4). The mixture was prepared and incubated at 37°C for 5 minutes. The Sample reagent ratio was 1:100, and the absorbance of the sample (Ax) and the standard (As) was read against a blank reagent at 510 nm.

Table (2-4): Procedure of total cholesterol

Pipette	Blank(µl)	Sample(µl)	Standard(µl)
Reagent (A)	1000	1000	1000
Water	10		
Sample		10	
Standard			10

Calculation:

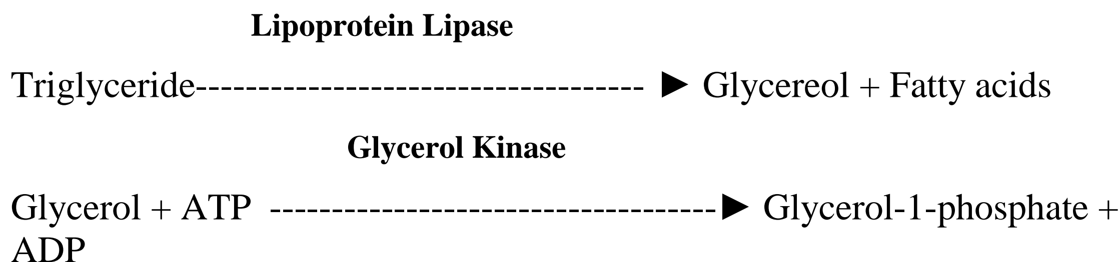
Serum/plasma:

Cholesterol mg/dl = Ax/As× 200 (standard value).

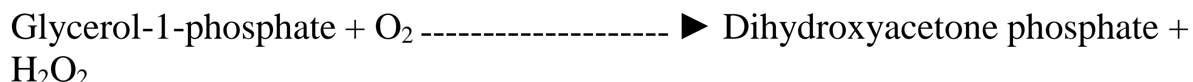
2.4.2.2. Measurement of Serum Triglyceride Concentration

Principle: Triglycerides are(TG) hydrolyzed by lipoprotein lipase (LPL) to produce glycerol and free fatty acids. The glycerol participates in a series of coupled enzymatic reactions, in which glycerol kinase (GK) and glycerol phosphate oxidase (GPO) are involved and H_2O_2 is generated. The hydrogen peroxide reacts with TOOS and 4-AAP to form a colored complex, whose color intensity is directly proportional to the concentration of triglycerides in the sample **(Fossati and Prencipe, 1982).**

A schematic representation of the reaction is shown in the following equations:



Glycerol Phosphate Oxidase



Glycerol-1-phosphate + O_2 --------------------- ► Dihydroxyacetone phosphate + H_2O_2

Peroxidase

H_2O_2 + TOOS + 4-Aminoantipyrine---------------► Colored Complex + H_2O

Table (2-5): Reagents used for triglycerides assay.

Reagent (A) **Volume =** **50/100/250/1000 ml**	Good buffer	100 mmol/L
	Magnesium chloride	15 mmol/L
	ATP	4 mmol/L
	4-AAP	1 mmol/L
	TOOS	0.1 mmol/L
	LPL (lipoproteinlipase)	2500 U/L
	POD (peroxidase)	1800 U/L
	GK (glycerol kinase)	1000 U/L
	GPO	5500 U/L
Standard **Volume = 10 ml**	Glycerol	200 mg/dl (2.28 mmol/l)

ATP, adenosine triphosphate; 4-AAP, 4-aminoantipyrine; LPL, lipoprotein lipase; POD, peroxidase; GK, glycerol kinase; GPO, glycerol phosphate oxidase

Reagents Preparation:

Liquid reagent, bring to room temperature (15-25 C) before use. The light color of the reagent (<0.050 O.D.) due to air or light does not affect their operation.

Procedure:

Concentrations of triglycerides were measured by using autoanalyzer chemistry and the procedure for blank, standard, and sample measurement is demonstrated in Table (2-6). The mixture was prepared and incubated at 37°C for 5 minutes. The Sample to reagent ratio was 1:100, and the absorbance of the sample (Ax) and the standard (As) were read against a blank reagent at 510 nm.

Table (2-6): Procedure of triglyceride.

Pipette	Blank(µl)	Sample(µl)	Standard(µl)
Reagent (A)	1000	1000	1000
Water	10		
Sample		10	
standard			10

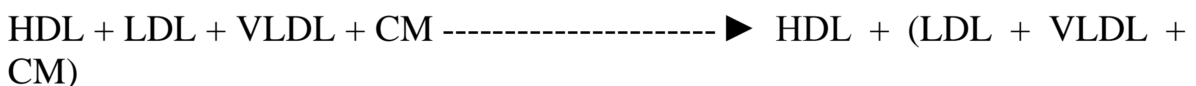
Calculation*:*

Triglycerides mg/dl = Ax/As × 200 (standard value)

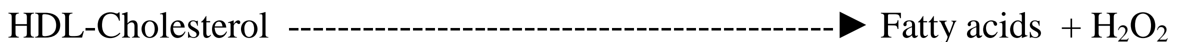
2.4.2.3. Measurement of Serum High-Density Lipoprotein

Principle: Specific polyanions in the first phase block the interfering lipoproteins (LDL, VLDL, chylomicrons), and a specific surface-active agent inhibits the coloration of VLDL, LDL, and chylomicrons in the second phase. The intensity of color produced is directly proportional to the HDL cholesterol in the sample **(Trinder, 1969)**.

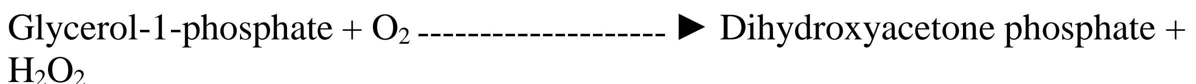
A schematic representation of the reaction is shown in the following equations:



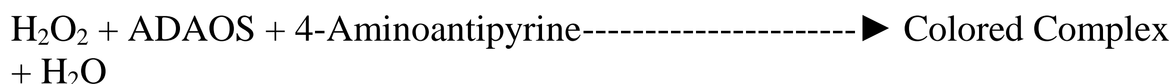
Cholesterol Oxidase, Cholesterol Esterase



Glycerol Phosphate Oxidase



Pereoxidase



H_2O_2 + ADAOS + 4-Aminoantipyrine----------------------► Colored Complex + H_2O

Table (2-7): Reagents used for high-density lipoprotein cholesterol assay.

Reagent (A) Volume = 90 mL	**Good Buffer Polianions 4-AAP**	**100 mmol/L 1mmol/L 4 mmol/L**
Reagent (B) Volume = 30	Cholesterol esterase Cholesterol oxidase Peroxidase HDAOS Detergent	800 U/L 500 U/L 1500 U/L 1 mmol/l 4 mmol/l

4-AAP, 4-aminoantipyrine; CHE, cholesterol esterase; CHOD, cholesterol oxidase.

Reagent Preparation:

Liquid reagents, bring to room temperature (15-25C) before use. Reconstitute the calibrator (included in the kit. 0026T) with 3.0 ml of distilled water.

Procedure:

Table(2-8): Procedure of high-density lipoprotein.

Pipette	Blank(µl)	Sample(µl)	Standard(µl)
Reagent (A)	300	300	300
Water	4		
Sample		4	
standard			4
Reagent (B)	100	100	100

Calculation:

HDL (mg/dl) = (Ax-Abx) / (Ac-Abc) × Calibrator Value mg/dl × 0.02586 = mmol/l

2.4.2.4. Calculation of Low-density Lipoprotein Cholesterol concentration.

Low-density lipoprotein cholesterol (LDL-C) was measured by an indirect method using the Friedewald equation: **(Friedewald *et al*., 1972)**

LDL-cholesterol (mg/dl) = Total cholesterol – (HDL–cholesterol +VLDL cholesterol).

2.4.2.5. Calculation of very low-density lipoprotein cholesterol concentration.

Very low-density lipoprotein cholesterol(VLDL-C) = TG/5

(VLDL-C) could be measured in mg/dl **(Friedewald *et al*.,1972)**.

2.4.3. Measurement of Oxidative Stress and antioxidant

2.4.3.1. Determination of Serum Lipid Peroxidation (MDA)

Principle:

Lipid peroxidation in sera was evaluated by thiobarbituric acid reative substances (TBARS). TBARS test gives a basic, reproducible, and standardized tool for measuring lipid peroxidation in serum. The 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 colorimetrically 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.

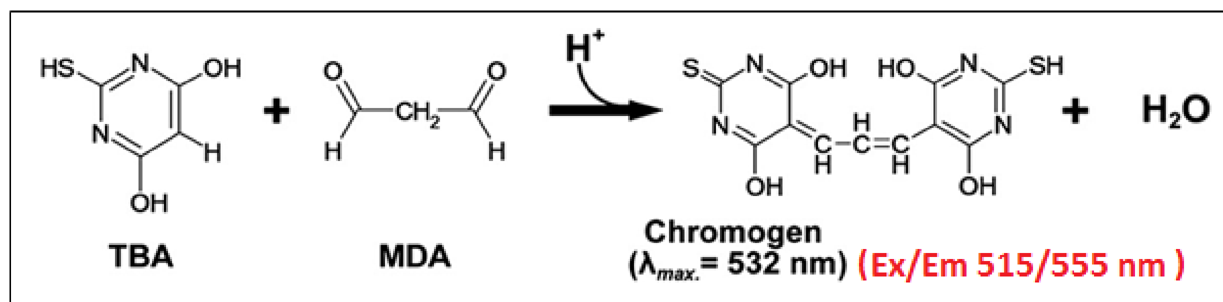


Figure : (2.2) Scheme of the adduct MDA–$(TBA)_2$.

Procedure:

1- 100 µl of sample was added to the test tube and 2ml of working solution which prepare as following:

0.514 of TBA, 25 g of TCA and 0.5 ml of 1M HCl mixed with 190 ml of D.W. Then we added 1 g of SDS and completed the volume to 200 ml.

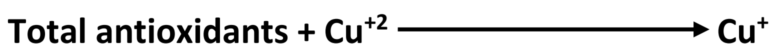
2- The sample was vortexed and heated in a 90° C water bath for 50 min, and then allowed to cool.

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.

$$SampleMDA = \frac{Absorbance}{d \ X \ E} X \ D.F$$

2.4.3.2. Total Antioxidants Capacity Assay: The CUPRAC Method

Principle: (Apak et al., 2007).



Total antioxidants + Cu^{+2} → Cu^{+}

Cu^{+} + 2,9-dimethyl-1,10-phenanthroline → complex (λ_{max} at 450 nm)

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 completing 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 Torolox.

Table (2.9): Procedure of TAC assessment.

Reagents	Test	STD	Blank
Copper(II) chloride solution	1ml	1ml	1ml
Sample	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 were mixed by vortex and incubated for 30 minutes at 37°C, after that the absorbance was read on a spectrophotometer at 450 nm.			

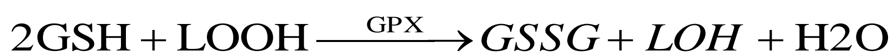
Calculation:

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

2.4.3.3. Assay of Glutathione Peroxidase (GPx) Activity

Principle: (Rotruck, *et al*.,1973)

Glutathione peroxidase catalyzes the following reaction:



$$2\text{GSH} + \text{LOOH} \xrightarrow{\text{GPX}} GSSG + LOH + \text{H2O}$$

The decrement of reduced glutathione concentration can be monitored by Ellman’s reagent [5,5'-Dithio-bis-(2-nitro benzoic acid) (DTNB)].

