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Association between Growth Differentiation Factor-15, Zonulin and osteocalcin in Cardiovascular Diseases with/without Non-Alcoholic Fatty Liver Disease of 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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Association between Growth Differentiation Factor-15, zonulin and osteocalcin in Cardiovascular Diseases with/without Non-Alcoholic Fatty Liver Disease of Iraqi Patients

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Dedication

With love, I dedicate this work to my biggest supporter,

Father

and to my source of inspiration,

Mother

For my sisters,

and all my lovely family

Aliaa Ahmed

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Summary

The most common cardiovascular disease (CVD), coronary artery disease (CAD), is one of the leading causes of death worldwide and has lately increased in prevalence in low- and middle-income counteries. It is brought on by atherosclerosis, which results in a reduction in blood flow to the distal myocardium by narrowing the arterial lumen.

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease and is the hepatic manifestation of metabolic syndrome. NAFLD is linked to both liver-related and extrahepatic morbidity. Among extrahepatic consequences, cardiovascular disease (CVD) is the leading cause of death in NAFLD patients. Coronary artery disease (CAD) is the most common clinical manifestation of CVD. Epidemiological studies show a relationship between CAD and NAFLD.

This study aimed to investigate the role of biochemical markers; Growth differentiation factor 15, osteocalcin, and zonulin as early diagnostic markers of CAD and NAFLD.

A Case-control study approach was conducted on 180 subjects with age ranging between (40-70) years involving 135 patients and 45 apparently healthy controls in Kerbala Governorate. The subjects were collected throughout the period from October 2022 to January 2023. The patients were divided into three groups, (45) coronary artery with non-alcoholic fatty liver, (45) coronary artery, and (45) non-alcoholic fatty liver. The patients of CAD and NAFLD were exposed to a medical examination for signs and symptoms by a specialized doctor. Serum biomarker levels were measured for the following parameters: GDF-15, osteocalcin, and zonulin levels were measured using the ELISA technique.; Measurement of Iipid profile and liver function test levels in human serum was performed using autoanalyzer chemistry. The association between biochemical markers and disease severity

was evaluated. The efficiency of the predicting value was assessed using the receiver operating characteristic (ROC) curve.

The observed results indicated that GDF-15 levels were shown significantly increased levels in the CAD with NAFLD cases compared to the CAD and NAFLD cases separately. The levels were (273.18 ng/L); (223.78 ng/L) and (237.34 ng/L) respectively. Also, zonulin showed higher levels in CAD+NAFLD group, the levels were (94.80 ng/ml); (73.42 ng/ml) and (63.42 ng/ml) respectively. On the other hand, patients showed significantly decreased levels of osteocalcin compared to the control group. Results also illustrated the receiver operating curve (ROC) and AUC analysis for the markers as possible diagnostic parameters. Zonulin level had shown good diagnostic performance for prediction CAD+NAFLD patients compared to the control group. The optimal diagnostic points for predicting CAD+NAFLD by GDF-15 was (sensitivity = 79%, specificity = 91%) at a level = 250.605 , osteocalcin (sensitivity = 88%, specificity = 60%) at a level = 8.732 and zonulin (sensitivity = 93%, specificity = 83%) at a level =69.79.

In conclusion, the increased serum level of GDF-15 in NAFLD and CAD functions as an early predictor marker for cardiovascular risk, non-alcoholic fatty liver, and appears to be a direct participant in the atherosclerotic process andmetabolic changes. The decreased level of osteocalcin in the NAFLD and CAD Patients groups might be returned to the potential role for improving endothelial function and reducing the pathological mechanisms that promote the development of atherosclerosis in patients with NAFLD. This effect is often a result of improved metabolic outcomes; however, whether there is a direct osteocalcin/vascular interaction is yet to be fully elucidated. The present results revealed that increased serum zonulin levels in NAFLD and coronary artery disease patients groups denote the possible role in the pathogenesis of NAFLD occurrence and progression.

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Abbreviations	Complete Names
AC	Atherogenic coefficient
ACD	Atherosclerotic cardiovascular disease
ACS	Acute coronary syndrome
AF	Atrial fibrillation
AHA	American hospital association
AIP	Atherogenic index of plasma
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AT	Adipose tissue
AUC	Area under curve
BMI	Body mass index
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CCTA	Coronary computed tomography angiography
CHD	Coronary heart disease
C-index	Cholesterol index
CMR	Cardiac magnetic resonance
cOC	Carboxylated osteocalcin
CR.I	Castelli's risk index I
CR.II	Castelli's risk index II
CT	Computed tomography
CVD	Cardiovascular disease
DNL	De novo lipogenesis
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
FFA	Free fatty acid
GDF-15	Growth Differentiation Factor 15
GSH	Glutathione
HCC	Hepatocellular carcinoma
HDL-C	High-density lipoprotein cholesterol
HF	Heart failure
HRP	High-risk plaque
HRV	Heart rate variability
HTN	Hypertension
ICA	Invasive coronary angiography
IHD	Ischemic heart disease
IR	Insulin resistance
LAD	Lift anterior descending

LCX	Lift circumflex
LDL-C	Low-density lipoprotein cholesterol
LFTs	Liver function test
LPS	Lipopolysaccharides
MDA	Malondialdehyde
MI	Myocardial infarction
MRI	Magnetic resonance imaging
MS	Metabolic syndrome
NADH	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver
NASH	Non-alcoholic steatohepatitis
NCD	Non-communicable disease
NEFA	Non-esterified fatty acids
NRS	Napkin ring sign
NSTEMI	Non-ST-elevation myocardial infarction
OCN	Osteocalcin
OS	Oxidative stress
PAD	Particularly arterial disease
PCI	Percutaneous coronary intervention
POD	Peroxidase
RCA	Right coronary arteries
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SCD	Sudden cardiac death
SD	Standard deviation
SIHD	Stable ischemic heart disease
SMC	Smooth muscle cell
SOD	Superoxide dismutase
STEMI	ST-elevation myocardial infarction
T2D	Type 2 diabetes
TB	Total bilirubin
TC	Total cholesterol
TG	Triglycerides
TGF-β	Transforming growth factor-β
TNF- α	Tumor necrosis factor-alpha
tOC	Total osteocalcin
ucOC	Undercarboxylated osteocalcin
VLDL-C	Very low-density lipoprotein cholesterol
WC	Waist circumference
WHO	World health organization

Chapter One

Introduction and Literature Review

1. Genral Introduction

Coronary Artery disease (CAD) is a complicated disease that causes a high rate of death and hospitalization in the general population around the world (**Benjamin *et al*., 2019; Knuuti *et al*., 2020)**. The disease caused approximately one-third of deaths worldwide **(Mozaffarian *et al*., 2016),** contributing to about deaths of 17.7 million people annually all over the world **(Qammar *et al*., 2020)**. CAD is a disorder in which the myocardium receives insufficient blood and oxygen. It is caused by blockage of the coronary ar- teries, which causes an oxygen demand-supply mismatch **(Rehman *et al*., 2021; Shahjehan *et al*., 2021)**.

The global prevalence of nonalcoholic fatty liver disease (NAFLD) is estimated to be 25% and continues to rise worldwide in the setting of the obesity epidemic. This increase is especially concerning because NAFLD is often a progressive disease that can be associated with significant complications such as liver cirrhosis, hepatocellular carcinoma, and an increase in liver-related and overall mortality **(Mundi *et al*., 2020)**.

Recent epidemiological research has identified a close relationship between these two global health problems, NAFLD and CVD. The progression of NAFLD displays the closest correlation with CVD, followed by extra-hepatic cancers and other liver-associated complications **(Wijarnpreecha *et al*., 2021)**. CVD represents a leading cause of death in the general population, with a prevalence of at least 40% among patients with NAFLD **(Younossi *et al*., 2016)**. NAFLD is a predictor and a risk factor for the development of CVD, as it increases the risk of morbidity and mortality and impacts the progress to other extrahepatic manifestations **(Targher *et al*., 2021)**.

1.1. Cardiovascular Diseases

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels **(Pagidipati and Gaziano, 2013)**. About one-third of deaths globally are caused by cardiovascular disorders **(Mozaffarian *et al*., 2016)**. Ischemic heart

disease (IHD) is the most common cardiovascular disease **(Roth *et al*., 2017)**. IHD is also known as coronary artery disease (CAD) and atherosclerotic cardiovascular disease (ACD), and it has two clinical manifestations: myocardial infarction and ischemic cardiomyopathy. An increasing percentage of people with non-fatal IHD have persistent impairments and poor quality of life **(Moran *et al*., 2014)**. It is anticipated that the rising incidence of IHD will continue, in part because of aging populations and in part because obesity, diabetes, and metabolic syndrome are becoming more and more common **(Desa, 2019)**.

Cardiovascular diseases are chronic illnesses that progressively worsen over time and go long periods asymptomatic **(Francula-Zaninovic and Nola, 2018)**. They are a group of diverse illnesses with atherosclerosis most frequently acting as their primary underlying cause of development **(Wong *et al*., 2022)**. The primary factor contributing to cardiovascular-related deaths globally is atherosclerosis **(Tang *et al*., 2021)**. It is a thickening and hardening of the arterial wall, accompanies aging, and is related to major adverse impacts on the cardiovascular system and various other diseases **(Mitchell and Powell, 2020)**. Elevated plasma cholesterol level (>150 mg/dL) is a major cause of the development of atherosclerosis **(Helvaci *et al*., 2019)**.

The cardiovascular system can experience a wide range of issues, such as endocarditis, rheumatic heart disease, and irregularities in the conduction system. Cardiovascular diseases were classified into **(Lopez *et al*., 2022)**:

1. Coronary artery disease (CAD): Also known as coronary heart disease (CHD), this condition is brought on by a reduction in myocardial perfusion, which can lead to angina, myocardial infarction (MI), and/or heart failure. It is responsible for between one-third and fifty percent of CVD cases.
2. Cerebrovascular disease (CVD): This category includes transientischemic attacks (TIA) and stroke.

3. Peripheral artery disease (PAD) is an arterial condition that affects thelimbs and may cause claudication.
4. Aortic atherosclerosis, which includes abdominal and thoracic aneurysms.

1.2. Coronary Artery Disease

The most common cardiovascular disease (CVD) is coronary artery disease (CAD), in which atherosclerosis causes narrowing of the artery lumen, resulting in diminished blood flow being supplied to the distal myocardium, i.e. ischemia. It is the leading cause of death worldwide and its prevalence has increased in low- and middle-income countries in recent years **(Alizadehsani *et al*., 2021)**.

Coronary artery disease occurs when at least one of the left anterior descending (LAD), left circumflex (LCX) and right coronary arteries(RCA) are stenotic **(Yildirim *et al*., 2019)**.

Heart and blood vessels are related to CVD diseases. Atherosclerosis is the main reason for CVD disease. It results due to blockage of a blood vessel by the deposition of plaque on the inner wall. The deposit of cellular waste products, fatty substances, cholesterol, calcium, and fibrin results in the development of plaque. High levels of cholesterol in the blood, diabetes, high blood pressure, and smoking are some main reasons that cause atherosclerosis. Cardiovascular also caused by stress and sleep apnea. In CAD, oxygen-rich blood flow is opposed by deposited plaque along the arteries. On rupture of plaque, blood clots appear and cause thrombosis. It leads to Myocardial infarction (MI) **(Sharma & Acharya, 2019)**.

Coronary artery disease is caused primarily by plaque formation within the intima of the vessel wall **(Shao *et al*., 2020)**, with plaque being defined as a fatty material growing inside the intima along with severe inflammation, especially if the inflammation is chronic. This in turn causes difficulties in supplying the cardiomyocytes with enough blood, oxygen, and nutrients **(Badimon *et al*., 2012)**. Clinical study demonstrates CAD into three categories. Single vessel coronary artery disease (SVCAD), Double Vessel coronary artery disease (DVCAD), and

Triple vessel coronary artery (TVCAD). The prediction of coronary artery disease depends upon the severity of left ventricular dysfunction and the number of diseased arteries. It also reported that therapy of SVCAD by medication had good outcomes while DVCAD and TVCAD did not **(Khan *et al*., 2020)**.

1.2.1. Classification of Coronary Artery Disease

1. Stable ischemic heart disease (SIHD)
2. Acute coronary syndrome (ACS)
3. ST-elevation MI (STEMI)
4. Non-ST elevation MI (NSTEMI)
5. Unstable angina **(Shahjehan & Bhutta, 2022)**

1.2.2. Stable Ischemic Heart Disease

Stable ischemic heart disease 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. The definition of angina has essentially remained the same over the years, and anginal pain is described as constricting discomfort that usually occurs in the front of the chest but may radiate to the neck, shoulders, jaw, or arms **(Jabbour & Curzen, 2023)**.

The sensation of discomfort is frequently described as pressure, tightness, or heaviness; it can also be strangling, restricting, or scorching. Shortness of breath may accompany angina, and chest discomfort may also be accompanied by less-specific symptoms such as fatigue or faintness, nausea, burning, restlessness, or a sense of impending doom.

Chest pain lasting only a few seconds is unlikely to be caused by CAD. Most often, the discomfort lasts only a few minutes or less, with most cases lasting less than ten minutes. A connection to exercise is an essential quality. When exercise increases, such as when walking uphill, against the breeze, or in cold weather, symptoms typically begin or become worse and diminish quickly within a short period of time after these causative variables subside **(Knuuti and Revenco, 2020)**.

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, elderly age, and diabetics. Management of SIHD includes both non-pharmacologic and pharmacologic interventions. Lifestyle modifications include smoking cessation, regular exercise, weight loss, good control of diabetes and hypertension, and a healthy diet. Pharmacologic interventions include cardioprotective and antianginal medications **(Shahjehan & Bhutta, 2022)**.

Every patient should get guideline-directed medical therapy (GDMT) which includes low-dose aspirin, beta-blocker, as-needed nitroglycerin, and moderate to high-intensity statin. If symptoms are not controlled with this, beta-blocker therapy should be titrated up to heart rates 55-60, and the addition of calcium channel blocker and long-acting nitrates should be considered **(Bahit *et al*., 2018)**. Ranolazine could also be added to relieve refractory anginal symptoms. If maximal GDMT has failed to relieve angina, cardiac catheterization should be done to visualize the coronary anatomy and a decision should be made for percutaneous coronary intervention (PCI) or coronary artery bypass graft (CABG) based on the patient profile **(Katz & Gavin, 2019)**.