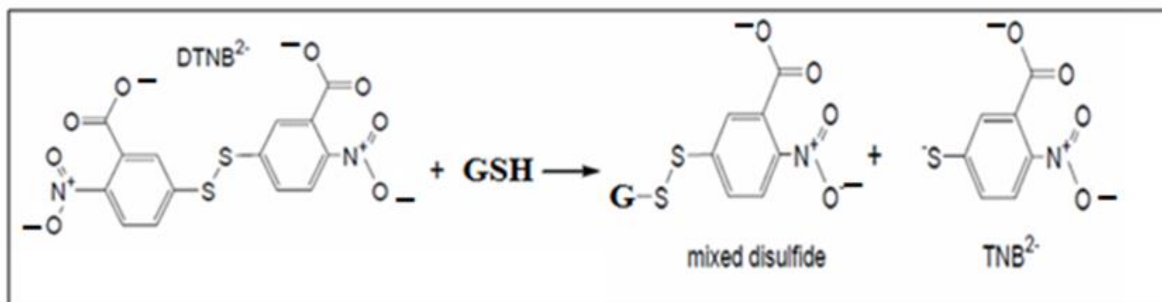


Figure: (2.3) Scheme of the adduct GPX.

Reagents:

1. Solution A: (0.4 M NaH2PO4) Dissolve 55.6 g of NaH2PO4 in 1L of water.
2. Solution B: (0.1 M Na2HPO4) Dissolve107.12 g of Na2HPO4 in 1L of water.

3. Sodium phosphate buffer (pH 7.0) (0.4 M): prepared by mixing 39 of solution A and 61 ml of solution B and dilute to 200 ml with D.W. which contain 0.0744 g EDTA.
4. Sodium azide (10mM): Dissolve 0.06501g of NaN_3 in 100ml of D.W.
5. Reduced glutathione (4 mM): prepared by dissolving 0.1228 gm of a GSH in a final volume of 100 ml of 0.4M EDTA solution.
6. Tert- butylhydroperoxide (2.5mM)
7. Na_2HPO_4 (0.4 M): Dissolve 5.68 g of Na_2HPO_4 in 100ml of D.W.
8. Sodium nitrate (0.1%)
9. DTNB {19.8 mg in 100 ml 0.1% sodium nitrate}

Table (2.10): Procedure of GPX assessment.

Reagents	Test	STD	Blank
Sodium phosphate buffer	400µL	400µL	400µL
Sodium azide	100µL	100µL	100µL
Reduced glutathione	200 µL	200 µL	------
D.W.	200 µL	250 µL	450 µL
Sample	50 µL	-------	------
Tert- butylhydroperoxide	200 µL	200 µL	200 µL
Mix by vortex and incubate for 10 minutes at 37°C, after that, the reaction was terminated with 0.5 ml of 10% TCA and Centrifuge for 15 minutes at 3000 xg, then remove 2 ml of supernatant in a clean tube , and add			
Na_2HPO_4	3ml	3ml	3ml
DTNB	1ml	1ml	1ml

The color developed was read at 412 nm trough 3 min.

Calculation:

$$\text{The residue reduced GSH in test tube} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD$$

Se-dependent glutathione peroxidase activity (μmol of glutathione utilized/min) = Conc. of GSH in STD - Conc. of GSH in test * D.F.

$$\text{Se - GPX activity (μmol of GSH utilized/min)} = \frac{\text{Conc. of GSH in STD - Conc. of GSH in test}}{\text{time(10min)}} * D.F.$$

2.4.3.4. Determination of Selenium in Serum

Four standard solutions (2.5, 5, 7.5, and 10) of the element were prepared as mentioned above which were used for drawing calibration curves as shown in Figure (2.4). Procedures: small amount of samples of 20μl is injected into a small graphite tube, which can then be heated by a wide range of temperatures to vaporize and atomize the analyte. The concentrations of Selenium in samples were measured directly and continuously beyond the measuring of standard solutions depending on the calibration curve. The conditions of Selenium determination are listed in Table (2-10).

Table (2.11): Ideal condition for Selenium determination

Variable	Ideal condition
Atomizer	Graphite Furnace
Fuel	Argon gas
Lamp current	35 mA
Wavelength	196 nm
Slit width	0.7 nm
Lighting mode	BGC-D2
Sample size	20 μl

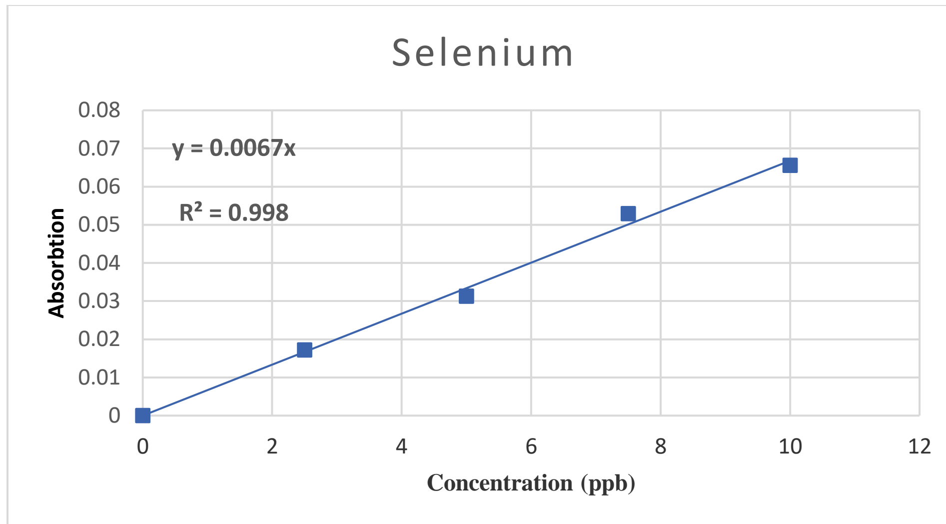


Figure (2.4): Standard curve for Selenium determination

2.4.4. Measurement of Mesencephalic Astrocyte-Derived Neurotrophic Factor

The levels of Measurement of Mesencephalic Astrocyte-Derived Neurotrophic Factor (MANF) in human Serum were determined by using ELISA technique

Table (2-12): Reagents used for MANF assay.

Reagents	Quantity	Reagents	Quantity
Coated ELISA plate	12-Well * 8Tubes	Washing concentrate (30X)	20ml
Standard dilution	3ml	Instruction	1
Chromogen solution A	6ml	Seal plate membrane	2
Chromogen solution B	6ml	Hermetic bag	1
Streptavidin-HRP	6ml	Stop solution	6ml
Standard solution(3600pg/ml)	0.5ml	Anti MANF antibodies labeled with biotin	1ml

Test principle

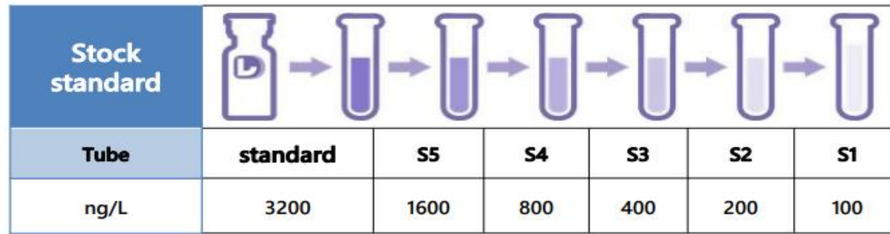
This kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Mesencephalic astrocyte-derived neurotrophic factor (MANF). Add Mesencephalic astrocyte-derived neurotrophic factor(MANF)to the wells, which are pre-coated with Mesencephalic astrocyte-derived neurotrophic factor(MANF)monoclonal antibody and then incubate. After that, add anti-MANFantibodies labeled with biotin to unite with streptavidin-HRP, which forms an immune complex. Remove unbound enzymes after incubation and washing. Add substrates A and B. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of Human Mesencephalic astrocyte-derived neurotrophic factor (MANF)are positively correlated.

Table (2-13): Standards used for MANF assay.

1800pg/ml	Standard No.5	120µl Standard diluents + 120µl Original Standard
900pg/ml	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
450pg/ml	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
225pg/ml	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
112.5pg/ml	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

Assay procedure

1. All samples and standard solutions were prepared at room temperature.



Stock standard						
Tube	standard	S5	S4	S3	S2	S1
ng/L	3200	1600	800	400	200	100

2. A volume of 50μl standard solutions were added in wells of standard.

3. A volume of 10μl sample and then 40μl sample diluent were added in each well of samples.

4. The plate membrane was sealed, Shaker gently to mixed them up. and then incubated at 37°C for 60 minutes.

5. The seal plate was removed carefully, the liquid was drained, and shake off the remaining liquid. Each well was full with washing solution. Drained the liquid after 30 seconds of standing. Then this procedure was repeated five times.

6. A volume of 50 μl of HRP-conjugate reagent was added to each well, covered with an adhesive strip, and incubated for 60 minutes at 37°C.

7. Each well were aspirated and washed, repeating the process four times for a total of five washes. each well were Washed by filling with 400μl

8.Wash Solution by using auto washer. Complete removal of liquid at each step is essential to good performance. After the last wash, removed any remaining Wash Solution by aspirating. Inverted the plate and blotted it against clean filter paper.

9. A volume of 50μl Chromogen solution A and 50μl chromogen solution B were added to each well. Gently mixed and incubated for 15 minutes at 37°C.

10. A volume of 50µl Stop Solution was added to each well. The color in the wells should changed from blue to yellow.

11. The Optical Density (O.D.) was read at 450 nm using a microtiter plate reader within 15 minutes.

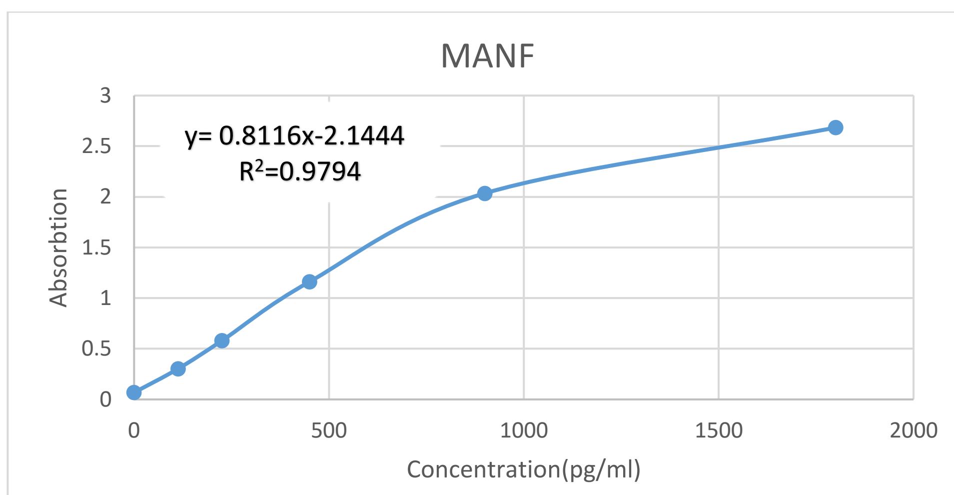


Figure (2.5): Standard curve for MANF determination

Calculation of results

Make concentration of standards the abscissa and OD value the ordinate. Draw the standard curve on the coordinate paper. According to the OD value of the sample, locate its corresponding concentration (which is the concentration of the sample); or calculate the linear regression equation of the standard curve according to the concentration of the standard and the OD value. Then substitute with the OD value of the sample to calculate its concentration.

2.4.5. Measurement of liver function test

2.4.5.1. Measurement of Total Bilirubin

Principle: In an acid medium, total bilirubin reacts with diazotized to form a pink diazo compound (azobilirubin), whose intensity is proportional to the

concentration of bilirubin present in the sample. Direct bilirubin consists of glucuronic acid conjugated derivates, is water soluble, and reacts directly. Total bilirubin is obtained by the presence of a solubilizing agent that cleaves the bond with albumin.

Table (2-14): Reagents used for total bilirubin assay.

Reagent (A) BIL-T Volume = 50/100 ml	**Sulphanilic acid** **Hydrochloric acid** **Solubilizing agent**	**46 mmol/L** **270 mmol/L** **10 mmol/L**
Reagent (B) BIL-T Volume = 25 ml	Sodium nitrite	143 mmol/L

Reagent preparation: A volume 6.250 ml from reagent A was mixed with 0.125 ml from reagent B.

Result Calculation:

Total bilirubin mg/dl = (Ax-Abx) × 20 at 546 nm

2.4.5.2. Measurement of Alkaline Phosphatase

Principle: P-Nitrophenylphosphate is hydrolyzed by phosphatases to phosphate and p-nitrophenol. All solutions should be prepared with fresh, doubly distilled water and also the dilutions should be prepared very accurately. Only fresh serum free from hemolysis should be used. Tygon tubing should be used for connections. The assays are carried out with a cam set at 40–1/2, that is, at the rate of 40 assays per hour and a ratio of sample to washing fluid of 1:2. The sample collector tube is then immersed for 30 seconds in the sample and

60 seconds in the washing fluid. Read off the enzyme activity in the samples (serum) in U/l from the standard curve.

Table (2-15): Reagents used for Alkaline phosphatase assay.

Reagent (A) ALP **Volume = 40/80 ml**	**Buffer DEA** **Magnesium chloride**	**1 mol/l** **0.5 mmol/l**
Reagent (B) ALP **Volume = 10 ml**	P-Nitrophenylphosphate	10 mmol/l

Reagent Preparation: 4 ml from reagent A was mixed with 1 ml from reagent B.

2.7.5.3 Measurement of ALT/GPT

Principle:

Principle: In the presence of a-ketoglutarate, alanine is transformed into pyruvate and glutamate by ALT/GPT in the sample. In the presence of NADH and lactate dehydrogenase, pyruvate is converted into lactate and NAD. NADH oxidation in time unit, measured at 340 nm, is proportional to ALT/GPT concentration in the sample.

Table (2-16): Reagents used for Alanine aminotransferase assay.

Reagent (A) ALT **Volume = 40/80 ml**	**Tris buffer ph 7.8L-** **alanine** **LDH**	**100 mmol/L** **500 mmol/L** **1000 U/L**
Reagent (B) ALT Volume = 10/40/80 mL	NADH α- ketoglutarate	0.18 mmo/l15 mmol/L

Reagent Preparation: mixed 4 ml from reagent A with 1 ml from reagent B.

2.4.5.4. Measurement of AST/GOT

Principle: In the presence of α-ketoglutarate, AST in the samples transforms aspartate into oxalacetate and glutamate. In the presence of NADH and malate dehydrogenase, oxalacetate is converted into malate and NAD.

Table (2-17): Reagents used for Aspartate aminiotransferase assay.

Reagent (A) AST Volume = 40/80 mL	**Tris buffer 7.8L- aspartate LDH MDH**	**80 mmol/L 200 mmol/L 600 U/L 400 U/L**
Reagent (B) AST Volume = 10/40/80 ml	NADH α- ketoglutarate	0.18 mmol/112 mmol/l

Reagent Preparation: 4 ml from reagent A was mixed with 1 ml from reagent B.

2.4.5.5. Measurement of Albumin

Principle:

In a pH 3.8 buffered solution, the albumin present in the sample reacts with bromocresol green (BCG) and causes a color change. The color intensity is proportional to the albumin concentration present in the serum or plasma.

Table (2-18): Reagents used for Albumin assay.

Reagent (A) ALB Volume = 50/100/250 ml	Buffer ph 3.8 BCG	100 mmol/17 mmol/l
Standard ALB Volume = 5 ml	Bovine Albumin	3 g/l

2.5 Statistical Analysis:

Information from the questionnaire from all participants were entered into a data sheet and were assigned a serial identifier number. Multiple entries were used to avoid errors. The data analysis for this work was generated using The Statistical Package for the Social Sciences software, version 28.0 (IBM, SPSS, Chicago, Illinois, USA), and the Real Statistics Resource Pack software for Mac (Release 7.2) of the resource pack for Excel 2016. Copyright (2013 – 2020) (1). Descriptive statistics were performed on the participants' data of each group. Values were illustrated by n (%) for categorical. The distribution of the data was checked using the Shapiro-Wilk test as numerical means of assessing normality.

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

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

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

Chapter Three
The Results

3. Results

3.1 Demographic and clinical characteristics

A total of 120 participants were included in current study 60 patients and 60 control, which were divided into subgroups based on Age, gender, and BMI. The participant demographic characteristics of the study groups were summarized in Figurs (3.1)(3.2)(3.4). (21.66%) of the age range of patients was (40 -50) years old, (45%) of the patients were within (51- 60) years, while (33.34%) of the patients were within the age range (More than 60). The gender distribution of the participants was (75%) male and (25 %) female. The smoker distribution of the participants was (41.6%) and non smoker (58.4%).

Also, the analysis of data illustrated 18.33% patients were having Metabolic syndrome, 83.33% patients were having a sedentary lifestyle, and 61.66% of the patients group were having fatty liver all as shown in figure (3.2).

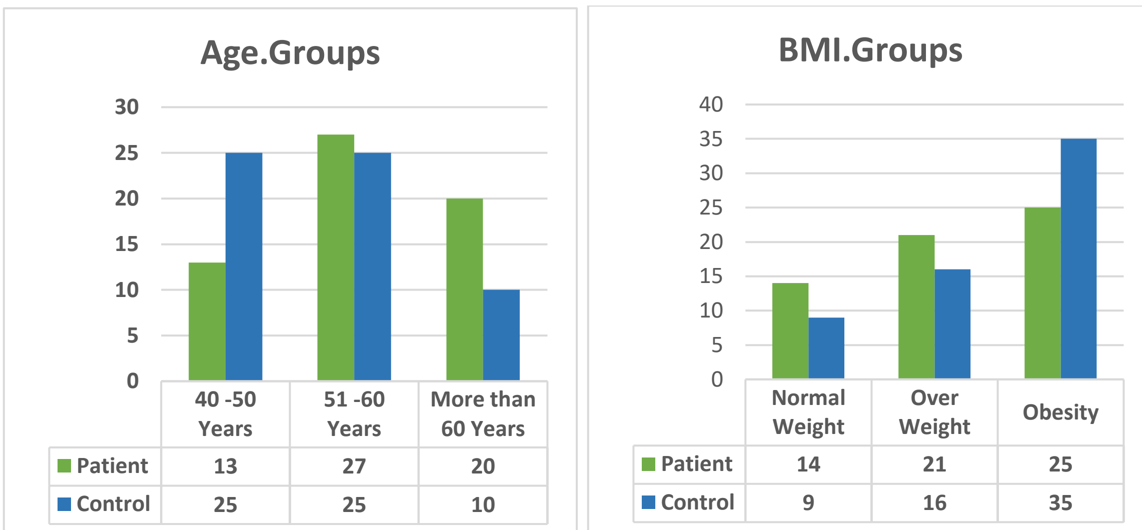


Figure 3.1: Distribution of patient samples according to age groups and BMI groups

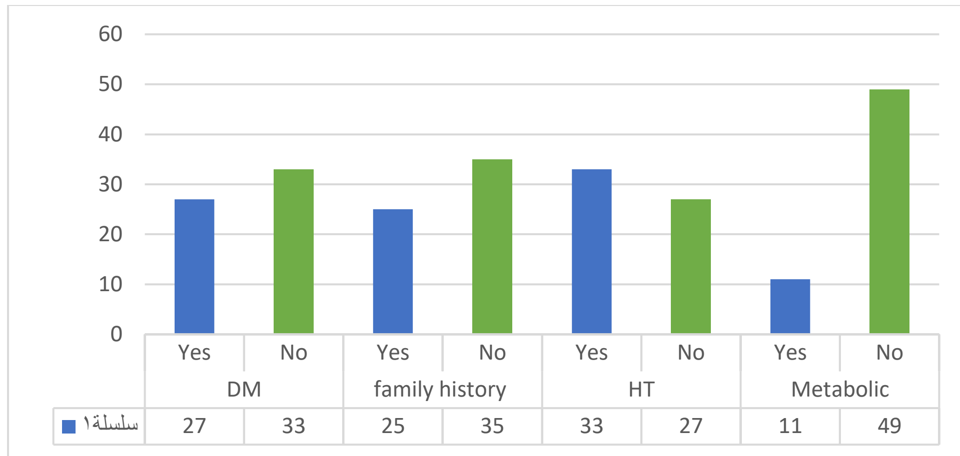


Figure 3.2: Distribution of patients according to the history diabetes mellitus, family history, hypertension, metabolic syndrome

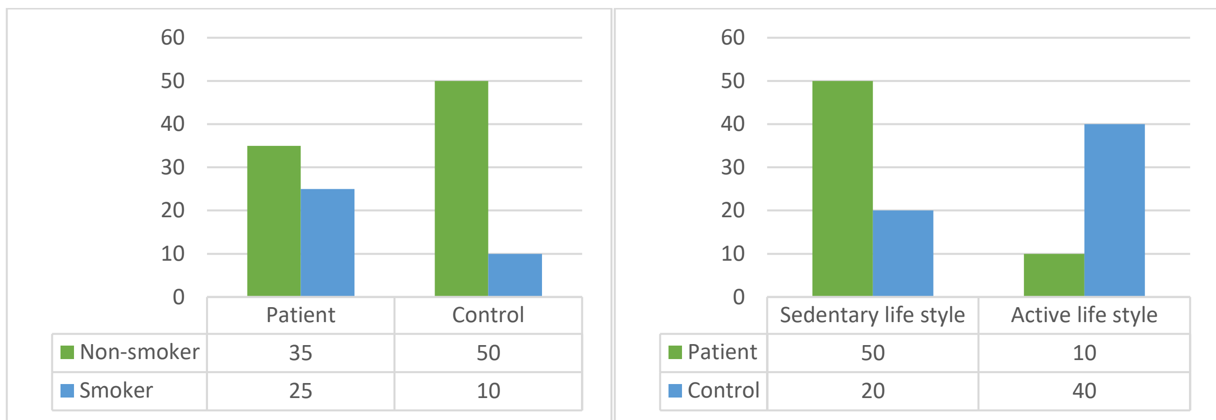


Figure 3-3: Distribution of patients according to smoker and life style

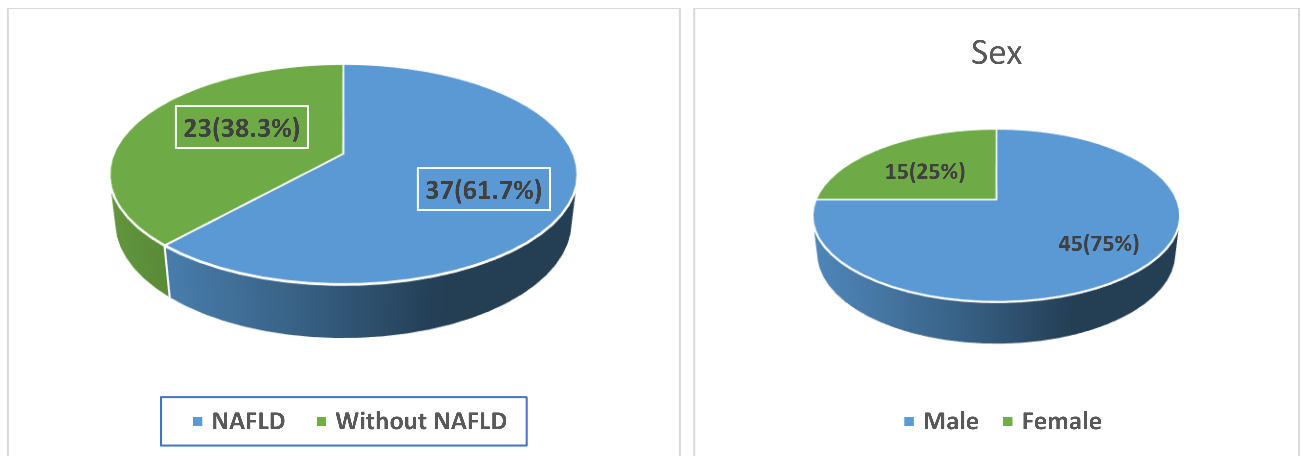


Figure 3.4: Distribution of patients with coronary artery disease (NAFLD and without NAFLD) and Sex (n= 60).