1.2.2.1. Pathogenesis

Two key factors in the pathogenesis of atherosclerosis are cholesterol deposition and chronic inflammation **(Malekmohammad *et al*., 2019)**. Atherosclerosis has three significant stages including fatty streak formation, induction of atheroma, and atherosclerotic plaques as showed in figure (1-1) **(Yan *et al*., 2020)**.

The vessel wall consists of a monolayer of endothelial cell (EC) that border the luminal blood flow. Underlying this is a largely acellular layer consisting of glycosaminoglycans and collagen, termed the “intima”. Next are layers of smooth muscle cells (SMC), termed the “media,” and finally, a fibrous layer

termed the "adventitia." Atherosclerosis is initiated in large part by the accumulation of certain plasma lipoproteins, including low-density lipoproteins (LDLs) and remnants of triglyceride-rich lipoproteins, in the intimal region of the vessel **(Björkegren & Lusis, 2022)**.

As mentioned previously, the initial event for atherogenesis is lipid retention in the intima of arteries enabled by endothelial dysfunction from insult-induced damage. Next, the trapped LDL is modified by enzymes and oxygen radicals, which subsequently stimulate endothelial cells to express adhesion molecules and vascular SMCs to release chemokines and chemoattractants, which recruit "inflammatory" monocytes and T cells into the developing plaque. Monocytes differentiate in situ into macrophages and uptake oxidized LDL and foam cells are formed **(Beverly & Budoff, 2020)**.

Yellow foam cells aggregate on the arterial walls and cause the development of fatty streaks. A fibrous atherosclerotic plaque cap is formed from the fatty streak during the migration of Smooth Muscle Cells (SMCs) from media to the intima and SMC proliferation. At advanced stages of atherosclerosis, Macrophages and T lymphocytes of fibrous atherosclerotic plaque cap secrete proteolytic enzymes such as metalloproteinase to reduce the stability of the fibrous cap and lyse the fibrous cap extracellular matrix. The breakdown of fibrous cap collagen content leads to the coagulation process, blood clot formation, thrombus formation, and blockade of the arteries**(Malekmohammad *et al*., 2021)**.

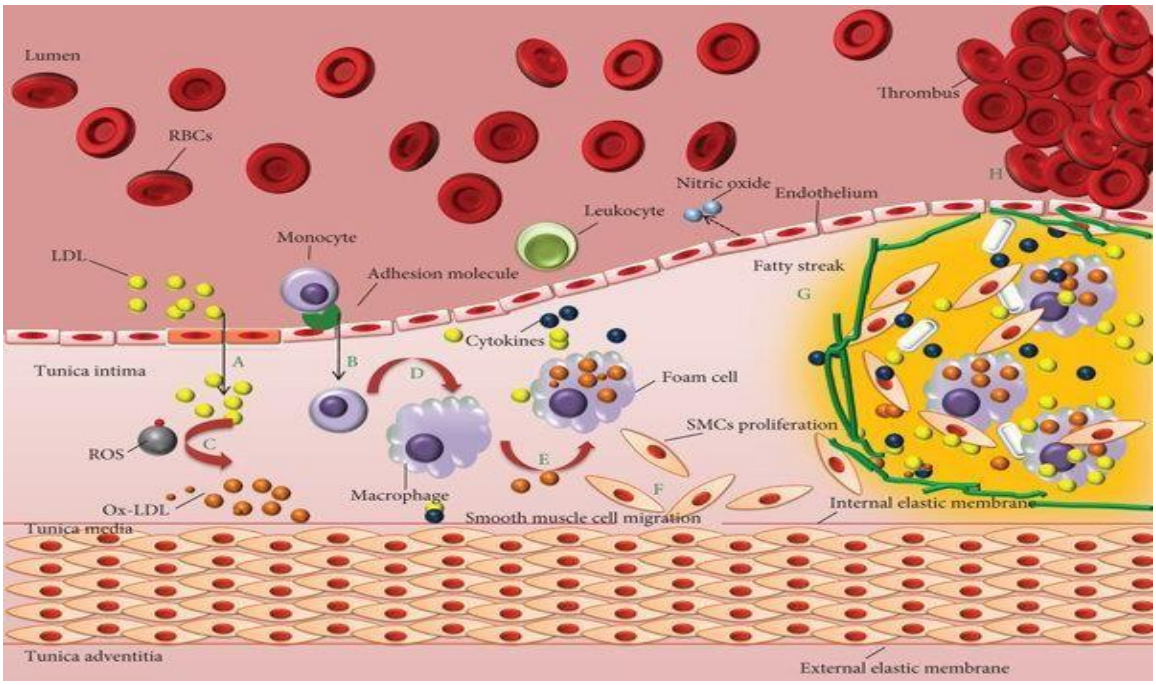


Figure (1-1): Pathogenesis of atherosclerosis. (Khatana *et al*., 2020).

1.2.2.2.Prevalence

Cardiovascular diseases (CVD) are the leading cause of death worldwide. They were accountable for 17.8 million deaths worldwide in 2017 **(Benjamin *et al*., 2018)**. This makes up more than 30% of global deaths. Ischemic heart diseases are the most common cardiovascular cause of death, followed by a stroke. Ischemic heart disease ranked first as a cause of years of life lost (YLL) and disability-adjusted life-years (DALYs) between 1980 and 2017 **(Hay *et al*., 2017; Roth *et al*., 2018)**. As per the World Health Organization (WHO) Health profile report of 2017, CVD accounted for 33.2% of total deaths in Iraq in 2015 with coronary heart disease being the most common cause, followed by a stroke. The common risk factors for CVD among the Iraqi population were age, sedentary lifestyle (46%), hypertension (24%), obesity (27%, more in females), and smoking (32.3% were self-reported as passive smokers) **(WHO, 2017)**. Women have long been disadvantaged in terms of CVD. Women's health research and public education have gone more toward maternal and child health **(Woodward, 2019)**.

1.2.2.3. Risk factors

Risk factors for coronary artery disease are classified into modifiable and non-modifiable risk factors.

A) Modifiable Risk Factors

- **Hypertension:** Hypertension (HTN) is a chronic medical condition characterized by elevated blood pressure (BP) in the arteries. It makes the heart work harder to circulate blood through the blood vessels. The World Health Organization (WHO) estimated that around 62% of cardiovascular diseases (CVDs) and 49% of ischemic heart diseases are attributable to high BP in the world. More than a quarter of the world's population had HTN in 2000, and by 2025, it may increase to 1.56 billion **(Suchitra *et al*., 2018)**. One major problem with HTN is its high mortality rate.

  According to the global report on the epidemiology of HTN, CVDs are responsible for the largest proportion of non-communicable disease (NCD) deaths in the world (48%) and raised BP is one of the leading behavioral and physiological risk factors to which 13% of global deaths are attributed **(Lloyd-Jones & Levy, 2012)**. The other problem is death due to complications of the disease. According to a study on causes of death in 2008, of 17 million CVD deaths every year, complications of HTN account for 9.4 million deaths. HTN is responsible for at least 45% of deaths due to heart disease and 51% of deaths due to stroke **(Legese & Tadiwos, 2020)**.

- **Hyperlipidemia:** Globally, dyslipidemia has been shown to be an independent predictor of many cardiovascular and cerebrovascular events, which led to recent advocacy towards dyslipidemia prevention and control as a key risk factor and its prognostic significance to reduce the burden of stroke and myocardial infarction (MI) **(Alloubani *et al*., 2021)**. Thus, the reduction of LDL cholesterol in those populations, particularly in initial cholesterol concentrations, can reduce the risk of vascular diseases. However, the impact

of using lipid-lowering drugs, such as statins, has been demonstrated in several studies as an important factor in decreasing the mortality and morbidity rates of patients with stroke and CVD **(Alloubani *et al*., 2021)**.

- **Diabetes mellitus:** Diabetes mellitus is a major cause of cardiovascular disease, chronic kidney disease, peripheral arterial disease, and strokes. In patients with diabetes, uncontrolled blood glucose levels are associated with high cardiovascular morbidity and mortality. Sufficient attention to traditional risk factors could yield further substantive reductions in adverse events in the diabetic population **(Kamishima *et al*., 2019)**. Diabetes is a powerful risk factor for CAD. Cardiovascular disease causes death in 75%-80% of diabetic adults and out of these deaths 75% of deaths are due to CHD. Patients with type 2 diabetes are found to have increased LDL, triglycerides, and decreased HDL. Diabetic women have a 3-7 times risk of having CHD disease as compared to men who have 2-3 times increased risk **(Ullah *et al*., 2022)**.

- **Obesity:** A wealth of clinical and epidemiological evidence has linked obesity to a broad spectrum of cardiovascular diseases (CVD) including coronary heart disease (CHD), heart failure (HF), hypertension, cerebrovascular disease, atrial fibrillation (AF), ventricular arrhythmias and sudden cardiac death (SCD). Obesity has been also linked to obstructive sleep apnea and other hypoventilation syndromes, which adversely affect cardiovascular function **(Koliaki *et al*., 2019)**.

- **Smoking:** Tobacco smoking is a major risk factor for CAD. Since the average cigarette contains a complex and changing mix of poisonous compounds with various pathological effects, the exact mechanisms leading to CAD remain unknown. Currently, it is known that smoking leads to atherosclerosis through endothelial dysfunction and damage, plaque vulnerability with increased risk of rupture, increased inflammatory and thrombotic state, and

increased blood pressure **(Said *et al*., 2019)**. Tabaco smoking is also under the influence of genetic factors, including several SNPs associated with smoking initiation, heaviness, and cessation **(Liu *et al*., 2019)**.

- **Poor diet**: The association between saturated fat and coronary heart disease has been a journey. Initially, thought to be a significant causative factor in the development of coronary heart disease, more recent reviews have cast more doubt on this association, placing more of an emphasis on the re-emergence of refined sugars as the main risk factor **(Temple, 2018)**.

  Research has more clearly shown that trans fat increases the risk of cardiovascular disease, through adverse effects on lipids, endothelial function, insulin resistance, and inflammation. Every 2% of calories consumed from trans fat was associated with a 23% higher CAD risk **(Arnett *et al*., 2019)**.

  More recent studies and systematic review articles have focused on red and processed meat consumption. These articles have revealed a consistently higher risk of coronary heart disease and cardiovascular events ranging from 15% to 29% higher risk with red meat and 23% to 42% higher risk with processed meat consumption. Most studies included approximately 50 to 100 grams per day of consumption **(Bechthod *et al*., 2019)**.

  One article indicated no significant association between processed meats and overall mortality, however, added that the combined intake of red and processed meats was associated with a 23% higher risk of overall mortality **(Bechthod *et al*., 2019)**.

- **Sedentary lifestyle**: Exercise is a protective factor in preventing the development of CAD. A 2004 case-control study performed in 52 countries, representing all continents, and involving 15,152 cases and 14,820 controls

revealed a population-attributable risk of 12.2% that physical inactivity has on myocardial infarction.

Several observational studies have shown that individuals who self-select for exercise have lower morbidity and mortality. Mechanisms for this include enhanced production of endothelial nitrous oxide, more effective deactivation of reactive oxygen species, and improved vasculogenesis **(Brown *et al*., 2020)**.

B) Non-modifiable Risk Factors

- **Age:** CAD prevalence increases after 35 years of age in both men and women. The lifetime risk of developing CAD in men and women after 40 years of age is 49% and 32%, respectively **(Brown *et al*., 2020)**
- **Sex:** Among patients with stable ischemic heart disease and acute coronary syndromes, women have a lesser extent of coronary atherosclerosis than men, despite a consistently higher risk profile including older age at presentation and a greater frequency of comorbidities **(Reynolds *et al*., 2020)**. There are sex differences in the prevalence and effect of cardiovascular risk factors. For example, among patients aged≥65 years, low high-density lipoprotein cholesterol is a stronger predictor of cardiovascular mortality in women than in men **(Norris *et al*., 2020)**.

  Testosterone and estrogen are the main sex hormones in men and women, respectively, and studies have shown that they have important roles in cardiovascular health and disease as showed in figure (1-2). Estrogen is a potential cardio-protectant that can be used to treat heart disease in women. Although estrogen is specific for women, it is also present at low levels in men and evidence indicates that abnormally high estrogen levels may contribute to cardiovascular disease in men **(Bajelan *et al*., 2019)**.

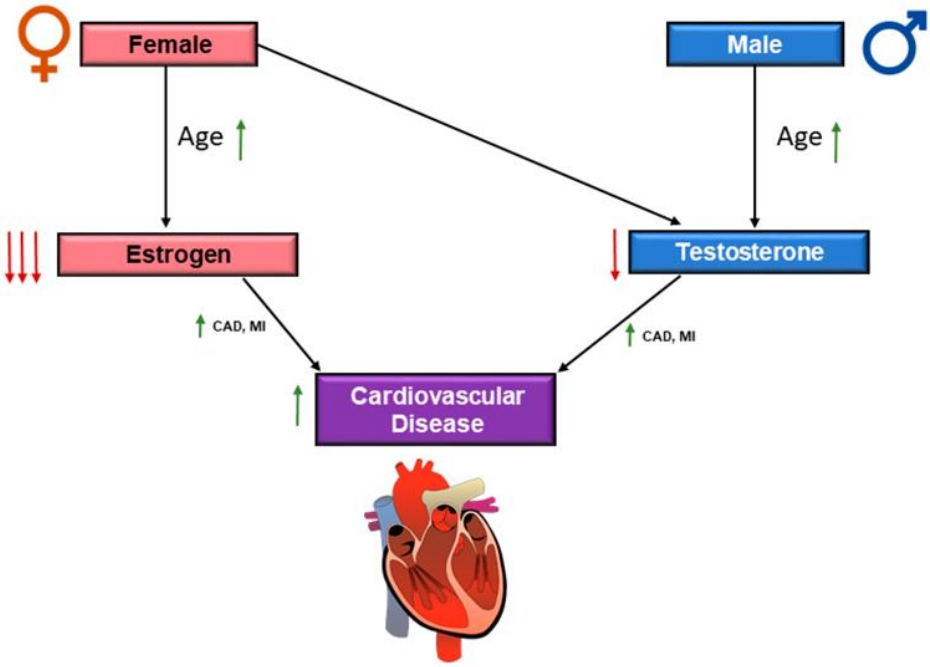


Figure (1-2): Schematic diagram of the influence of sex hormones on cardiovascular disease (CVD) (Rodgers *et al.*, 2019).

- **Family History:** Family history is also a significant risk factor. Patients with a family history of premature cardiac disease younger than 50 years of age have an increased CAD mortality risk. A separate article indicated that a father or brother diagnosed with CAD before 55 years of age, and a mother or sister diagnosed before 65 years of age are considered risk factors **(Brown *et al.*, 2020)**.

1.2.2.4. Diagnosis

The diagnostic approach in a patient with suspected obstructive CAD can be described as a series of successive steps. An initial step is to assess the symptoms and signs to exclude patients with possible unstable angina or other forms of acute coronary syndrome. In other patients, and to evaluate the patient's general condition and quality of life. Comorbidities and other possible causes of the symptoms that potentially influence therapeutic decisions are considered **(Knuuti & Revenco, 2020).**

- **Electrocardiogram (ECG):** A common way to detect angina is to use an electrocardiogram (ECG). The standard 12-lead ECG is the most useful tool for the diagnosis and prognosis of patients with angina pectoris. The reliability of diagnosis can be further improved by signal processing techniques and biomedical analysis. To obtain ECG data more conveniently, a heart rate test application for mobile devices such as smartphones is being actively developed. Short-term heart rate variability (HRV) analysis may help monitor dynamic changes in cardiac autonomic activity **(Zhang & Xu, 2023)**.

  Recently deep learning methods have been used to solve medical problems related to ECG **(Hong *et al.*, 2020; Somani *et al.*, 2021)**. Deep learning methods can fill the limitations of traditional disease diagnosis, improving performance and generalization by reducing pre-processing and feature extraction **(Ting *et al.*, 2019)**.

- **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 also is used in an outpatient setting as part of stress testing. In addition to diagnostics, it also has a role in therapeutics, for example, pericardiocentesis could be performed with the needle guided by echocardiography. This test is user-dependent and could be costly compared to ECG **(Sicari & Cortigiani, 2017)**.

- **Coronary Computed Tomography Angiography (CCTA):** Coronary computed tomography angiography (CCTA) has evolved as an accurate, noninvasive alternative to invasive coronary angiography (ICA) for determining the presence and anatomic extent of CAD in intermediate-/high-risk patients **(Trost *et al*., 2022)**.

  Coronary computed tomography angiography allows plaque visualization, which is not available during routine invasive coronary angiography (ICA) without intravascular imaging. High-risk plaque (HRP) features on CCTA, including low-attenuation plaque (LAP), positive remodeling (PR), and the Napkin ring sign (NRS), have been previously recognized as potentially valuable in identifying patients at increased risk of cardiovascular events. However, the potential utility of these features may be limited in routine practice by inter-observer variability, particularly amongst less experienced CCTA practitioners **(Salem *et al*., 2023)**.

- **Cardiac Catheterization:** Cardiac catheterization is the gold standard and most accurate modality to evaluate ischemic coronary heart disease. It is however an invasive procedure with associated complications. Not everyone is a candidate for the procedure. In non ACS settings, patients with intermediate pretest probability for CAD are usually the right candidates for it. In the ACS setting, all STEMI patients and selected NSTEMI patients get an emergent cardiac catheterization. This procedure is done in a cardiac catheterization lab, is expertise dependent, and is done under moderate sedation. There is contrast exposure in the procedure which could cause serious allergic reactions and kidney injury **(Shahjehan & Bhutta, 2022)**.

1.2.2.5. Complications

The most feared complication from CVD is death and, as explained above, despite multiple discoveries in the last decades CVD remains in the top leading causes of death all over the world owing to the alarming prevalence of CVD in the population. Other complications as the need for longer hospitalizations, physical disability, and increased costs of care are significant and are the focus for healthcare policymakers as it is believed they will continue to increase in the coming decades **(Benjamin *et al*., 2018)**.

1.3. Liver Diseases

Persistent liver damage occurring in the context of metabolic (non-alcoholic fatty liver), viral hepatitis B or C, cholestatic, toxic (alcohol), or genetic (hemochromatosis, Wilson disease, etc.) diseases, usually causes a chronic inflammatory reaction. Similar to a misdirected wound-healing reaction, chronic inflammation can cause scarring of the liver **(Weiskirchen *et al*., 2019)**. Liver disease accounts for approximately two million deaths per year worldwide, one million due to complications of cirrhosis and one million due to viral hepatitis and hepatocellular carcinoma (HCC) **(Asrani *et al*., 2019)**.

1.3.1. Non-Alcoholic Fatty Liver

Non-alcoholic fatty liver is defined by the imagistic or histological presence of hepatic steatosis and the absence of other secondary causes of hepatic fat accumulation such as significant alcohol consumption and other causes **(Dumitrascu & Neuman, 2018)**. Histologically, NAFLD is considered present when ≥5% of the liver cells contain fat and is considered severe when ≥30% of liver cells contain fat on liver biopsy **(Ismaiel & Dumitraşcu, 2019)**.

This disease is used as an umbrella term for the different histological and clinical subtypes of a fatty liver , ranging from hepatocellular steatosis or fatty liver disease to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and carcinoma **(Mirmiran *et al*., 2019)**.

The rising rates of obesity and type 2 diabetes worldwide are paralleled by a rise in the global prevalence of NAFLD **(Loomba *et al*., 2021)**. NAFLD is estimated to afflict one billion individuals globally and may be present in approximately 25% of the world population **(Younossi *et al*., 2019)**.

There is considerable variability in the prevalence of NAFLD across the various geographic regions in the world. The Middle East and South America have the highest and Africa have the lowest prevalence of NAFLD. In the United States, up to 80 million individuals may have NAFLD **(Estes *et al*., 2018)**. The prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing worldwide. In some patients with NAFLD, isolated steatosis can progress to advanced stages with non-alcoholic steatohepatitis (NASH) and fibrosis, increasing the risk of cirrhosis and hepatocellular carcinoma. Furthermore, NAFLD is believed to be involved in the pathogenesis of common disorders such as type 2 diabetes and cardiovascular disease **(Stefan *et al*., 2019)**.

1.3.1.1. Signs and Symptoms

The majority of individuals with NAFLD are asymptomatic, and the disease may remain silent until it has progressed to cirrhosis. The most common symptoms noted at initial referral among individuals with NAFLD include right upper quadrant pain and fatigue. Affected people may have an echogenic liver on ultrasound or evidence of liver fat based on an imaging test that is noted incidentally or as part of a workup for right upper quadrant pain **(Chalasani *et al.*, 2018)**. Liver-related serum tests typically reflect a hepatocellular pattern of enzyme elevations with serum alanine aminotransferase (ALT) higher than serum aspartate aminotransferase (AST). When advanced fibrosis, cirrhosis, and portal hypertension have developed, the platelet count declines gradually over the years **(Loomba & Adams, 2019)**. Decreased serum albumin and an increased prothrombin time are noted in individuals with cirrhosis who manifest evidence of synthetic dysfunction.

Most of the patients with NAFLD have normal or near-normal LFTs. The ALT typically falls (and AST may rise) as fibrosis progresses to cirrhosis. Patients with abnormal LFTs should be simultaneously evaluated for alcohol abuse, drug-induced liver injury, viral hepatitis, autoimmune liver disease, hemochromatosis, celiac disease, and Wilson's disease in patients <45 years old **(Sharma & Arora, 2020)**.

1.3.1.2. Pathogenesis

The metabolic mechanisms leading to NAFLD reflect an imbalance of energy metabolism in the liver: excess energy, mostly in the form of carbohydrates and fat, entering the liver relative to the ability of the liver to oxidize this energy to CO_2 or export it as very-low-density lipoproteins (VLDLs). The result is a net accumulation of energy in the liver as triglycerides, which can explain the widespread presence of NAFLD in obese individuals and in those with lipodystrophy, where surplus energy is stored in the liver because of the deficiency in white adipose tissue lipid storage **(Loomba *et al*., 2021)**.

Although excess consumption of any food can lead to the development of NAFLD, mono- and disaccharides, especially fructose, sucrose, and high-fructose corn syrup, which is ubiquitous in processed foods, can activate programs of hepatic de novo lipogenesis that further exacerbate NAFLD. Furthermore, fructose is metabolized almost exclusively by the liver, and therefore dietary fructose is funneled into the liver and mostly metabolized to triglycerides by de novo lipogenesis (DNL) **(Samuel & Shulman, 2018)**.

Skeletal muscle insulin resistance, one of the earliest defects associated with metabolic syndrome and prediabetes, can also promote the development of NAFLD through increased hepatic DNL and hypertriglyceridemia by diverting ingested glucose away from skeletal muscle glycogen synthesis and into the liver for DNL**.** Development of hepatic insulin resistance, where insulin activation of glycogen synthase is impaired **(Petersen & Shulman, 2018)**, would also be expected to redirect glucose into lipogenic pathways and further promote NAFLD figure (1-3).

Consistent with this hypothesis, mice lacking hepatic glycogen synthase manifest hepatic insulin resistance but increased hepatic lipogenesis and NAFLD **(Irimia *et al.*, 2017)**.

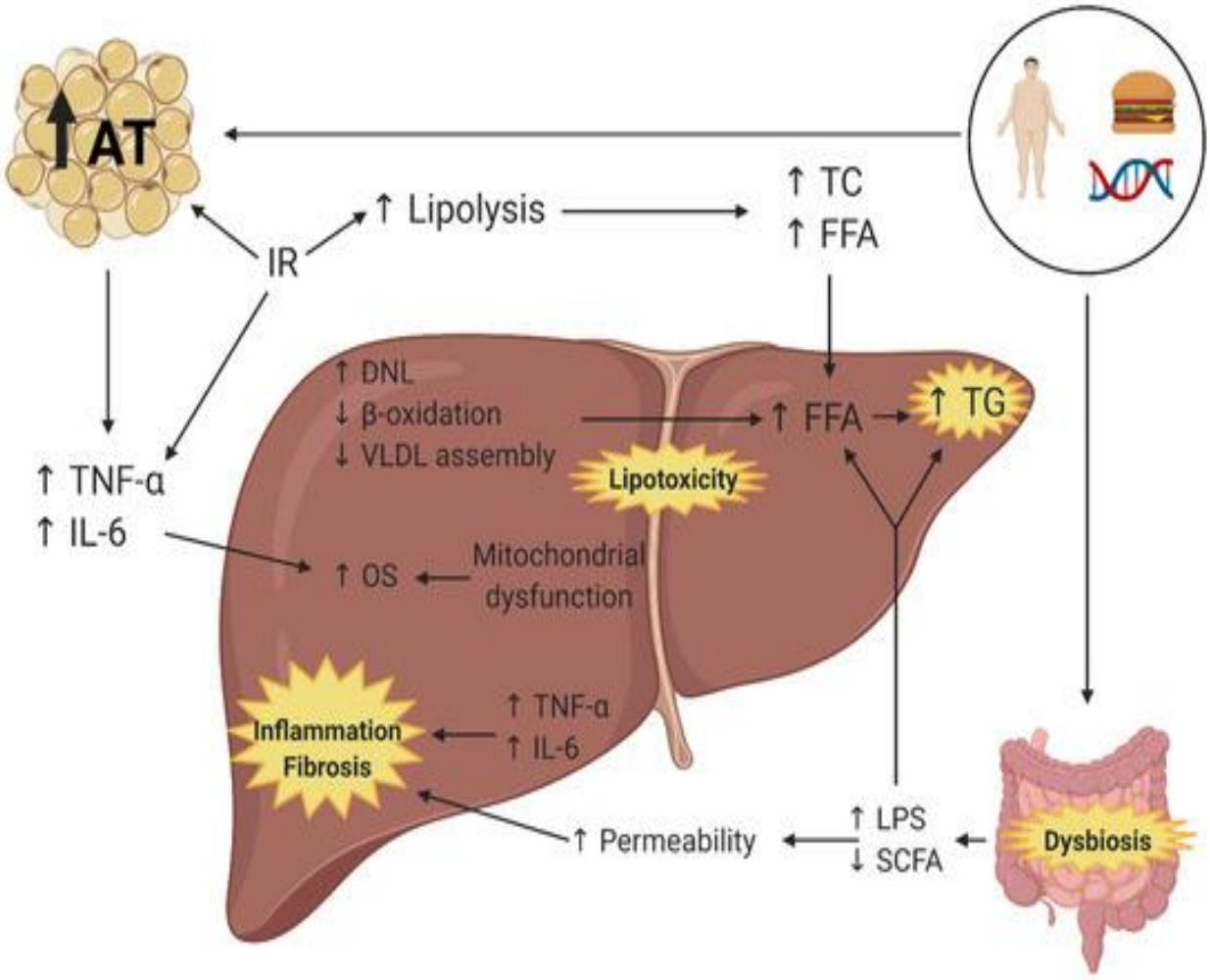


Figure (1-3): Pathophysiology of non-alcoholic fatty liver (Castillo *et al.*, 2021).

1.3.1.3. Prevalence

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease affecting approximately 20–30% of adults and 10% of adolescents in Western developed countries. The condition is common in the Middle East, with a prevalence of 24.8% in Saudi Arabia and 23.7% in the United Arab Emirates **(Gu *et al.*, 2019)**.

However population-level data from four European primary care databases of over 18 million patients found that only 0.7% of patients had a recorded diagnosis

of either NAFLD or NASH **(Spiers *et al*., 2022)**. Steatosis is also prevalent in young people, with a recent UK study finding that 20% of young people aged 22–26 years had steatosis, and 1 in 40 had fibrosis, on transient elastography **(Abeysekera *et al*., 2020)**. By 2025, it is estimated that NAFLD will be the leading cause of liver failure and the leading indication for liver transplantation worldwide **(Haldar *et al*., 2019)**. There is no clear evidence that morbidity and mortality (both cardiovascular and liver) increase with a progressive disease that is driven by the degree of inflammation and fibrosis as well as the development of T2D and continued weight gain **(Moolla *et al*., 2020)**.

In the general population, a prevalence of NAFLD ranging between 17% and 33% was estimated, whereas, in obese and/or diabetic individuals, this prevalence reached 75% **(Rosato *et al*., 2019)**.