3.2 Biochemical Investigation in patients and control groups

Generally, patients with coronary were shown an increasing range level of the MDA when compared to the control groups, while the range level of MANF, TAC, GPX, and Selenium were decreased compared to the control. Results indicated a significant difference in MDA levels among groups. The mean level of MDA in the patient was presented at (2.43±0.90), while the mean level of MDA in the control (2.15±0.51) and the mean levels of MANF in the control were (348.62±143.50) which was significantly higher than the patient group (287.58±76.71),($p \leq 0.001$). The Distribution of serum levels of MANF, TAC, GPX, and Selenium in patients compared to the control group were (287.58±76.71),(567.33±167.64),(177.47±51.25) and (60.14±16.7071) for the control and (348.62±143.50),(773.33±191.13),(359.82±115.69),(.41±24.16) for patient presented in figure (3.5).

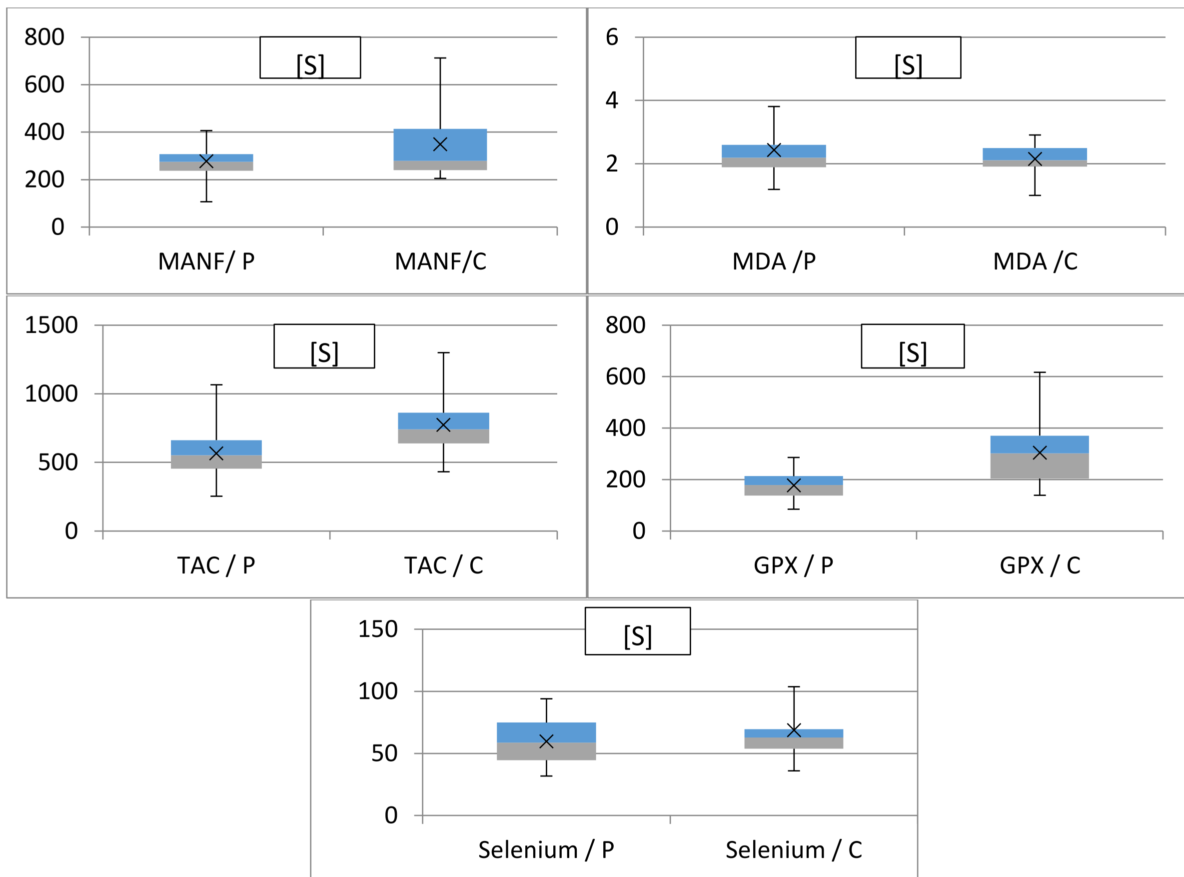


Figure 3.5: Boxplot of the difference in the levels of (MANF, MDA, TAC, GPX and Selenium) in patients groups and control groups (T-test was used S= significant at p ≤ 0.05, NS= Non-significant).

Figure (3.6) illustrates the mean level of the lipid profile in the Patients and control groups, Results were shown that the levels of Chol, TG, HDL, and LDL were decreased markedly in the patients group compared to the control, all parameters are highly statistically significant p values were <0.001.

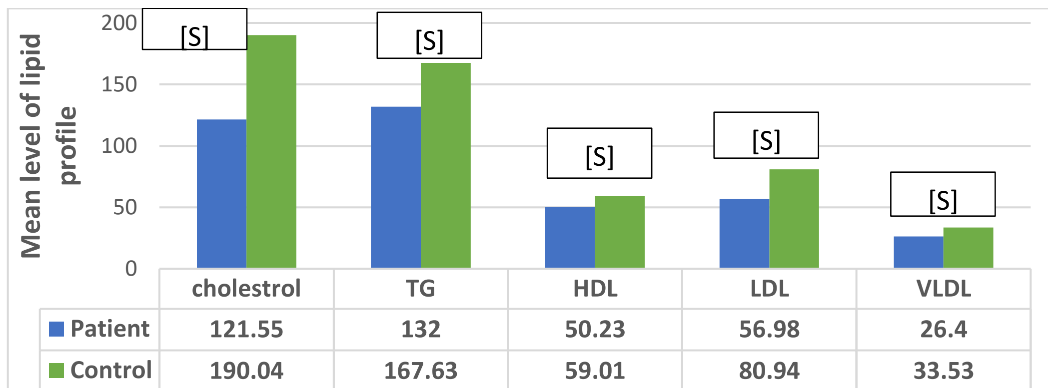


Figure 3.6: Difference between mean levels of Lipid profile in patients with CAD and control groups (T-test was used S= significant at p ≤ 0.05, NS= Non-significant).

Figure (3.7) illustrates the mean level of the liver function test in the Patients and control groups. Results were shown that the levels of Albumin, ALT, ALP, AST, and TSB were increased in patients group compared to the control, p values >0.05, non-significant.

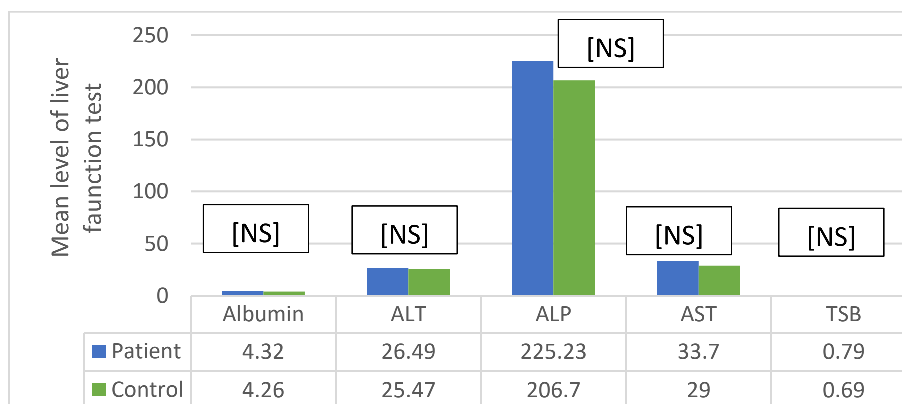


Figure 3.7: Difference between mean levels of Liver function test in patients with CAD and control groups (T-test was used S= significant at p ≤ 0.05, NS= Non-significant).

3.3 Difference between the levels of (MANF, MDA, TAC, GPX and Selenium) patients with medicated and non-medicated sub-groups

Generally, patients with coronary artery disease according to medicated (lowering lipids) were shown an increasing range level of the MANF, TAC, and GPX when comparing with non-mediated groups, while the range levels of MDA and Selenium were decreased compared to with non-mediated

Results indicated a significant difference in MDA and Selenium levels among groups, The means, and standard deviations were presented at (2.34±0.74) and (58.61±15.99) respectively. The mean levels of MANF in the control were (229.73±54.51) which was significantly higher than for the non-medicated group (287.58±76.71), (p≤ 0.001). The Distribution of serum levels of MANAF, TAC, and GPX in medicated compared to the non-medicated group was (296.48 ±76.12),(569.35 ±174.46) and (181.46 ±51.17) respectively, presented in Figure (3.8).