1.3.1.4. Risk factors

Clinically, NAFLD patients tend to be obese, with insulin resistance and/or type 2 diabetes, dyslipidemia, hypertriglyceridemia, and hypertension, which are all risk factors for cardiovascular diseases (CVDs) **(Li *et al*., 2014)**. The prevalence of NAFLD in patients with components of MetS is quite high **(Chan *et al*., 2013)**. For instance, NAFLD has been reported in over 76% of type 2 diabetics **(Portillo-Sanchez *et al*., 2015)**. Furthermore, over 90% of severely obese patients undergoing bariatric surgery have NAFLD **(Yamazaki *et al*., 2015)**.

1.3.1.5. Stages of Non-Alcoholic Fatty Liver

There are four different clinical phases described for NAFLD as showed in figure (1-4). Phase one is characterized by simple steatosis and is considered harmless. Some patients progress to Phase two developing inflammation and ballooning (NASH). Phase three is defined by the presence of NASH with persistent inflammation resulting in liver fibrosis (scarring), which is considered the strongest predictor of liver-related events in NASH patients. Over time, this 3rd stage can lead to a more serious condition, such as liver cirrhosis (Phase four) or even cancer,

where a liver transplant is the only therapy option. In addition to liver-specific pathology, a diagnosis of NASH is also associated with increased cardiometabolic risk and represents the leading cause of death for these patients **(Fraile *et al.*, 2021)**.

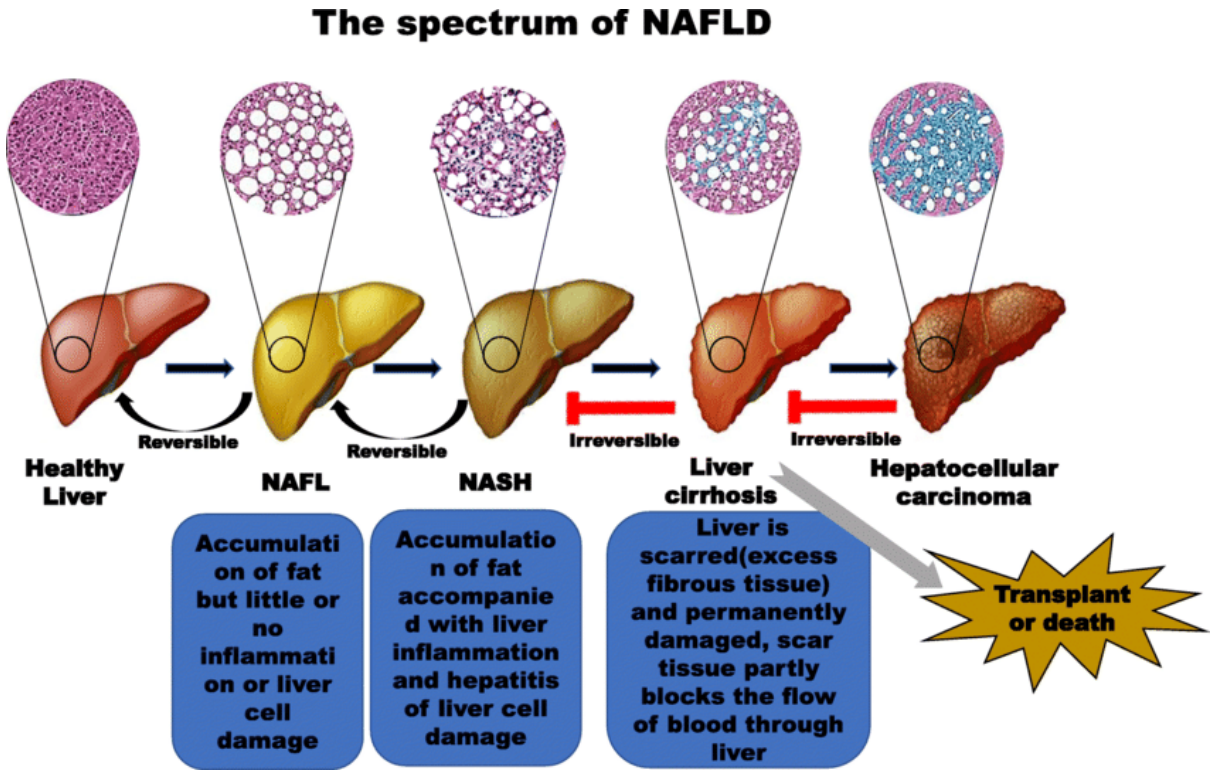


Figure (1-4): The spectrum of NAFLD progression (Wang *et al.*, 2020).

1.3.1.6. Diagnosis

Non-alcoholic fatty liver can be diagnosed by imaging studies such as ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI), the presence of NASH still requires a liver biopsy to identify the presence and location of its features such as inflammation, hepatocyte ballooning, Mallory-Denk bodies, and early fibrosis. The clinical challenge is then to identify the subset of patients with NASH and fibrosis, who are at higher risk of disease progression. Liver biopsy may be the gold standard investigation for determining fibrosis stage in NAFLD, but it is invasive, costly, carries a risk of complications and, probably most importantly, is subject to sampling and inter- and intra-observer variability **(Davison *et al.*, 2020)**.

Therefore, intensive efforts have been exerted to develop noninvasive biomarkers for the detection of the degree of fibrosis in patients with NAFLD. These biomarkers include clinical and biochemical parameters such as body mass index (BMI), presence of diabetes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), AST/ALT ratio, and HOMA-IR, among others. Some scoring systems derived from the aforementioned and some other parameters have been validated to reflect the degree of liver fibrosis noninvasively. The NAFLD, FIB4, BARD, and NIPPON scores are some examples of these scoring systems **(Bayrak, 2020)**.

1.3.1.7. Treatment

Despite the promising results from research with new pharmacological strategies, drug treatment by itself is likely, not sufficient, if the unhealthy lifestyle is not corrected. Lack of physical activity is one critical factor having an impact on the outcome of hepatic diseases. Undoubtedly, exercise can be very beneficial, even in the advanced stages of liver fibrosis or cirrhosis. It has been shown that in response to exercise a large number of endogenous active mediators are produced that endorse anti-inflammatory effects. Therefore, it is not surprising that physical activity and aerobic exercise of moderate intensity for at least 20 to 60 minutes on at least 5 days a week in combination with resistance training performed thrice weekly has entered into the practical recommendations for the prevention and treatment of NAFLD **(Tacke & Weiskirchen, 2021)**

Weight loss of 1 kg/week is recommended for overweight and obese subjects suffering from NAFLD and NASH **(Ullah *et al*., 2019)**. There is currently no effective pharmacological therapy for the treatment of NAFLD. Weight loss, achieved through lifestyle changes including diet modifications and exercise, remains the most effective strategy for NAFLD management **(Rives *et al*., 2020)**.

1.4. Mechanisms linking NAFLD and CAD

Non-alcoholic fatty liver is associated with liver-related and extrahepatic morbidity-mortality. Among extrahepatic complications, cardiovascular disease (CVD) is the primary cause of mortality in patients with NAFLD. The most frequent clinical expression of CVD is coronary artery disease (CAD).

The available findings strongly support the fact that NAFLD and CAD are two conditions closely related to MS. Similar to NAFLD being named the hepatic MS manifestation, we could say that CAD is its cardiac manifestation, closely related to the former. Consistent data showed that CA D has a high prevalence among patients with NAFLD, leading to an increased mortality. NAFLD is significantly associated with clinical and subclinical CAD, independently of the conventional cardiometabolic risk factors.

Many putative mechanisms are considered relevant in NALFD-related CAD, including genetics, inflammation, oxidative stress, lipotoxicity, atherogenic dyslipidemia, or gut microbiota. Key questions for future research refer to the complex mechanisms linking NAFLD to CAD, the nature of optimal personalized lifestyle modification and appropriate pharmacologic approaches for both conditions and to whether NAFLD-directed therapeutic strategies can also reduce CVD risk **(Cazac *et al*., 2022)**.

It is therefore obvious that NAFLD and CVD share several common risk factors, e.g., obesity, T2DM, dyslipidemia, and physical inactivity, supporting the idea of a shared pathogenesis**(Liu and Lu, 2014)**. In turn, insulin resistance is associated both with NAFLD and with endothelial dysfunction and ASCVD **(Puchner *et al*., 2015)**.

1.5. Growth Differentiation Factor-15

1.5.1. Definition

Growth differentiation factor 15 (GDF-15), also known as macrophage inhibitory factor-1, is a member of the transforming growth factor-β (TGF-β)

cytokine superfamily. Study has found that GDF-15 is a stress-responsive cytokine that is only highly expressed in the placenta and prostate **(He *et al*., 2020)**, and rarely expressed in myocardial tissues under normal physiological conditions.

1.5.2. Structure and Secretion

Growth differentiation factor 15 circulates as a 25kDa dimer linked by a single inter-chain disulfide bond. It is synthesized as a 308aa peptide consisting of a signal peptide, propeptide, and mature peptide. The membership of GDF-15 in the TGF-β superfamily is conferred by high sequence homology and a conserved 9 cysteine region. Eight of the 9 cysteines in the conserved domain form a cysteine knot, which functions to stabilize the mature GDF-15 monomer. The orientation observed in the crystal structure of GDF-15 is unique amongst all 9 cysteine TGF-β superfamily members in that cysteines 1 and 2 and cysteines 3 and 7 form disulfide bonds, whereas in the other 9 cysteine members cysteine 1 pairs with cysteine 3 and cysteine 2 pairs with cysteine 7 **(Lockhart *et al.*, 2020)**.

The expression and secretion of GDF-15 can be strongly induced in the case of cardiovascular injury, such as pressure overload, myocardial infarction, heart failure, and atherosclerosis **(Wang *et al*., 2019b)**. In recent years, a series of clinical studies have reported that high circulating GDF-15 levels are an independent predictive factor for several cardiovascular diseases, in patients with atherosclerosis and acute coronary syndrome and that investigating the levels of GDF-15 could improve risk prediction beyond traditional risk factors and biochemical indicators **(Wang *et al*., 2019b; Shang *et al*., 2019)**.

Similar to many other proteins, GDF-15 is regulated at the level of transcription, translation, and even translocation in the cell. It is synthesized as a pro-GDF-15 dimer in the cytoplasm, and subsequently, cleaved and secreted as the mature dimer GDF-15. In addition, the pro-peptide GDF-15, a cleavage product, and unprocessed pro-GDF-15 dimer can bind to the extracellular matrix that can act as a deposit site. The circulating serum levels of only the mature GDF-15 can be easily measured and

are very low in humans; however, they are dramatically increased in a large number of diseases, including cancer, cardiovascular disease, liver and kidney diseases, and tissue damage. In addition, the serum levels of GDF-15 are very high during pregnancy **(Baek & Eling, 2019)**.

A recent study further validated that GDF-15 is associated with cardiovascular and noncardiovascular death (eg, cancer morbidity) in stable CAD patients with and without a previous cancer diagnosis. The predictive value persists even a decade later, and the findings support the hypothesis that GDF-15 is not a consequence of cardiovascular disease or a passive biomarker of the disease process, but plays an active role in the pathophysiology of atherosclerosis and CAD **(Wang *et al*., 2019a)**.

Elevated GDF-15 has been shown to promote inflammation and angiogenesis, the clinical and experimental studies support the physiological and pathophysiological role of the GDF-15 system in atherosclerosis and CAD **(Wang *et al*., 2019a)**.

1.6. Osteocalcin

1.6.1. Definition

Osteocalcin (OCN), the most abundant non-collagenous protein in bone, is produced and secreted almost exclusively by osteoblasts during bone formation. Human OC is a 5.7 kilo-Dalton (KDa) peptide containing 49 amino acids (mouse OC has 46 amino acids). During post-translational modifications, OCN can be carbonylated at its 17th, 21st, and 24th glutamine (Glu) residues into carboxylated OC (cOC) **(Lin *et al*., 2020).**

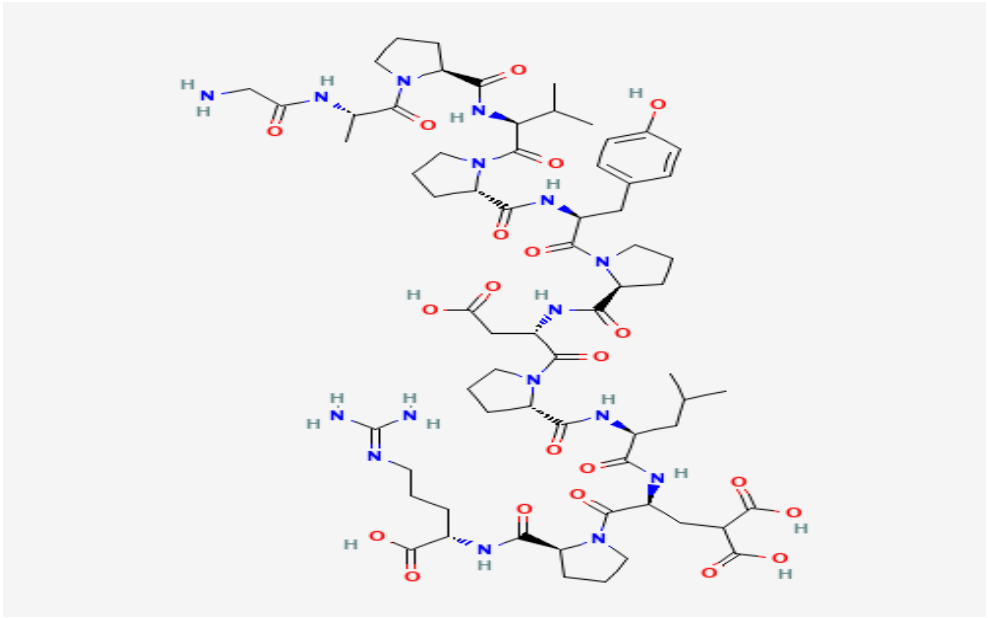


Figure (1-5): Chemical Structure of osteocalcin (National Center for Biotechnology Information, 2023).

1.6.2. Process and scretion

osteocalcin secreted by osteoblasts contains three γ-carboxyglutamic acid residues that confer high affinity to the bone hydroxyapatite matrix. During bone resorption by osteoclasts, the acidic environment promotes the carboxyl groups on OCN to be removed, decreasing its affinity for hydroxyapatite and therefore promoting its release into circulation **(Mizokami *et al*., 2017).**

In humans, apart from its associations with bone remodeling and fractures, circulating OCN has been documented to be inversely associated with adiposity, plasma glucose, insulin resistance, and metabolic syndrome severity **(Lin *et al*., 2018).** Five longitudinal studies evaluated the relationship between total OCN and the incidence of diabetes; three reported a significant or near-significant inverse association **(Shu *et al*., 2016; Urano *et al*., 2018; Massera *et al*., 2018)**, and the other two showed that OCN was not a risk factor for diabetes **(Hwang *et al*., 2012; Zwakenberg *et al*., 2015)**.

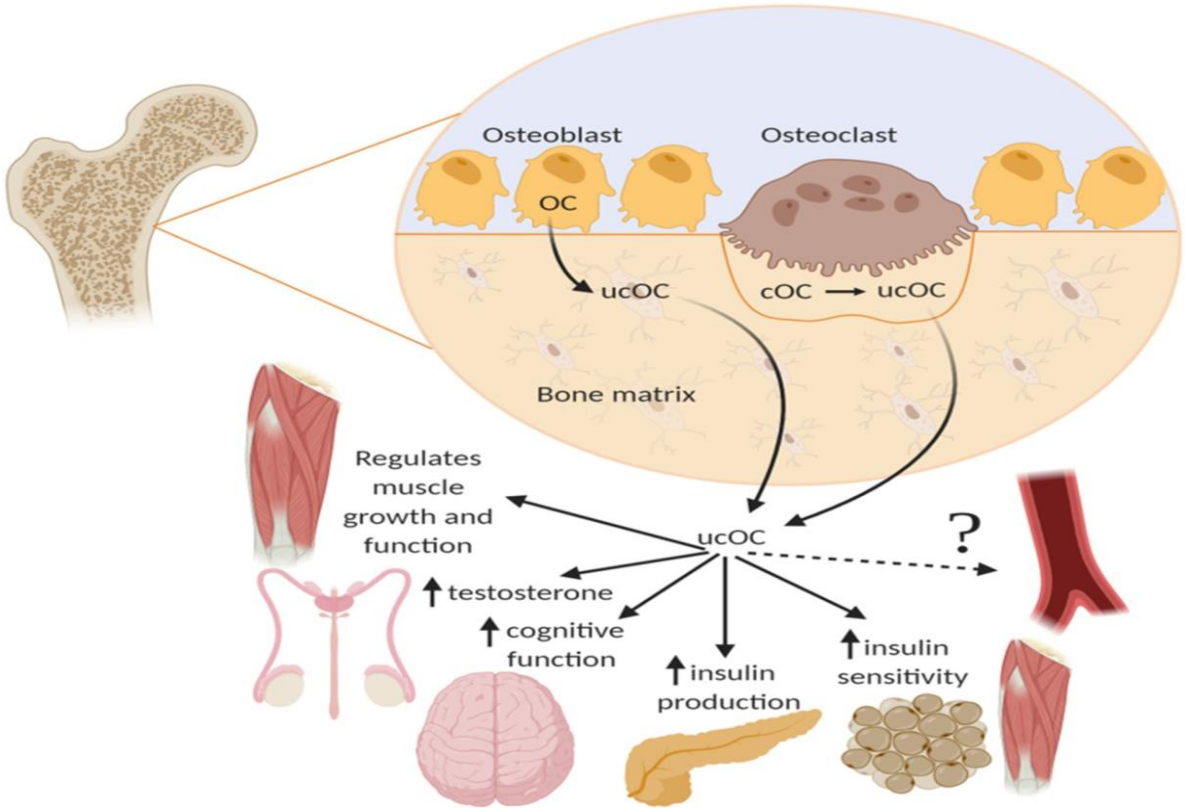


Figure (1-6): Biological functions of ucOC outside of the skeleton (Tacey *et al*., 2021).

OCN exists primarily in two forms; undercarboxylated osteocalcin (ucOC) and carboxylated osteocalcin (cOC), with each form, suggested to have distinct functions. Total OCN (tOC), which is a combination of circulating ucOC and cOC, is more often reported because it is easier to measure. ucOC is thought to be the bioactive form of OCN, involved in the regulation of glucose homeostasis and energy metabolism, figure (1-6)**(Dirckx *et al*., 2019; Rossi *et al*., 2019; Lin *et al*., 2020)**.

1.7. Zonulin

1.7.1. Definition

Zonulin is the protein that elevated permeability in the layer of epithelial of a Small bowel. It is the just physiographic mediator called that elevate gut permeability by modulation the intercellular tight junctions, whereas adequate role of the tight junctions is maintaining normal physiologic operations in the intestinal tract **(Fasano, 2012)**.

1.7.2. Structure and Scretion

Zonulin, a 47 kDa protein, is involved in the regulation of the paracellular permeability in the intestine. The protein is secreted by the liver, the intestine, and several other tissues circulates in the blood, and binds to receptors on the enterocytes in the ileum and jejunum. Binding to these receptors leads to reversible modulation of intercellular tight junctions and thereby increased small intestinal paracellular permeability **(Aasbrenn *et al*., 2020).** Zonulin may also impact the blood-brain barrier through similar mechanisms **(Rahman *et al*., 2018)**.

Zonulin can be measured in the blood and feces. High serum zonulin (s-zonulin) has been interpreted as increased intestinal permeability **(Leech *et al*., 2019)**. Measured with the available enzyme-linked immunosorbent assay (ELISA) methods, s-zonulin has been associated with obesity and high energy intake in cross-sectional studies. S-zonulin has also been associated with the comorbidities of obesity, such as fatty liver disease and diabetes mellitus **(Lin *et* al., 2018)**.

Some conditions were linked to increased zonulin levels, representing their associations with impaired gut permeability **(Leech *et al*., 2019).** Non-coeliac gluten sensitivity **(Barbaro *et al*., 2020),** inflammatory bowel syndrome **(Singh *et al*., 2019)**, ankylosing spondylitis **(Ciccia *et al*., 2017)**, and arthritis **(Tajik *et al*., 2020)** also have been linked to changes in intestinal permeability. Besides that, non-communicable diseases, such as obesity **(Żak-Gołąb *et al*., 2013)** and cardiovascular diseases **(Loffredo *et al*., 2020)** were correlated to zonulin levels.

Zonulin is considered as a physiological modulator of intestinal permeability and a surrogate marker of the dysfunctional gut barrier. Certain gut microbes, in particular pathogens, might induce the release of zonulin from the gut suggesting a mechanistic link between alterations in the gut microbiota and gut barrier function **(Ahmadi *et al*., 2020)**.

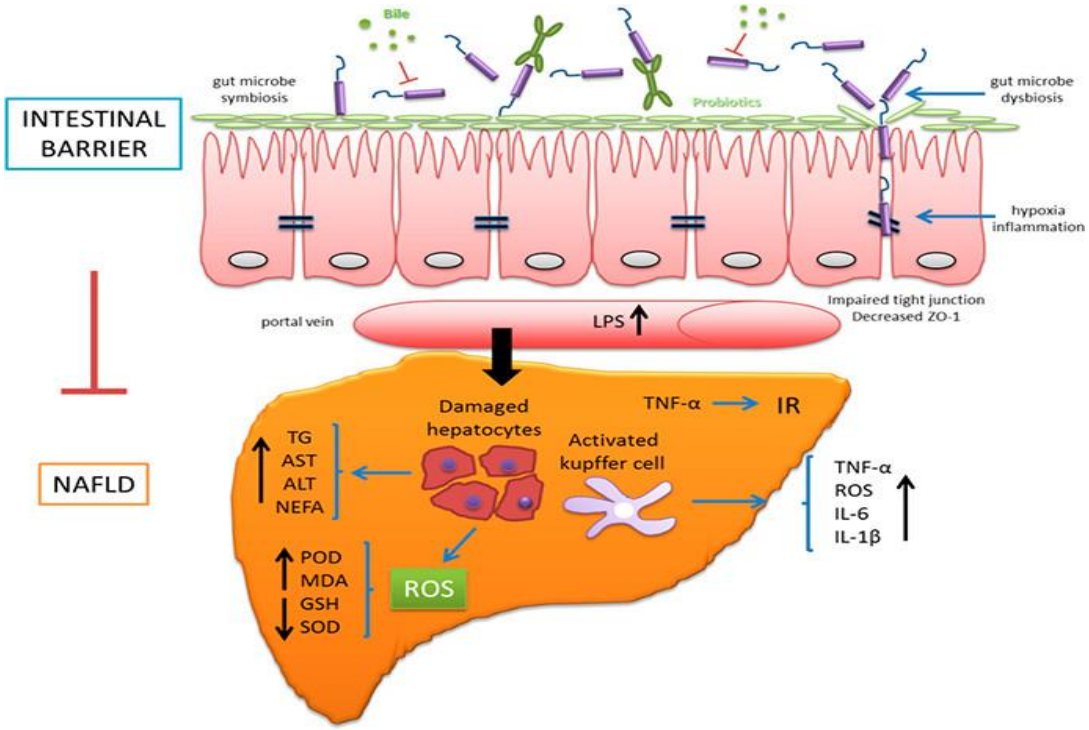


Figure (1-7): Role of Zonulin and intestinal permeability in non-alcoholic fatty liver pathogenesis (Cui *et al*., 2019).

Aim of the study

This study has some goals to achieve

1. Assessment of serum levels of growth differentiation factor 15, osteocalcin, and zonulin in patients with coronary artery disease and with non-alcoholic fatty liver disease.
2. Study the correlation analysis between serum of growth differentiation factor15, osteocalcin, and zonulin levels in CAD and NAFLD with other biochemical parameters like lipid profiles, and liver enzymes in patients groups.
3. Study the receiver operating characteristics (ROC) curve for showing the best marker to diagnose CAD and NAFLD.

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Study Design and Ethical Approval

A case-control study was conducted in the Department of Biochemistry, College of Medicine, University of Karbala. The subjects were collected throughout the period from October 2022 to January 2023. This study was conducted according to the World Medical Association Declaration of Helsinki. It's approved by the Ethical Committee at college of Medicine in Karbalaa University. Informed consent was obtained from participants after elaborating the plan of the study. Samples were obtained after consent from patients or the patient's relatives.

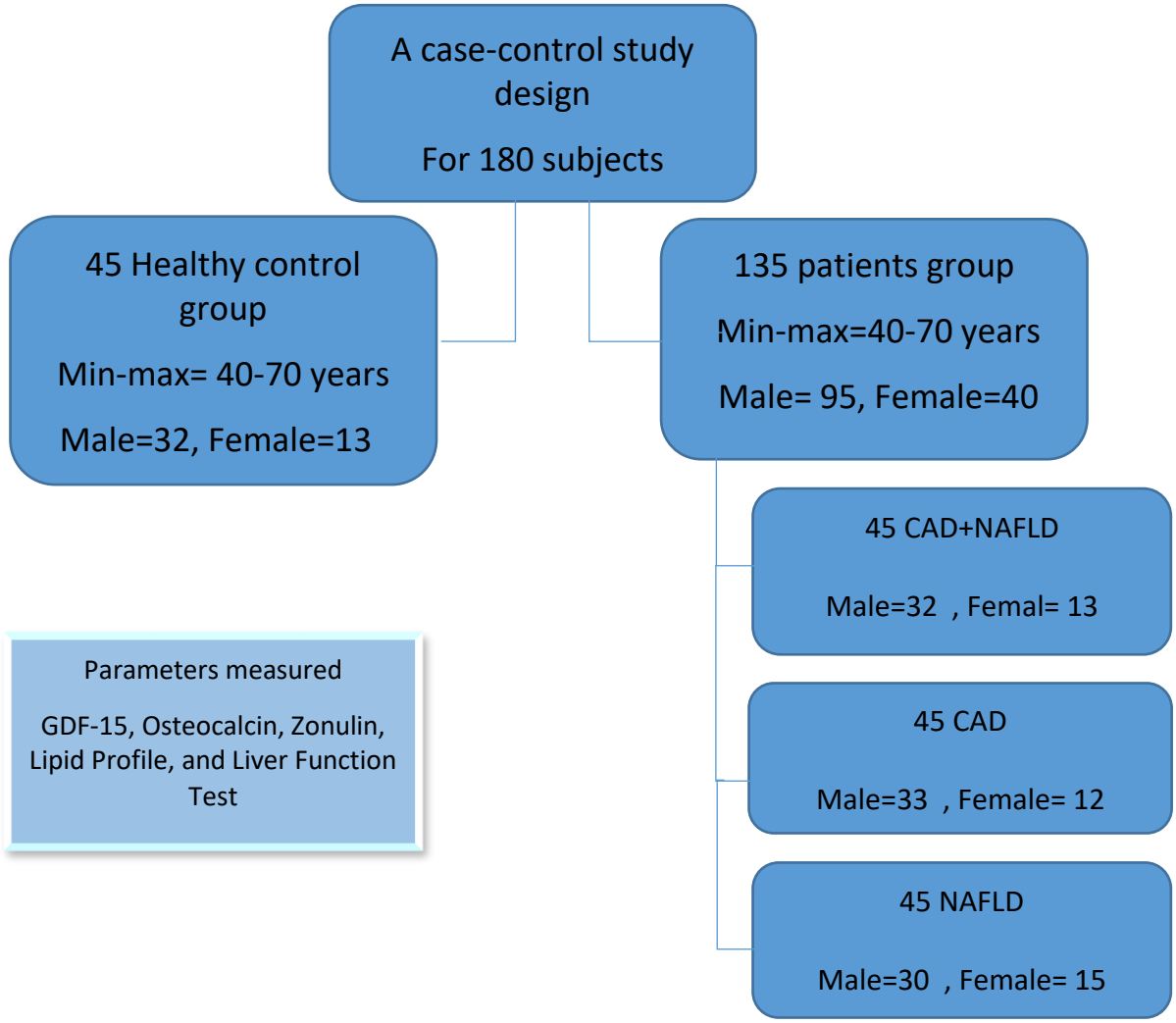


Figure (2-1): Flow chart of study design.