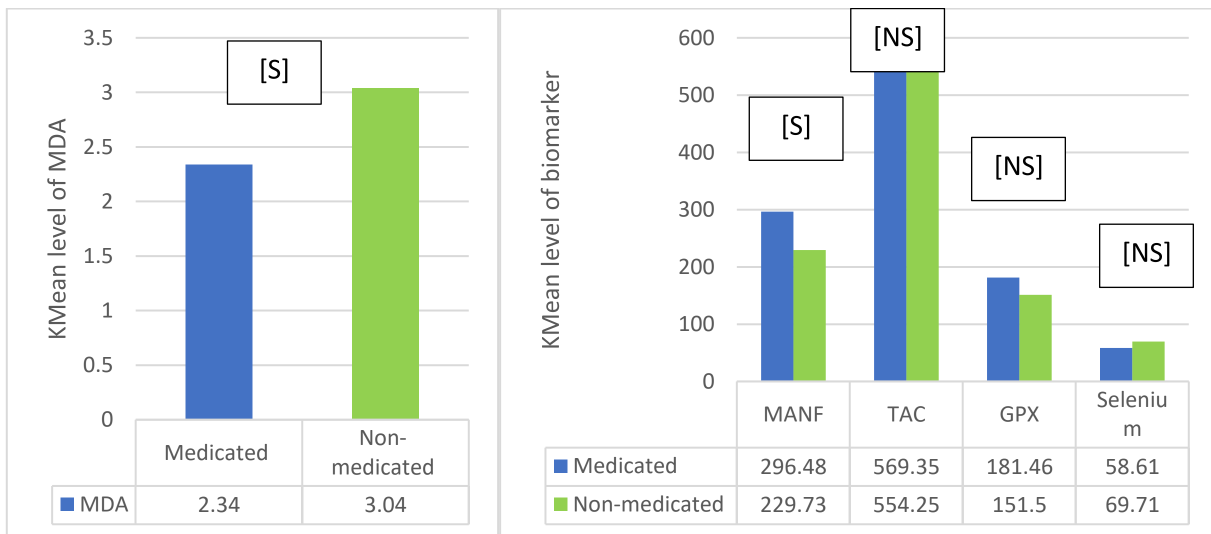
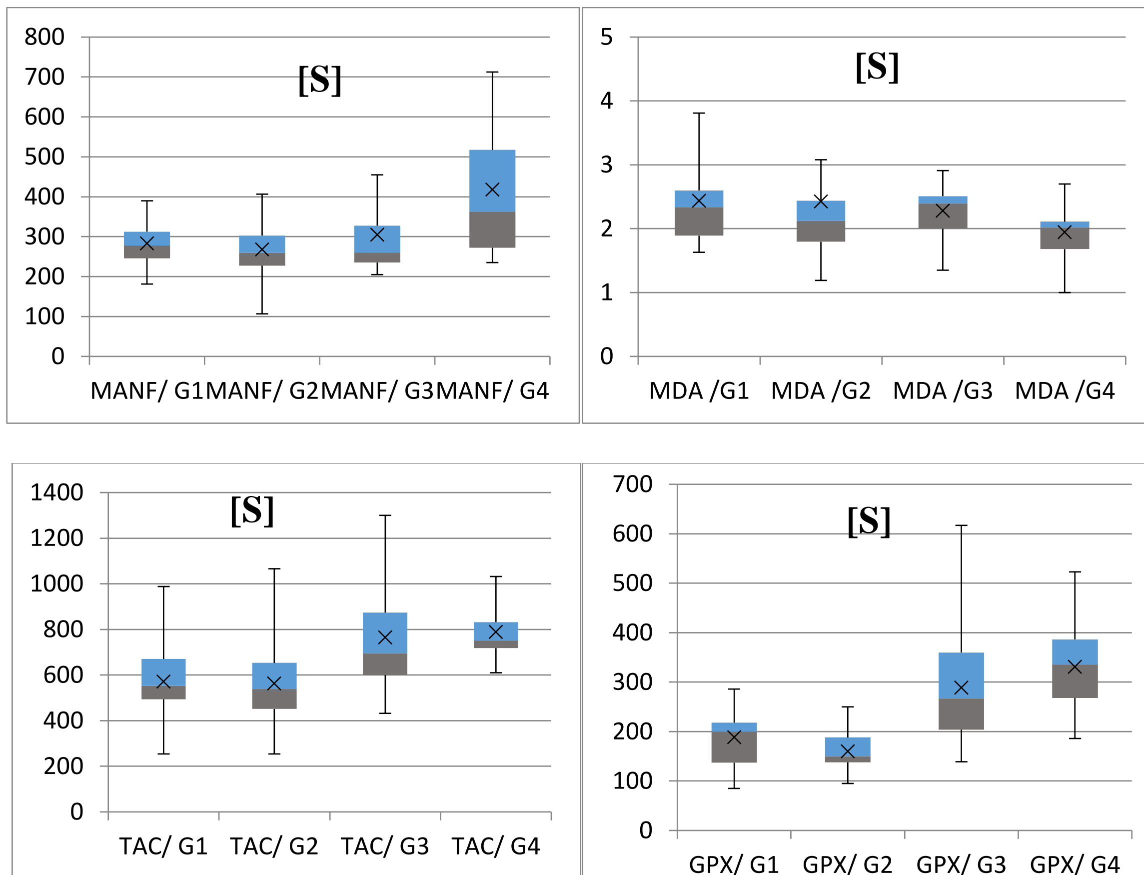


Figure 3.8: Difference between mean levels of (MANF, MDA, TAC, GPX and Se) in medicated and non-medicated (lowering lipid) coronary artery disease (T-test was used S= significant at p ≤ 0.05, NS= Non-significant).

3.4 Difference between the levels of MANF, MDA, TAC, GPX, and Selenium in the patients and control sub-groups

Generally, by using box plots patients with coronary artery disease and fatty liver, patients with coronary artery disease without fatty liver, control with NAFLD without CAD, and control without NAFLD and without CAD, patients with NAFLD were shown an increasing mean level of the MDA when comparing to the control groups, while the mean level of MANF, TAC, GPX, and Selenium were decreased compared to the control groups. Results indicated a significant difference in MDA levels among groups, showed in figure (3.9).



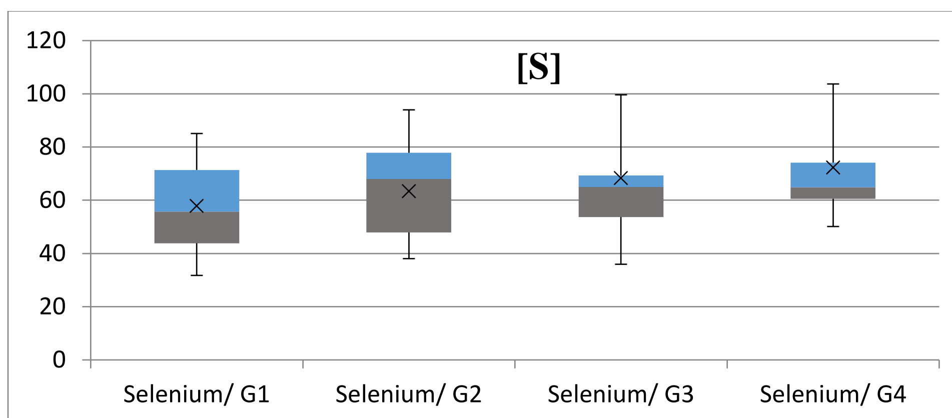


Figure 3.9: Box plot Difference between mean levels of (MANF, MDA, TAC, GPX and Se) in sub-groups (ANOVA-test was used S= significant at p ≤ 0.05, NS= Non-significant). (G1=coronary artery disease with fatty liver, G2 = coronary artery disease without fatty liver, G3 = Non-coronary artery disease with fatty liver, G4 = Non-coronary artery disease without fatty liver

3.5. Difference in the levels of MANF, MDA, TAC, GPX, and Selenium according to Sex

Table (3.1) illustrates the mean level of the biochemical in the Patients between male and female. Results showed that the levels of **MANF**, MDA, GPX increased markedly in the female, while the mean level of TAC and Selenium increased markedly in the male but all biomarkers were non-statistically significant, p-values were >0.05.

Table 3.1. The effect of Sex and serum Biomarkers levels in Coronary arty disease in the patient

Parameters	Sex (N=60)		P value
	Male Mean ±SD (N=45)	Female Mean ±SD (N=15)	
MANF	277.64±61.24	278.16±37.00	0.084 [NS]
MDA	2.39±0.91	2.54±0.88	0.414 [NS]
T AC	573.60±175.96	548.53±143.54	0.710 [NS]
GPX	172.53±54.62	192.26±37.16	0.011 [NS]
Selenium	60.63±17.09	59.39±15.90	0.764 [NS]
T-test, Results are presented as mean ± SD, p<0.05 considered significantly different, [S]= Significant, [NS]= non-significant			

3.6. The effect of Age Groups on the Measured (MANF, MDA, TAC, GPX and Se) in the patients and control groups

Table (3.2) illustrates the mean level of the biochemical in the Patients for age.groups between two groups (40-55) years and (more than 55) years. Results showed that the levels of **MANF**, increased markedly in the groups (40-55)years more than anothers groups the mean level was (285.89 ±51.22) and (275.05±60.365) show highly statistically significant difference ($p < 0.05$),
On the other hands, Non statistical significant difference was found between other mean of biomarkers.

Table 3. 2:The effect of Age on serum levels of (MANF, MDA, TAC, GPX and Se) in Coronary artery disease patients.

LAB parameters	Age group =60		
	40 -55 Years Mean ±SD N=28	More than 55 Years Mean ±SD N=32	P value
MANF	285.89 ±51.22	275.05 ±60.365	**0.04[S]**
MDA	2.46 ±0.867	2.40 ±0.93	0.604[NS]
T AC	567.71±139.62	567.00 ±191.05	0.089[NS]
GPX	174.78 ±50.37	179.81 ±52.69	0.991[NS]
Selenium	60.65 ±18.19	59.70 ±15.58	0.157[NS]

3.7. The effect of BMI on the measured (MANF, MDA, TAC, GPX and Se) in the patients and control groups

Table (3.3) illustrates the mean level of the biochemical in the Patients for BMI.groups between two groups Non-obesity and Obesity. Results showed that the levels of MANF, increased markedly in the Non-obesity more than anothers groups the mean level was (281.16 ±64.47) and (272.96 ±42.20) show highly statistically significant difference ($p < 0.05$),

On the other hands, Non statistical significant difference was found between other mean of biomarkers.

Table 3.3:The effect of BMI on serum levels of (MANF, MDA, TAC, GPX and Se) in Coronary artery disease patients.

LAB parameters	BMI group =60		
	Non-obesity Mean ±SD N=35	Obesity Mean ±SD N=25	P value
MANF	281.16 ±64.47	272.96 ±42.20	**0.037[S]**
MDA	2.37 ±0.86	2.51 ±0.95	0.611[NS]
T AC	572.46±180.41	560.16 ±151.25	0.356[NS]
GPX	183.60 ±55.05	168.88 ±45.09	0.171[NS]
Selenium	57.11 ±18.01	62.49 ±16.42	0.788[NS]

3.8. Difference between the level of (MANF, ALP, ALT, AST, AlP, and ALT/AST ratio) in NAFLD patients and patients without NAFLD.

Generally, patients with NAFLD were shown an increasing range of levels of Albumin, ALT, ALP, AST and, TSB when compared to the control groups, while the range level of MANF decreased compared to the control.

Results were indicating a highly statistically significant difference in all biomarkers just **Albumin was non-significant.** The mean levels of ALT, ALP, AST, TSB, and **Ratio(ALT/AST)** in the NAFLD patients were (34.76 ± 10.77), (264.57 ± 49.61), (34.62 ± 6.33) (1.03 ± 0.46) and (1.23 ± 0.33) which was significantly higher than for without NAFLD group (29.26 ± 11.26) (202.00 ± 30.50) , (26.96 ± 5.53), (0.68±0.26) and (0.99 ± 0.33),($p \leq 0.001$), while the mean level of MANF in patients without NAFLD (305.25 ± 110.49) which was significantly higher than for NAFLD group, as shown in figure (3-10).