2.2. Subjects

2.2.1. Patients

The study was conducted on 135 patients (95 male and 40 female) aged between 40-70 years old as shown in fig (2-1). The patients were divided into three subgroups: 45 coronary artery with non-alcoholic fatty liver, 45 coronary artery, and 45 non-alcoholic fatty liver. They have been observed and diagnosed by specialist physicians. Patients were collected from Karbala Heart Center and Imam Hussein Medical City. A particular questionnaire form including descriptive information was designed and filled with each patient. The questionnaire included name, age, sex, under treatment, history of other diseases, and others(mentioned in Appendix).

Inclusion Criteria

All patients were subjected to full clinical history and clinical examination. The diagnosis of chronic coronary artery disease was identified based on signs and symptoms, evaluation of Echo, coronary computed tomography angiography, and Catheterization. The presence of fatty liver disease was confirmed by abdominal ultrasound.

Exclusion Criteria

Patients with acute coronary syndrome, alcoholic fatty liver, thyroid diseases, and acute heart failure

2.2.2. Control

A control group of apparently healthy 45 subjects (32 male and 13 female) was chosen from well-known volunteer participants. Participants have no chronic diseases or history of diseases.

2.2.3. Sample Collection

Five milliliters of venous blood were drawn from fasting subjects by using a sterile disposable syringe. Blood was put into a gel tube and left at room temperature for nearly 5 minutes for clotting, separated by using centrigufe , then separated into five Eppendorf tubes and stored at -20 °C in the deep freezer. Three tubes were used to measure serum zonulin, serum GDF-15, and serum osteocalcin by using the enzyme-linked immunosorbent assay (ELISA) technique. Two Eppendorf tubes were used to measure lipid profile, and liver function test by using Autoanalyser chemistry.

2.3. Chemicals and Kits

The kits used in this study are summarized in **Table (2-1)**

Table (2-1): Kits used in this study and their suppliers.

No.	Name of kit	Company	Country
1	Albumin Kit	GIESSE	**Italy**
2	ALP Kit	GIESSE	**Italy**
3	ALT Kit	GIESSE	**Italy**
4	AST Kit	GIESSE	**Italy**
5	ELISA test for GDF-15 in humn serum(cat no: YLA0250HU)	Shanghai YL Biont	**China**
6	ELISA test for zonulin in humn serum(cat no: EKHU-3002)	Melsin Medical	**China**
7	ELISA test for osteocalcin in humn serum(cat no: YL1183HU)	Shanghai YL Biont	**China**
8	HDL-Cholesterol Kit	GIESSE	**Italy**
9	Total Bilirubin Kit	GIESSE	**Italy**
10	Total Cholesterol Kit	GIESSE	**Italy**
11	**Triglycerides Kit**	**GIESSE**	**Italy**

2.4. Instruments and Lab Equipment.

Table (2-2): Instruments and Lab Equipment used in this study.

No.	Laboratory Equipment	Manufacturing Company	Country
1	Autoanalyser Chemistry	AFLO	China
2	Centrifuge	Hettich	Germany
3	Deep Freezer	LAB Tech	Korea
4	ELISA system/Micro plate reader	Bio Tek	USA
5	Eppendrof tubes 1.5ml	ATACO	China
6	Gel tube (5ml)	CMC Medical Devices	Spain
7	Vortex – mixture	Karlkole	Germany

2.5. Methods

2.5.1. Calculation of Body Mass Index

Weight in kilogram (kg) and height in meter (m) were recorded. Body mass index (BMI) was calculated by the following equation:

$$\textbf{BMI (kg/m}^2\textbf{) = weight / (height)}^2$$

Patients were classified into normal(18.5-24.9kg/m^2), overweight (25.0-29.9 kg/m^2), and obese($\geq$ 30.0 kg/m^2) depending on reference ranges demonstrated in table (2-3) **(Mendoza *et al*., 1996)**.

2.5.2. Determination of Lipid Profile

2.5.2.1. Determination of Total cholesterol level

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 Esterase

Cholesterol Ester ---------- ► Cholesterol + Fatty acids

Cholesterol Oxidase

Cholesterol ----------► Cholesta-4-ene 3-one + H_2O_2

Peroxidase

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

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 (2-5). 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).

2.5.2.2. Determination of 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:

Lipoprotein Lipase

Triglyceride ---------- ► Glycereol + Fatty acids

Glycerol Kinase

Glycerol + ATP ----------► Glycerol-1-phosphate + ADP

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 Magnesium chloride ATP 4-AAP TOOS LPL (lipoproteinlipase) POD (peroxidase) GK (glycerol kinase) GPO	100 mmol/L 15 mmol/L 4 mmol/L 1 mmol/L 0.1 mmol/L 2500 U/L 1800 U/L 1000 U/L 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 (2-8). 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)

2.5.2.3. Determination of High-density Lipoprotein Cholesterol concentration.

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:

Phosphotungstate, $MgCl_2$

HDL + LDL + VLDL + CM----------► HDL + (LDL + VLDL + CM)

Cholesterol Oxidase, Cholesterol Esterase

HDL-Cholesterol ----------► Fatty acids + H_2O_2

Glycerol Phosphate Oxidase

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

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

2.5.2.4. 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).

2.5.2.5. 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)**.

2.5.3. Calculation of Atherogenic Index

2.5.3.1. Atherogenic Coefficient

Atherogenic coefficient (AC) is the ratio of non-high-density lipoproteins cholesterol (non-HDL-C) to high-density lipoproteins cholesterol (HDL-C) **(Olamoyegun *et al*., 2016)**. It is a diagnostic alternative, which has been used in predicting the risk of developing cardiovascular events**(Bhardwaj *et al*., 2013)**.

AC = non-HDL-C / HDL-C

Non- HDL-C = TC – HDL-C

2.5.3.2. Atherogenic index of Plasma (AIP)

Atherogenic index of plasma (AIP) is an unconventional lipid ratio representing the logarithm of the molar ratio of TG to HDL-C **(Gómez-Álvarez *et al*., 2020)**.

AIP = log (TG/ HDL-C)

2.5.3.3. Castelli's Risk Indexes (I & II)

Castelli's risk indexes (I & II) also called cardiac risk indexes) are two lipid ratios, the CRI-I is the ratio of TC to HDL-C, while the CRI-II is the ratio of LDL-C to HDL-C. They were reported by William Castelli, at the end of the past century **(Castelli *et al*., 1983)**.

CRI-I= TC/ HDL-C ratio

CRI-II = LDL-C / HDL-C ratio

2.5.3.4. Cholesterol Index

Cholesterol index (C-index) is a simple index that predicts the probability of developing CAD with greater accuracy than the other indices **(Ulusoy, 2013).**

C- index= (LDL-C) – (HDL-C)

2.5.4. Determination of Liver Function Test

2.5.4.1. Determination of Albumin concentration.

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 (2-9): Reagents used for Albumin assay.

Reagent (A) ALB Volume = 50/100/250 ml	**Buffer pH 3.8 BCG**	**100 mmol/L 7 mmol/L**
Standard ALB Volume = 5 mL	Bovine Albumin	3 g/L

BCG, bromocresol green; ALB, Albumin

2.5.4.2. Determination of Aspartate aminotransferase Activity

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 (2-10): 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/l12 mmol/l

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

2.5.4.3. Determination of Alanine Aminotransferase Activity

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 T F 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 (2-11): 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.

2.5.4.4. Determination of Alkaline Phosphatase Activity

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 (2-12): 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.

2.5.4.5. Determination of Total Bilirubin concentration.

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 (2-13): 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

2.5.5. Determination of Human growth differentiation factor 15 by ELISA Kit

Reagents and materials

Table (2-14): Reagents used for GDF-15 assay.

Reagents	Quantity	Reagents	Quantity
Coated ELISA plate	12-Well	8 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(3200ng/L)	0.5ml	Anti-GDF-15 antibodies labeled with biotin	1ml

Test principle

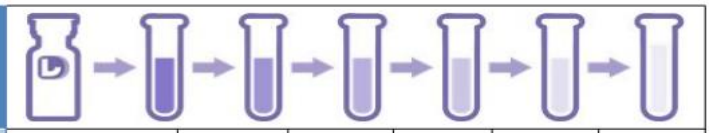
This kit uses enzyme-linked immunosorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human growth differentiation factor 15 (GDF-15). Add growth differentiation factor 15(GDF-15) to the wells, which are pre-coated with growth differentiation factor 15 monoclonal antibody, and then incubate. After that, anti-GDF-15 antibodies labeled was added 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 growth differentiation factor 15 (GDF-15) are positively correlated.

Table (2-15): Standards used for GDF-15 assay.

1600 ng/l	**Standard No.5**	**120µl Original Standard+120µl Standarddiluents**
800 ng/l	Standard No.4	120µl Standard No.5+120µl Standard diluents
400 ng/l	Standard No.3	120µl Standard No.4+120µl Standard diluents
200 ng/l	Standard No.2	120µl Standard No.3+120µl Standard diluents
100 ng/l	Standard No.1	120µl Standard No.2+120µl Standard diluents

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 was added in wells of standard.

3. A volume of 40µl sample and then 10µl GDF15 antibodies 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 was aspirated and washed, repeating the process four times for a total of five washes. Each well was washed by filling it with 400µl

8. Wash Solution by using the auto washer. Complete removal of the 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 of the wells should be changed from blue to yellow.

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

Calculation:

In Figure(2-2), standards' concentrations, the corresponding OD values, and the linear regression equation of the standard curve were calculated. Then according to the OD value of the samples, the concentration of the corresponding sample were calculated. Special software could be employed to calculate as well, and the data may be linearized by plotting the log of the GDF-15 concentrations versus the log of the OD and the best-fit line can be determined by regression analysis. As shown in Figure (2-3). This procedure will produce an adequate but less precise fit of the data.

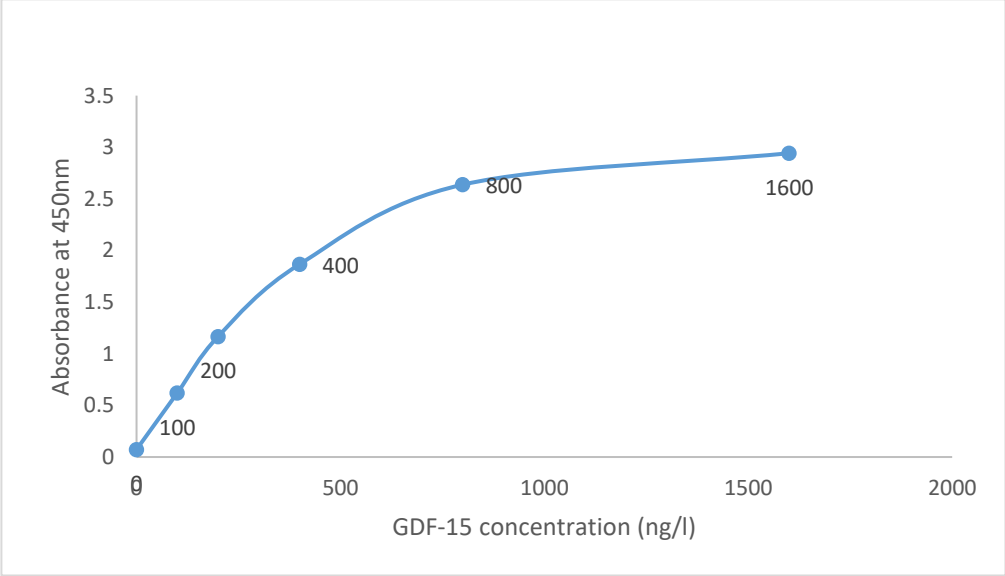


Figure (2-2): Standard curve of Human serum growth differentiation factor 15 concentration(ng/l).

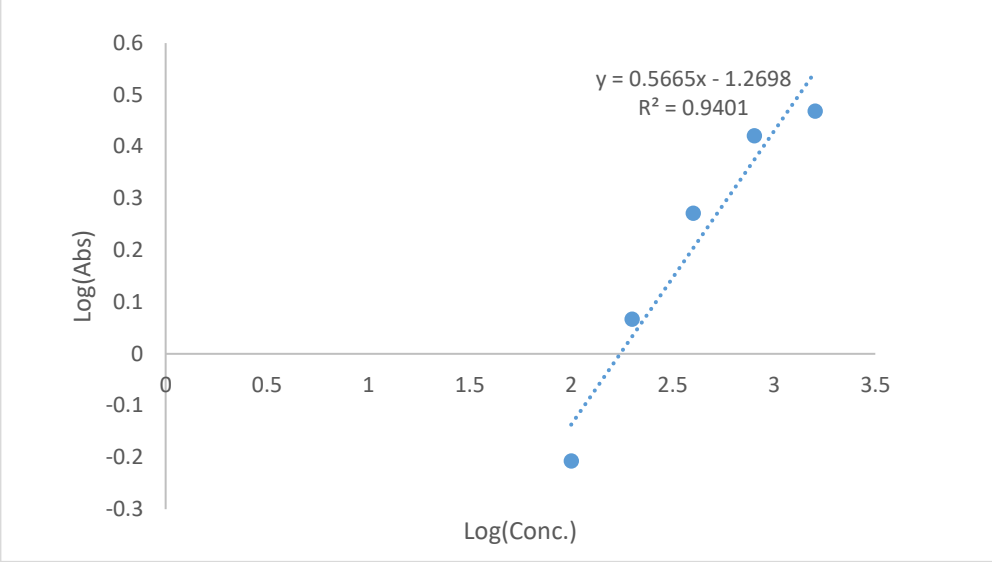


Figure (2-3): Log-log calibration curve of human serum growth differentiation factor 15 concentration(ng/l).

2.5.6. Determination of Human Osteocalcin/Bone gla protein by ELISA Kit

Assay procedure

*** Principle and procedure as mentioned in growth differentiation factor 15, section 2.4.5.**

Calculation:

In Figures(2-4), standards' concentrations and the corresponding OD values, the linear regression equation of the standard curve were calculated. Then according to the OD value of the samples, the concentration of the corresponding sample were calculated. Special software could be employed to calculate as well, and the data may be linearized by plotting the log of the osteocalcin concentrations versus the log of the OD and the best-fit line can be determined by regression analysis. As shown in figure (2-5). This procedure will produce an adequate but less precise fit of the data.

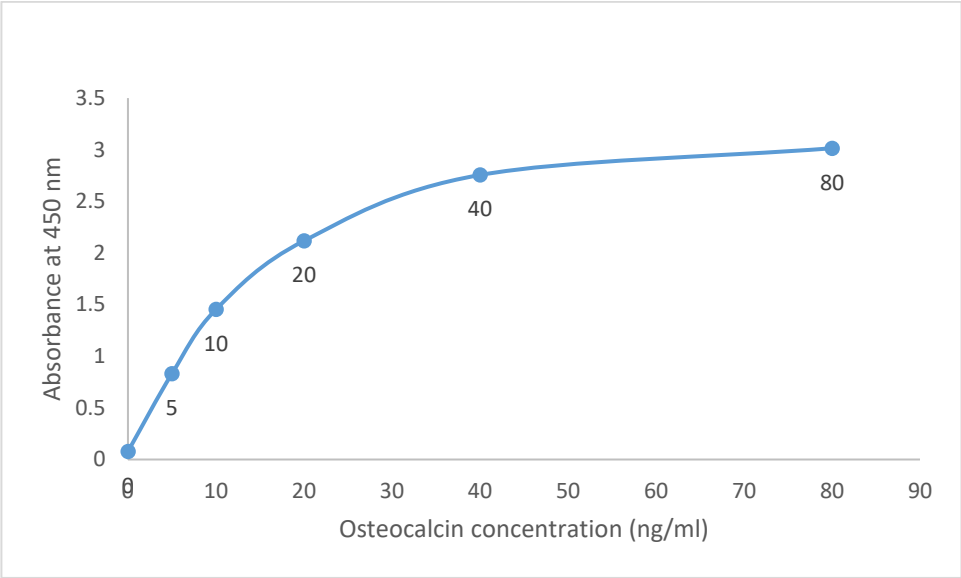


Figure (2-4): Standard curve of Human serum Osteocalcin concentration (ng/ml).

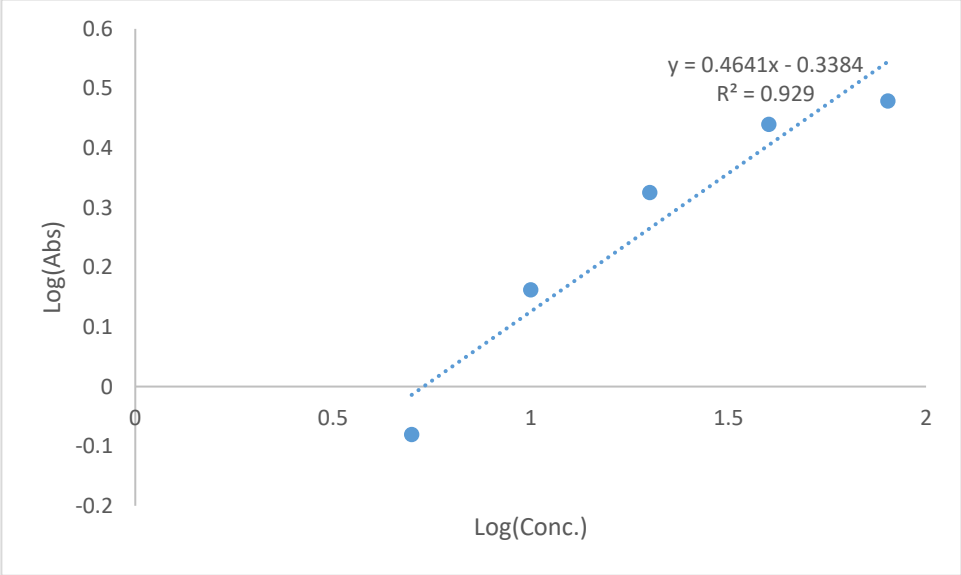


Figure (2-5): Log-log calibration curve of human serum osteocalcin concentration(ng/ml).

2.5.7. Determination of Human zonulin by ELISA Kit

Reagents and materials

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

Name	96 Determinations
Micro ELISA strip plate	12*8strips
Standards (1 set)	0.3ML X6
Sample diluent	6.0MLX1
HRP-Conjugate reagent	10.0MLX1
20X Wash solution	25MLX1
Chromogen Solution A	6.0MLX1
Chromogen Solution B	6.0MLX1
Stop Solution	6.0MLX1
Closure plate membrane	2
User manual	1
Sealed Bags	2

Test principle

This kit utilizes a double-Ab sandwich ELISA one-step process to assay Zonulin in human serum. Add standard, test sample, and HRP-labeled Zonulin antibodies to wells that are pre-covered with Zonulin Ab. After incubation and washing to eject the uncombined enzyme, added chromogen (A) and (B) solutions. The color of the liquid will change into blue. At the effect of acid, the color finally becomes yellow. The color alteration is estimated spectrophotometrically at a wavelength of 450 nm. The concentration of Zonulin in specimens is next measured by comparing the optical intensity of a specimen to the standard curve.

Assay procedure

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

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.

Calculation:

In figure(2-6), standards' concentrations and the corresponding OD values, the linear regression equation of the standard curve were calculated. Then according to the OD value of samples, the concentration of the corresponding sample were calculated. Special software could be employed to calculate as well.and The data may be linearized by plotting the log of the zonulin concentrations versus the log of the OD and the best fit line can be determined by regression analysis. As shown in figure (2-7). This procedure will produce an adequate but less precise fit of the data.

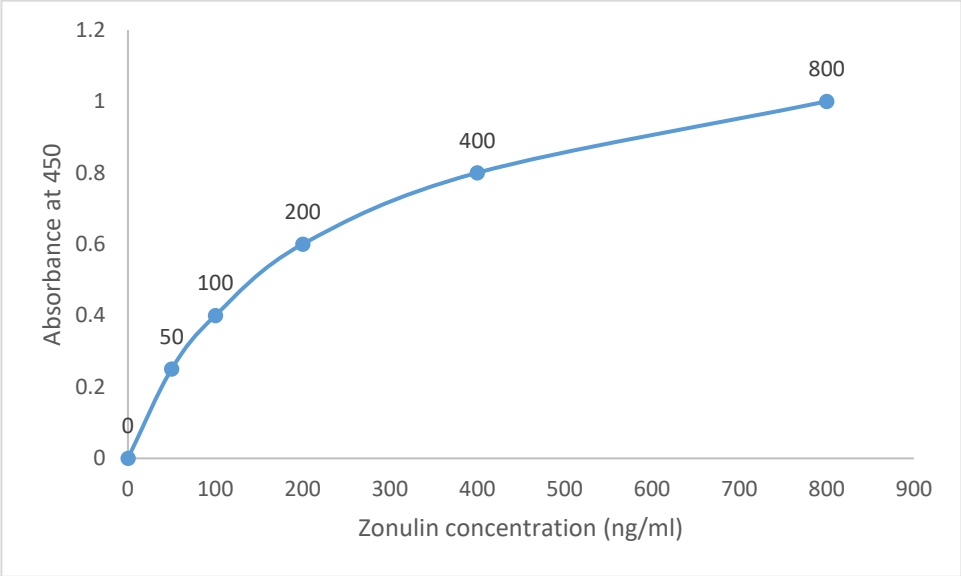


Figure (2-6): Standard curve of human serum zonulin concentration (ng/ml).

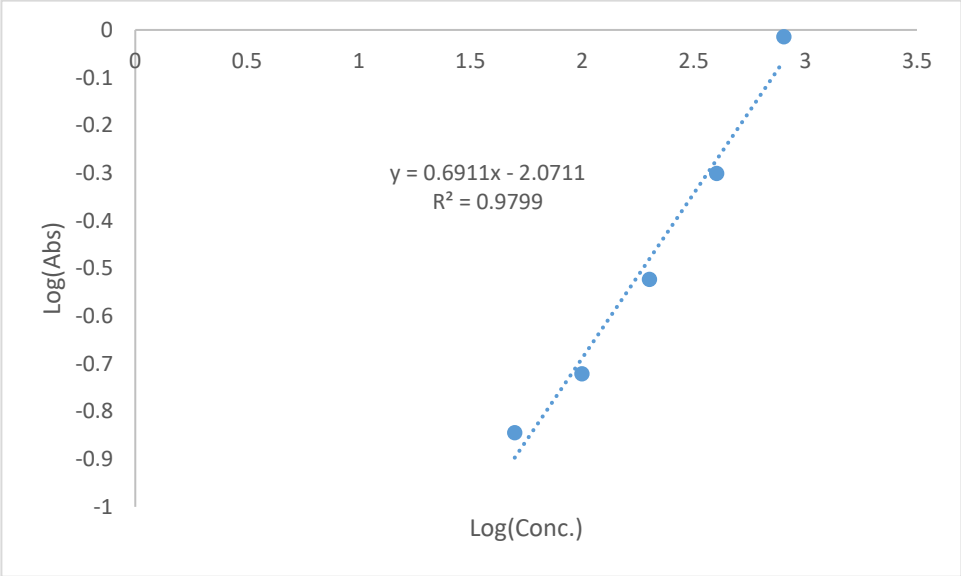


Figure (2-7): Log-log calibration curve of human serum zonulin concentration(ng/ml).

2.6. Statistical Analysis

Information from the questionnaire and all test results from study groups samples were entered into a data sheet. The data analysis for this work was generated using the Statistical Package for the Social Sciences software, version 24.0 (IBM, SPSS, Chicago, Illinois, USA)

Descriptive statistics were performed on the data of each group. Values were illustrated by n (%) for categorical, Scale variables were presented by mean ± standard deviation for normal data. The distribution of the data was checked using Shapiro-Wilk test as numerical means of assessing normality.

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.

Chapter Three

Results

3. Results

3.1. Demographic Characteristics

The demographic characteristics of patients & control groups were summarized in Table (3-1). The mean age of the patient group was (55.07) years old and for the control group (54.8) years old. Overall, the results indicated that most of the patient's samples were obese. Sex distribution among the studied groups was: 70.4% male, 29.6% female. The disease history of the patients was collected through a student self-report questionnaire, indicated that about 57.7% of them have DM and 53.3% have hypertension. Also, 45.1% of them were taking lipid-lowering agents.

Table (3-1): The demographic characteristics of patients and control groups.

Characteristics	Patient Group N(%)	Control Group N(%)
Total Number	**135**	**45**
Sex	Male 95 (70.4%)	32 (71.1%)
	Female 40 (29.6%)	13(28.9%)
DM(Yes/No)	(78/57)	-
HTN(Yes/No)	(72/63)	-
Smoking Status(Yes/No)	(54/81)	-
Family History of coronary artery diseae (Yes/No)	(49/87)	-
Sedentary Life Style(Yes/No)	(105/30)	(28/17)
Lipid-lowering agents(Yes/No)	(61/74)	-
	Mean ± SD	**Mean ± SD**
Age(years)	55.07 ±9.31	54.80±8.75
BMI(kg/m^2)	29.34 ±3.90	24.53 ± 2.87
WC(cm)	105.01 ±13.26	91.29 ± 7.27

DM: diabetes mellitus; HTN: hypertension; SD: standard deviation; BMI: body mass index, WC: waist circumference

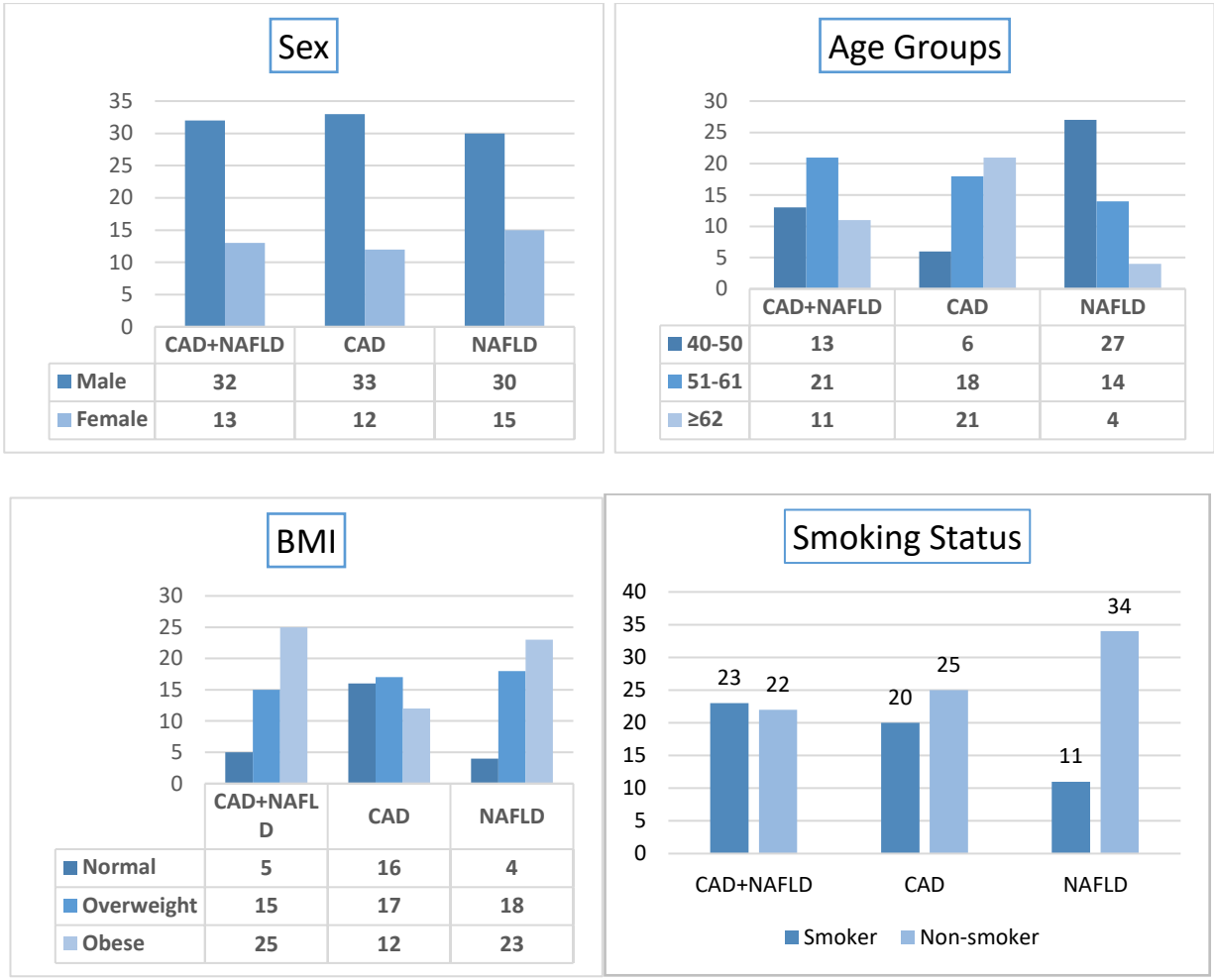


Figure (3-1): Distribution of patient samples according to sex, age group, BMI, and smoking status.