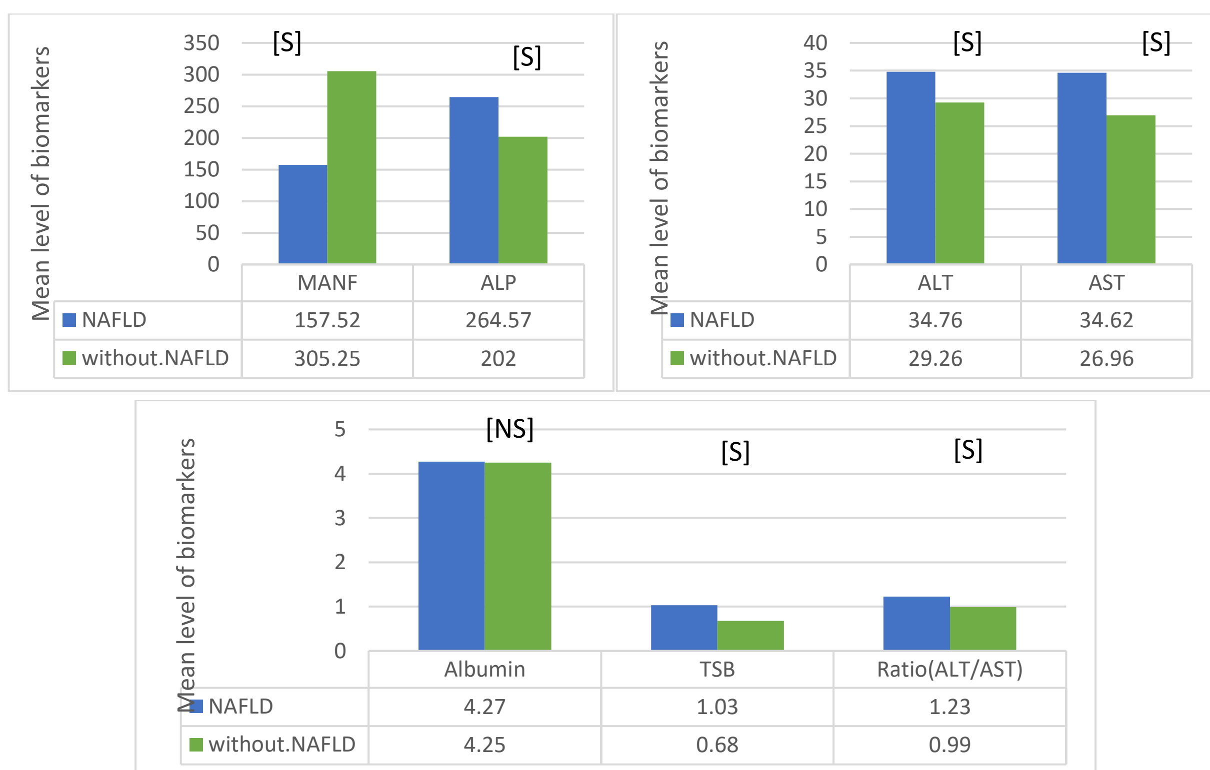


Figure 3.10: Results of the analysis of LFTs and MANF levels between patients with NAFLD and patients without NAFLD.

3.9. Relationship between the MANF and lipid profile:

By using correlation person test to assess the correlation between MANF and lipid profile, an inverse significant correlation had been found between MANF and all lipid profiles (-0.5, -0.4, -0.3, -0.4) (P <0.001), a significant inverse correlation with LDL (R= - 0.173, P=0.034), these findings are shown in (table 3.4).

Table 3.4: Correlations of MANF with lipids profile.

Biomarkers		***Correlation coefficient (r)***	***P (2-tailed)***
MANF,pg/ml	**TC, mg/dL**	-0.5	<0.001[S]
	TG, mg/dl	-0.4	<0.001[S]
	HDL-C, mg/dl	-0.3	0.09[NS]
	LDL-C, mg/dl	-0.4	0.036[S]
	VLDL-C, mg/dl	- 0.4	0.038[S]
p<0.05 considered significantly different, [S]= Significant, [NS]= Non significantTC: Total cholesterol ; TG: troiglyceride ; HDL-C : high density lipoprotein ; LDL-C : low denisty lipoprotien ; VLDL-C : very low density lipoprotien			

3.10: Odds ratio

Multinomial logistic regression was performed and forward logistic regression was adopted to analyze the results. The correlation coefficient was used for determining linear relationships between biochemical markers MANF, MDA, TAC, GPX and Se in patient groups compared to the control group. It was found that the biomarkers MDA in the patient was a risk factor for CAD and NAFLD and highly difference significant OR: 3.239; 95% CI: (0.504-20.821), while the MANF, TAC, GPX, and Selenium in the patient was a protective factor for CAD and NAFLD ((OR: 0.984; 95% CI: (0.973-0.996), (OR: 0. 999; 95% CI: (0.993-1.004), (OR: 0.974; 95% CI: (0.962-0.987) and (OR: 0.947; 95% CI: (0.904-

0.992). Finally, all biomarkers were shown to be significantly correlated with coronary artery disease with NAFLD except TAC, and all biomarkers were shown to be significantly correlated with coronary artery disease without NAFLD except TAC and Selenium.

Table 3.5: Estimation the Associated of the analyzed factors in Patients Compared to the control

Variable	Groups	OR (Lower – upper)	P value
MANF			
	CAD with NAFLD	0.984(0.973-0.996)	**0.007[S]**
	CAD without NAFLD	0.978(0.965-0.992)	**0.002[S]**
	No-CAD with NAFLD	0.989(0.981-0.997)	**0.008[S]**
	No-CAD without NAFLD	1[a]	-
MDA			
	CAD with NAFLD	3.239(0.504-20.821)	**<0.001[S]**
	CAD without NAFLD	2.87(0.425-19.399)	**<0.001[S]**
	No-CAD with NAFLD	3.627(0.629-20.909)	**<0.001[S]**
	No-CAD without NAFLD	1[a]	-
TAC			
	CAD with NAFLD	0.999(0.993-1.004)	0.617[NS]
	CAD without NAFLD	0.998(0.992-1.005)	0.641[NS]
	No-CAD with NAFLD	1.003(0.998-1.008)	0.287[NS]
	No-CAD without NAFLD	1[a]	-
GPX			
	CAD with NAFLD	0.974 (0.962-0.987)	**<0.001[S]**
	CAD without NAFLD	0.963(0.947-0.979)	**<0.001[S]**
	No-CAD with NAFLD	0.993(0.985-1.002)	0.124[NS]
	No-CAD without NAFLD	1[a]	-
Selenium			
	CAD with NAFLD	0.947(0.904-0.992)	**<0.021[S]**
	CAD without NAFLD	0.965(0.916-1.016)	0.171[NS]
	No-CAD with NAFLD	0.992(0.961-1.024)	0.622[NS]
	No-CAD without NAFLD	1[a]	-
p<0.05 [S]= Significant, [NS]= Non significant, 1[a] : reference category is Control			

3.11: Receiver Operating Characteristic Analysis (ROC)

Groups 2

- **ROC curve and AUC analysis for the MANF and MDA for Patients G2 (CAD without NAFLD) compared to the control group (G4)**

Results of the receiver operating curve (ROC) curve and AUC analysis for the MANF and MDA diagnostic parameters. MANF and MDA were shown a good performance for prediction patients compared to the control group G4 (No CAD without NAFLD), data are presented in Table (3.8).

For MANF levels: (sensitivity = 95.7%, specificity 91.3%) at a level = 198.1415 pg/ml, MDA levels (sensitivity 52.2%, specificity 73.9%) at a level = 2.11 µmol/l, the p-values of the AUC were <0.05 and highly statistically significant

Table 3.6: Receiver operating characteristic curve showing sensitivity and specificity of MANF & MDA in Groups 2 compared to the control group (G4)

Test Result Variable(s)	MANF	MDA
AUP	91.7%	60.8%
Sensitivity %	95.7%	52.2%
Specificity %	91.3%	73.9%
Youden index	0.87	0.261
Cut-off points	198.1415	2.11
CI (95%)	0.819-1.000	0.442-0.773
PPV	90%	70%
NPV	96%	58.82%
Accuracy	92%	65%
P value	**<0.001[S]**	0.210[NS]
S= significant at p ≤ 0.05, NS= Non-significant).		

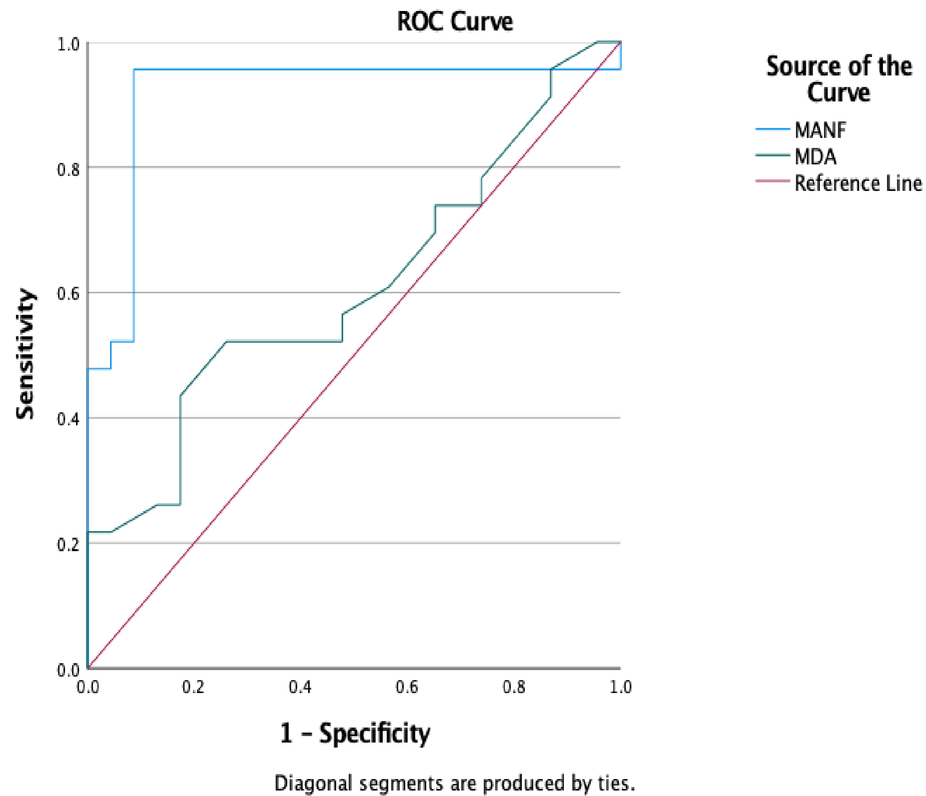


Figure 3.11: Receiver operating characteristics (ROC) curve analysis of MANF and MDA levels in the patient G2 (CAD and without NAFLD) with control groups (G4)

Groups 3

- **ROC curve and AUC analysis for the MANF and MDA for Patients G3 (No CAD with NAFLD) compared to the control group G4 (No CAD and without NAFLD)**

Results of the receiver operating curve (ROC) curve and AUC analysis for the MANF and MDA diagnostic parameters. MANF and MDA were shown a good performance for prediction patients G3(No CAD with NAFLD) compared to the control group G4, data are presented in table (3. 9).

For MANF levels: (sensitivity = 75%, specificity 91.3%) at a level = 194.527 pg/ml, MDA levels (sensitivity 64.9%, specificity 82.6%) at a level = 2.16 µmol/l, the p-values of the AUC were <0.05 and highly statistically significant

Table 3.7: Receiver operating characteristic curve showing sensitivity and specificity of MANF & MDA in Groups 3 compared to the control group G4

Test Result Variable(s)	MANF	MDA
AUP	95.5%	68%
Sensitivity %	75%	64.9%
Specificity %	91.3%	82.6%
Youden index	0.913	0.475
Cut-off points	194.527	2.16
CI (95%)	0.894-1.000	0.538-0.822
PPV	95%	70%
NPV	86%	58.82%
Accuracy	88%	65%
P value	**<0.001**[S]	0.020[S]
S= significant at p ≤ 0.05, NS= Non-significant).		

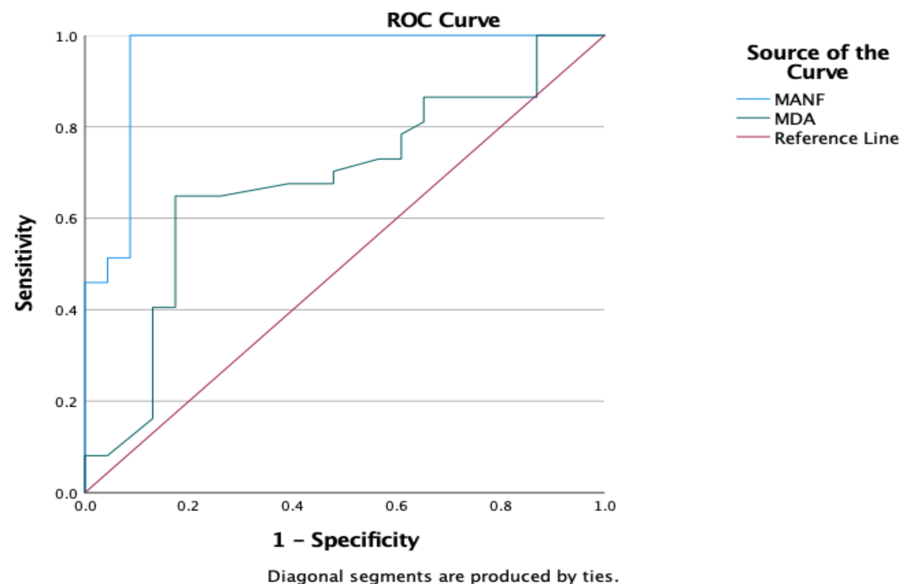


Figure 3.12: Receiver operating characteristics (ROC) curve analysis of MANF and MDA levels in the patient G3 (No CAD with NAFLD) with control groups G4

Chapter Four

Discussion

Discussion

The current study the researcher addressed chronic coronary artery obstructive disease with and without Non-alcoholic fatty liver disease because this disease is a leading cause of death worldwide, as this disease multiplies in adults over the age of 30 years **(Hosseini *et al*., 2021)**.

It is found in this that men are more affected than women, where the rate of injury with CAD of men 75% from all patients**,** and every three men correspond to the injury of one woman, as well as the rate of men with NAFLD is higher than women, where the proportion of men was 75.7 % and women 24.3 % as other studies have shown that men are higher incidence of coronary artery disease and NAFLD than women before menopause, due to the presence of estrogen, which is a protective factor for women before menopause. This is because estrogen is made from cholesterol, so cholesterol levels are moderate, but after menopause, estrogen production decreases, and thus cholesterol levels increase after menopause, which is considered a major cause of fat accumulation and blockage of the coronary arteries. women are more affected by CAD and NAFLD in older age **(Czajkowski *et al*., 1997; Lindquist, *et al*., 2003; Lonardo *et al*., 2019).**

The incidence of coronary artery disease also increases with age, as the current study showed that the greater the age, the greater the number of CAD infections, As for the age after 60 years, the incidence of the disease decreased Perhaps we need a larger number of samples. as the percentage of injuries increases with age, as shown in previous studies **(Cox *et al*., 2022).**

Through the current study, the risk of chronic coronary artery occlusion disease increases as the body weight increases, i.e. obesity, as people with

obesity have a greater risk of developing chronic coronary artery disease than people whose weights are normal, as obesity is one of the risk factors for coronary artery disease, and this is confirmed by previous studies **(Kenchaiah *et al*., 2002)**.

Through the current study, It is noticed the effect of risk factors for coronary artery disease, including type II diabetes, and it is found that there are several people with chronic coronary artery disease suffering from type II diabetes This indicates that diabetes is a risk factor for coronary artery disease. which are considered a risk indicator for CAD as other studies have shown **(Sewdarsen *et al*., 1991).**

It is noted through the current study that a number of chronic coronary artery patients have a family history of the disease with a family member or a relative who was suffering from this disease, as the family history is considered a risk factor for coronary artery disease, the percentage of people who had a family history of the disease was about (41.7%) percent of the total number of patients with coronary artery disease in current study. as shown by previous studies **(Malakar *et al*., 2019)**.

It is also found in that approximately 55% of chronic coronary artery patients had chronic hypertension, this may be due to the narrowing of the arteries or the accumulation of fat on the walls of the blood vessels, which causes high blood pressure, which are the same reasons that lead to blockage of the coronary arteries, and chronic hypertension is also a risk factor that affects and increases the risk of chronic coronary artery disease and is proven among the risk factors in previous studies **(Huma *et al*., 2012).**

Several chronic coronary artery patients had metabolic syndrome, and studies are confirming that metabolic syndrome is a risk factor for both chronic coronary artery disease and non-alcoholic fatty liver disease (**Bhalwar, 2020; Furman, 2004).**

About 78.33% of coronary artery patients were smokers, as smoking is a major cause of cardiovascular disease **(Zhang, 2010).**

Lifestyle had a significant impact on current study in increasing the risk of chronic coronary artery disease, where the proportion of patients was inactive people **(83.33%)** and this is a major reason that indicates that inactive people are at risk factor for having chronic coronary artery disease. Inactive people with multiple cardiac risk factors are more likely to develop MI and this is consistent with other studies **(Giri *et al*., 1999)**.

In current study, dealt with non-alcoholic fatty liver disease and its relationship to chronic coronary artery disease because it is an important topic and widely spread recently **(Cotter, & Rinella, 2020).**

The current study examined the effect of non-alcoholic liver fat disease on coronary artery disease. It found that 61.7 % of chronic coronary artery patients suffer from NAFLD, and this large percentage indicates an association between or a risk factor for coronary artery disease, and this is what a previous study showed in 2019 that NAFLD is associated with cardiovascular disease **(Stahl *et al*., 2019).** Another study in 2021 showed that the pathophysiology behind NAFLD association with cardiovascular disease is not fully understood and may include other pathways besides insulin resistance **(Ndrepepa, 2021).**

As for lipids profile and their relationship to chronic coronary artery disease, the results of lipid tests were low in CAD patients the current study, because most patients take lipid-lowering treatments within the treatment protocol for coronary artery patients such as statins and other treatments before undergoing percutaneous coronary intervention PCI surgery. as studies have shown (**Sirtori, 2014; Soppert,** ***et al*****., 2020**).

The study also addressed the effect of oxidative stress represented by the level of **Malondialdehyde** MDA the final product of fat oxidation (lipide peroxidation) and have an increase in its levels in patients compared to control as the oxidative stress induced by uncontrolled production of ROS and altered antioxidant systems has a key role in the pathogenesis of ACVDs, such as CAD, HF, or heart valve disorders among others. Additionally, the fact that the heart is a highly oxidative organ makes it more susceptible to the accumulation of lipid peroxidation and lipoxidation products contributing to the onset of ACVDs. Indeed, the excessive production of ROS stimulates the oxidative modification of LDL and the loss of HDL cardioprotective properties, increasing the inflammatory response and deregulating cell growth and vascular tone. and core-aldehydes promote the inflammatory process underlying atherosclerosis, macrophage recruitment endothelial dysfunction, extracellular matrix deposition, and platelet aggregation accelerating atherosclerosis and compromising atherosclerotic plaque stability. In the case of heart failure, both lipid peroxidation products and the further adducted proteins have been associated with cardiac hypertrophy, apoptosis, or contractile dysfunction, thus playing a role in the development and progression of the pathology as a previous study showed (**Gianazza** ***et al*****., 2019).**

Through the current study, it has seen the effect of antioxidants and its relationship with coronary artery disease, where we have noticed low levels of antioxidants represented by (GPX, TAC, Se). This is because the decrease in antioxidant levels leads to an imbalance between the oxidant represented by MDA and antioxidant and thus leads to an increase in free radicals ROS causing the disease (**Valencia-Perez *et al*., 2015).** A previous study found that patients with cardiovascular disease had lower GPX levels compared to healthy people **(Čolak, *et al*., 2020**). There are other studies that have shown that TAC level is decreased in CAD **(Demirbag *et al*., 2005).** As for selenium, studies have not yet found any effect on coronary artery disease (**Rath *et al*., 2021**). The main function of primary antioxidants is to donate hydrogens to the lipid-free radical, which turns itself into a free radical. The antioxidant free radical then can react with other lipid peroxide radicals or other antioxidant free radicals to finish the reaction. Several primary antioxidants are endogenous in food systems **(Buettner, 1993).** It has been found lower GPx concentrations in obese versus non-obese patients but non significant. This is confirmed by previous studies **(Ghayour-Mobarhan *et al*., 2008)** It has been observed in these studies that the results between BMI and antioxidant levels were contradictories. Although the relationship between increased oxidative stress and the development of disease and complications in obesity has been revealed in numerous studies, There are limited studies investigating the relationship between the dietary antioxidant levels and serum TAC levels of the obese individuals of which a study in 2020 corresponds to our current study **(Beşağil *et al*., 2020).** As for selenium, the results of the current study, that obesity did not affect the level of selenium, but another study in Brazil that showed the influence of obesity in selenium deficiency, role of the environment as a determinant factor for selenium deficiency. There is potentially a promising role for selenium

supplementation in Brazilian patients with obesity, especially for those using statins **(Watanabe *et al*., 2021).**

When comparing the results of patients taking blood lipid-lowering treatments with patients without treatments, It has been found that antioxidant levels were higher with treatment than with patients without treatment but nonsignificant and MDA levels had decreased in patients with treatment than in patients without treatment, and this indicates that lipid reduction therapy reduced lipid oxidation and reduced bad cholesterol by reducing the release of free radicals and thus reduced the association of antioxidants with free radicals, so the level of antioxidant increased in patients. With treatment, this increase was noticeable and statistically significant as previous studies have shown). Excessive ROS can be generated in vascular cells from NAD(P)H oxidase (Nox), nitric oxide synthases (NOS) uncoupling, and mitochondria, which cause oxidative modifications of low-density lipoprotein (LDL) **(Cachofeiro *et al*., 2008**; **Valencia-Perez *et al*., 2015**).

It has been also noticed that the level of MANF was higher in patients who take lipid-lowering therapy than in patients without treatment. The study expects through these results that lipid-lowering therapy also affects the level of MANF, which increases and improves the level of MANF, but there is a need for more studies on this topic.

As for the effect of obesity on markers, it was noticed through the results of this study that the level of MDA was not affected by weight gain by comparing the groups with BMI, while another study that MDA levels increase with BMI **(Chaves *et al*., 2021).**

As for antioxidants, it has been noticed non affected in antioxidant levels by increasing BMI, while showed another study that obesity leads to a decrease in oxidative levels **(Chrysohoou *et al*., 2007).**

As for obesity, it has been found in the current study that the level of MANF is affected by obesity, as its level decreases in patients with obesity, as MANF levels in one study showed an inverse relationship with obesity, where the lower the level of MAMF, the greater the obesity and vice versa (**Wu *et al*., 2021**). While other studies have shown that the higher the level of MANF, the greater the obesity constitutes a noticeable **(Tang, & He, 2020).** There are studies that did not show any different between obese patients and the control group in MANF level (**Galli *et al*., 2019**). The results in current study showed that the level of MANF decreased with age, when comparing MANF levels for ages 40 to 55 and ages over 55 years. Other studies have also confirmed that the low level of MANF is associated with age, as its level decreased in old age (**Sousa-Victor *et al*., 2019; Yang *et al*., 2014**).

In general, it has been noticed through the current study a slight rise in levels of liver enzymes ALT, AST, ALP, and TSB compared to the control group, as liver enzymes are affected in all liver diseases, but they may be less affected or normal in fatty liver grade one The first stage and the first to rise from these enzymes is the Alanine aminotransferase ALT enzyme in non-alcoholic fatty liver disease and this is confirmed by previous studies **(Schindhelm *et al*., 2006; Su *et al*., 2019**).

The albumin test was not affected in the current study because the samples were from people with non-alcoholic liver fat grade I while a study in 2021 showed that albumin is reduced in liver disease and liver failure **(Chen *et al*., 2021).**

While the level of Mesencephalic Astrocyte-Derived Neurotrophic Factor MANF, which is a new marker, Through the results of the current study was lower in the patient's serum compared to control, As the lack of MANF level leads to the accumulation of liver fat. A previous study also confirmed that a decrease in the level of MANF contributed to age-related inflammation and inflammation, Liver damage and fibrosis are clinical manifestations in patients with non-alcoholic steatohepatitis, the fact that nonalcoholic hepatitis and low levels of MANF in the blood were confirmed by the current study (**Sousa-Victor *et al*., 2019**). Through the results in current study, it has been concluded that MANF is considered a protective factor against non-alcoholic fatty liver. Other studies have also confirmed that MANF is a protective factor against liver fat as in previous studies **(Cordero-Llana *et al*., 2015; Ezhilarasan, & Lakshmi, 2020; Glembotski, 2011).**

The statistical results indicated a highly statistically significant between non-alcoholic fatty liver disease patients and patients with CAD without NAFLD ALT, AST, TSB, ALP, and MANF. The ALT/AST ratio is also a marker for the identification of Non-alcoholic fatty liver disease **(Su *et al*., 2019; Adams *et al*., 2014).** This ratio showed the rate of the disease increases in older people and obese people and the level of MANF decreases, as obesity is one potential contributor to the buildup of liver fat, but in addition to diabetes, lifestyle, and other reasons were statistically significant in current study. This has been confirmed by many other studies in non-alcoholic fatty liver disease. As for the relationship of the level of MANF with lipids tests represented by TC, TG, LDL-C VLDL-C in this study was a negative relationship where the lower level of MANF the higher the levels of these markers and the higher the level of MANF the lower the levels of these markers **(Wang *et al*., 2014).**

The correlation of the results of all these markers with MANF was statistically significant. These findings are consistent with a previous study that says MANF levels are negatively associated with total cholesterol and LDL cholesterol (**Nasiri-Ansari *et al*., 2022; Day, and James, 1998; Buzzetti *et al*., 2016).**

It has been concluded from the results of current study that MANF prevents the deposition of fat in hepatocytes, and this was confirmed by a study in 2020 that MANF stopped the deposition of fat in hepatocytes **(He *et al.,* 2020).**

By evaluating the MANF levels of the samples, the results of the current study showed that the MANF level in the patients is generally below control, either by dividing the samples into four groups. **Group 1** samples of coronary artery patients who also suffer from non-alcoholic fatty liver disease, **Group 2** Coronary Artery Patients without NAFLD, **Group 3** NAFLD Patients without Coronary Artery Disease**, the 4 group** is controlled healthy people. The results of the MANF and MDA were statistically significant differences in the four groups, where MANF showed the highest value in group **4** group of healthy people while other groups showed a lower level in groups **1**, **2**, and **3** from this it was concluded that MAMF is a protective factor against in both NAFLD and CAD, while MDA was a risk factor for both CAD and NAFLD (**Gianazza *et al.,* 2019).** Through the results of the current study MANF levels were lower in the patient's serum compared to control, as the lack of MANF level leads to the accumulation of liver fat. A previous study also confirmed that a decrease in the level of MANF contributed to age-related inflammation and Liver damage, inflammation and fibrosis are clinical manifestations in patients with non-alcoholic steatohepatitis, as this study confirmed that nonalcoholic hepatitis is associated with low levels of MANF in circulation **(Sousa-Victor *et al*., 2019).**

Other studies have shown that MANF acts as an anti-inflammatory and apoptosis agent and MANF protects heart muscle cells from apoptosis to alleviate the severity of the disease (**Wang *et al*., 2020).** Another study showed that it can be predicted that MANF levels could act as a therapeutic agent for cardiovascular disease **(Ren *et al.,* 2021).** As for ROC, the ROC results for MANF and MDA in **G2** (CAD only) and **G3** (NAFLD only) showed high specificity and sensitivity, so they are considered strong diagnostic markers for both diseases CAD and NAFLD.

Chapter Five

Conclusion & Recommendation

5. Conclusions and Recommendations

5.1. Conclusions

1. The results showed the effect of non-alcoholic fatty liver disease on coronary artery disease. In the current study, it has been found that 61.7 % of chronic coronary artery patients suffer from NAFLD, and this large percentage indicates an association between or a risk factor for coronary artery disease.
2. The results presented here contribute to the determination of the functions of the physiological MANF protein in protecting against non-alcoholic fatty liver and chronic coronary artery disease, as the lack of the level of the MANF protein helps the accumulation of liver fat and also causes increased cell death.
3. The results of the study showed that MDA is a risk factor in both CAD and NAFLD and the anti-oxidant markers GPX, TAC, Se, and MANF showed a protection factor against both CAD and NAFLD and were statistically significant except TAC.
4. Results of the receiver operating curve (ROC) curve and AUC analysis for diagnostic parameters. MANF and MDA were shown a good performance for prediction patients compared to the control group for MANF levels: (sensitivity = 95.7%, specificity 91.3%) at a level = 198.1415, while MDA levels: (sensitivity 52.2%, specificity 73.9%) at a level = 2.11, the p-values of the AUC were <0.05 and highly statistically significant.

5.2. Recommendations & Future Work

1. There is a still need more number of patients for studying the relationship of fat-lowering therapies such as statins with MANF levels, i.e. measuring MANF levels with patients Taking fat-lowering therapies and comparing them to people without taking this treatment.

2. There is a still need for studying on the use of MANF protein as a treatment against non-alcoholic fatty liver, coronary artery disease, inflammation, and metabolic diseases.

3. There is a still need for studying the effect of selenium levels on coronary artery disease and study the relationship between them.

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Appendices

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((Experimental Data))

Effect of Mesencephalic Astrocyte-Derived Neurotrophic Factor and Oxidative Imbalance in Coronary Artery Diseases with/without Non-Alcoholic Fatty Liver in Iraqi Patients

Sample NO:		
Inclusion Criteria: Chronic Coronary Syndrome,		
Exclusion Criteria: Alcoholic Fatty Liver, Acute Coronary Syndrome, Acute Heart Failure		
Risk Factor Of Coronary Artery Disease		
Age:		
Gender:		
Family History:		
Diabetes Mellitus	Yes	No

Hypertension	Yes	No
Smoking	Yes	No
Sedentary Life Style	Yes	No
Investigation		
Ultra Sound:		
Mesencephalic Astrocyte-Derived Neurotrophic Factor		
Total Antioxidant Capacity		
Glutathione Peroxidase		
Malondialdehyde Selenium		
Lipid Profile		
Total Cholesterol:		
HDL-Cholesterol:		
Triglycerides:		
LDL-Cholesterol:		
VLDL-Cholesterol:		
Liver Function Tests		
Albumin:	ALT:	
TSB:	AST:	
ALP:		
Height:	Weight:	
Waist:	BMI:	
Medication:		
Other Notes:		

الخلاصة

مرض انسداد الشريان التاجي هو سبب رئيسي للوفاة في جميع أنحاء العالم في البلدان المنخفضة والمتوسطة الدخل. يحدث في الأعمار الصغيرة والكبيرة. بمرور الوقت، يتضاعف هذا المرض لدى البالغين الذين تزيد أعمارهم عن 30 عامًا. يشير مرض الشريان التاجي (CAD) ، المعروف أيضًا باسم مرض القلب الإقفاري (IHD) ، إلى مجموعة من المتلازمات وثيقة الصلة الناتجة عن اختلال التوازن بين الطلب على الأكسجين في عضلة القلب وإمدادات الدم.

يُعد مرض الكبد الدهني غير الكحولي (NAFLD) مشكلة صحية عامة تؤثر على ما يصل إلى ثلث السكان البالغين في العالم. لقد وثقت العديد من الدراسات الجماعية باستمرار أن NAFLD (خاصة في أشكاله الأكثر تقدمًا) يرتبط بارتفاع مخاطر الوفيات لجميع الأسباب وأن الأسباب الرئيسية للوفاة بين مرضى NAFLD هي أمراض القلب والأوعية الدموية (CVDs).

تهدف الدراسة الى التعرف عن مدى تأثير مرض الكبد الدهني غير الكحولي وعلاقته بمرض انسداد الشريان التاجي، وتقييم مستويات MANF وما هو تأثيرها على كل من مرض الكبد الدهني الغير الكحولية وامراض الشرايين التاجية.

صممت هذه الدراسة للتحري عن حالة انسداد الشريان التاجي المزمن وتضمنت الدراسة 120 عينة (ذكور وإناث) وتم جمع عينات السيرم من مركز القلب في كربلاء / مديرية صحة كربلاء - كربلاء - العراق تتراوح أعمارهم بين 40 إلى 73 سنة. كان عدد مرضى الشرايين التاجية 60 مريضاً وعدد الأصحاء 60 وكان عدد المرضى 37 مصاباً بمرض الكبد الدهني غير الكحولي و 23 مريضاً بدون مرض الكبد الدهني غير الكحولي في كل من مجموعة المرضى والسيطرة. تم الاحتفاظ بمصل الدم عند -20 درجة مئوية. تم تحديد عامل التغذية العصبية المشتق من الخلايا النجمية، واختبار وظائف الكبد، وفحص الدهون والألبومين في الحالة المثلى في مختبر قسم الكيمياء والكيمياء الحيوية، كلية الطب، جامعة كربلاء باستخدام جهاز الكيمياء الحيوية الآلي لقياس وظائف الكبد وفحص الألبومين، بينما تم تحديد العلامات الحيوية MANF بواسطة تقنية الإليزا ساندويج ELISA Sandwich.

توصلت الدراسة الى حدوث انخفاض معنوي من مستوى **MANF** في مرض الكبد الدهني غير الكحولي، والتنكس الدهني، وأمراض الشرايين التاجية، وكانت النتائج تشير إلى وجود اختلاف كبير في مستويات مانف بين المجموعات، وزيادة مستويات MDA وانخفاض في المستويات في مجموعة مضادات الأكسدة GPX، TAC ، والسلينيوم. كان متوسط مستويات MANF في السيطرة (348.62 ± 143.50) وهو أعلى بكثير من مجموعة المرضى (287.58 ± 76.71)، (p 0.001). توزيع مستوى مصل MANF (348.62 ± 143.50)، مستوى MANF يقي من التهاب الكبد وتليفه، ويقلل من أمراض الشرايين التاجية المزمنة.

استخلصت الدراسة الى النتائج المعروضة هنا تساهم في تحديد وظائف بروتين MANF الفسيولوجي في الحماية من الكبد الدهني غير الكحولي ومرض انسداد الشريان التاجي المزمن، حيث أن نقص مستوى بروتين MANF يساعد على تراكم الدهون في الكبد ويؤدي أيضًا إلى زيادة موت الخلايا بسبب نقص الأكسجين.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء

كلية الطب

فرع الكيمياء والكيمياء الحياتية

تأثير عامل التغذية العصبية المشتق من الخلايا النجمية المتوسطة وعدم التوازن التأكسدي في أمراض الشريان التاجي مع / بدون الكبد الدهني غير الكحولي في المرضى العراقيين

رسالة ماجستير

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

من قبل

ابتهال حميد غازي

بكالوريوس علوم كيمياء

2011-2010

إشراف

الأستاذ الدكتور

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أيلول 2023 ميلادية

صفر 1445 هجرية