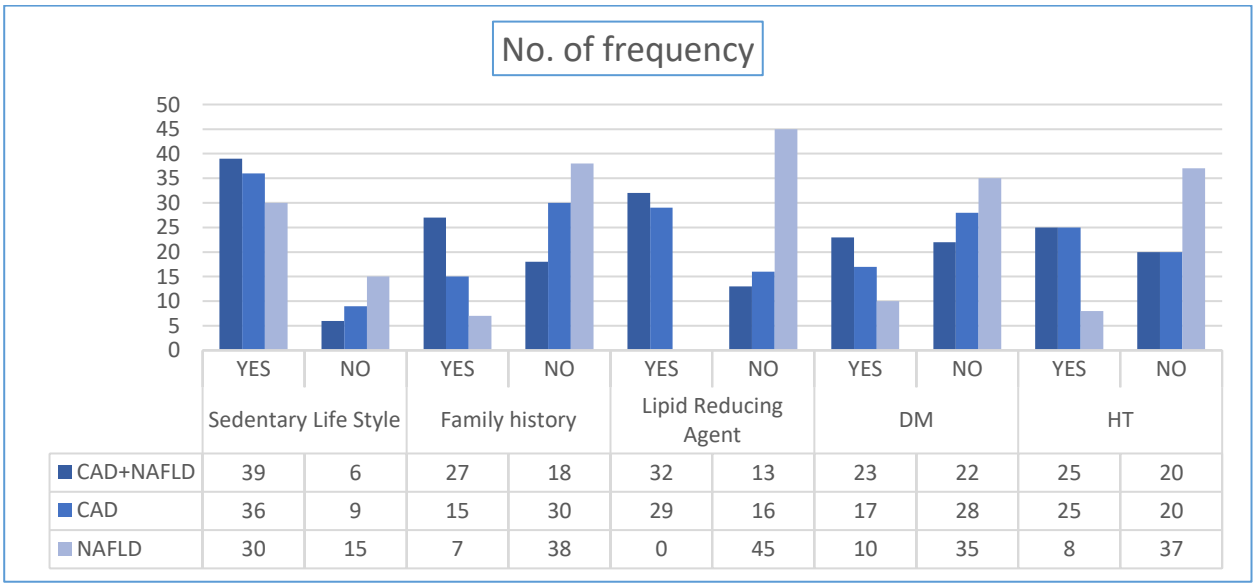


Figure (3-2): Distribution of patients samples according to the history of participants.

3.2. Examination of the distribution of data in the studied groups.

3.2.1. Anthropometric Characteristics

Table (3-2): The Comparison of Anthropometric Characteristics in patients and healthy control groups.

Parameters	CAD+NAFLD N=45 Mean±SD	CAD N=45 Mean±SD	NAFLD N=45 Mean±SD	Healthy N=45 Mean±SD	P-value
Age(Years)	54.93±8.07	56.89±6.763	55.24±9.170	54.80±8.92	**0.136[NS]**
BMI(kg/m^2)	30.01 ± 3.69	27.17±4.30	30.838±2.56	24.53±2.87	**≤0.001[S]a**
WC(cm)	109.33±17.50	99.87±10.94	105.82±7.93	91.29±7.27	**≤0.001[S]b**

ANOVA-test was significant at $p \leq 0.05$; N: number

SD: standard deviation; S: significant; NS: Non significant; by using Post Hoc test a: least significant difference between values in NAFLD and healthy; b: least significant difference between values in CAD+NAFLD and healthy

The Age of the groups shows no significant differences. BMI and WC of patients show extremely significant differences compared to the control group.

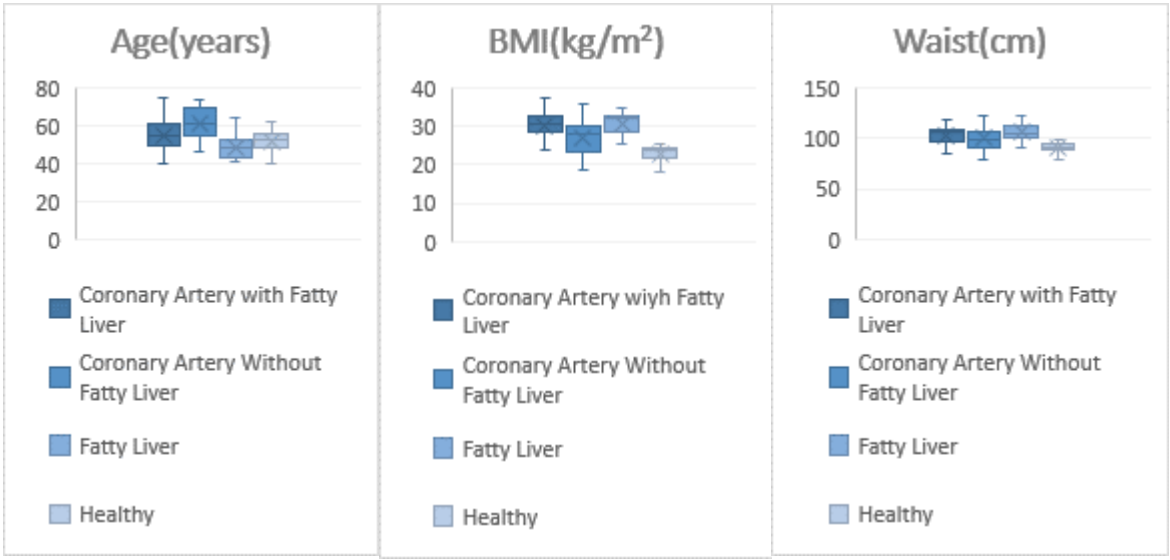


Figure (3-3): Boxplot of the Distribution of Age, BMI, and WC in patients and healthy groups.

3.2.2. Comparison of Lipid Profile between patients and healthy groups.

Table (3-3): The Comparison of Lipid profile levels in patients and healthy groups.

Parameters	CAD+NAFLD N=45 Mean±SD	CAD N=45 Mean±SD	NAFLD N=45 Mean±SD	Healthy N=45 Mean±SD	P-value
TC(mg/dl)	125.38 ±25.61	119.13±30.69	183.29±30.50	130.35±19.90	**<0.001[S]a**
TG(mg/dl)	148.60± 58.53	125.08±49.98	169.92±73.68	124.71±34.94	**0.071[NS]b**
HDL-C (mg/dl)	46.89± 3.43	47.42 ± 3.81	48.31± 2.17	56.76± 9.60	**<0.001[S]c**
LDL-C (mg/dl)	85.70± 22.37	90.66 ± 15.73	112.31±32.32	65.50 ± 19.70	**<0.001[S]d**
VLDL-C (mg/dl)	26.56±10.50	25.01± 9.99	33.98± 14.73	24.94 ± 6.98	**0.071[NS]e**

ANOVA-test was *: significant at $p \leq 0.05$; N: number; SD: standard deviation; S: significant; AC: atherogenic coefficient; AIP: atherogenic index of plasma; CR-I, CR-II: castelli's risk indexes; C-index: cholesterol index; by using Post Hoc test a: least significant difference between values in CAD and healthy; b: least significant difference between values in NAFLD and healthy; c: least significant difference between values in CAD+NAFLD and healthy; d: least significant difference between values in NAFLD and healthy; e: least significant difference between values in NAFLD and healthy.

Figure (3-4) demonstrated the distribution of serum levels of the lipid profile in patients' groups and the healthy control group. Patients were divided into three subgroups (coronary artery disease with fatty liver, coronary artery, and non-alcoholic fatty liver.

Total cholesterol levels were decreased markedly in a group of coronary artery, while TG was estimated to have great variability in patients compared to control. The mean levels of cholesterol in the patient's group were (125.38, 119.13, and 183.29) mg/ml respectively, while in the control group, the mean level of cholesterol was (130.35mg/mL). on the other hand, LDL in the patient's groups was (85.70, 90.66, and 112.31g/dL) compared to (65.50 mg/dL) in the healthy control group.

Furthermore, the mean differences of TG were also examined, and results indicated that there was an increase in the TG levels in patients compared to the healthy control group. The mean of TG in the control group (124.71) mg/dl was

significantly lower than in patient subgroups, (148.60, 125.08, and 169.92 mg/dL) respectively.

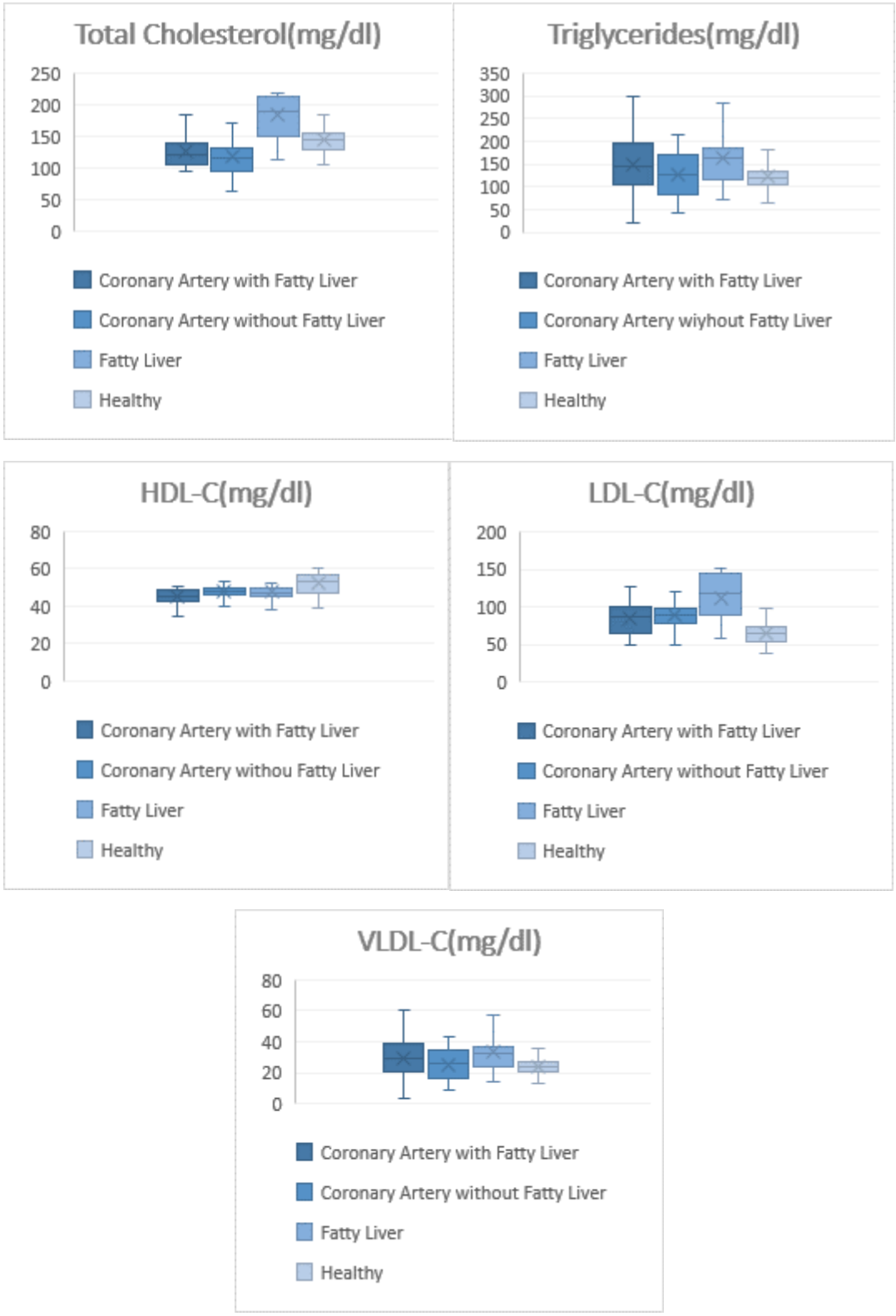


Figure (3-4): Boxplot of the Distribution of serum lipid profile in patients and Healthy groups.

3.2.3. Distribution of Atherogenic Index

Table (3-4): The Comparison of Atherogenic index levels in patients and healthy groups.

Parameters	CAD+NAFLD N=45 Mean±SD	CAD N=45 Mean±SD	NAFLD N=45 Mean±SD	Healthy N=45 Mean±SD	P-value
AC	1.78 ± 0.56	1.50± 0.60	2.87 ±0.71	1.61±0.57	**<0.001[S]a**
AIP	0.47± 0.22	0.38 ±0.20	0.51± 0.17	0.33±0.15	**<0.001[S]b**
CR-I	2.78± 0.56	2.50 ±0.60	3.87±0.71	2.61±0.57	**<0.001[S]c**
CR-II	1.89± 0.45	1.91 ±0.33	2.37±0.72	1.21±0.49	**<0.001[S]d**
C-index	40.48 ± 21.27	43.15±15.67	64.79±32.71	9.24±23.31	**<0.001[S]e**

ANOVA-test was *: significant at $p \leq 0.05$; N: number; SD: standard deviation; S: significant; AC: atherogenic coefficient; AIP: atherogenic index of plasma; CR-I, CR-II: castelli's risk indexes; C-index: cholesterol index; by using Post Hoc test a: least significant difference between values in CAD and NAFLD; b: least significant difference between values in NAFLD and healthy; c: least significant difference between values in CAD and NAFLD; d: least significant difference between values in NAFLD and healthy; e: least significant difference between values in NAFLD and healthy.

The levels of AC in patients groups were (1.78, 1.50, and 2.87) respectively, and (1.61) in the control group. Levels of AIP were (0.47, 0.38, 0.51, and 0.33) respectively. CR-I levels were (2.78, 2.50, 3.87, and 2.61), and for CR-II were (1.89, 1.91, 2.37, 1.21). levels of C-index were significantly higher in patients groups (40.48, 43.15, 64.79) compared to the control group (9.24).

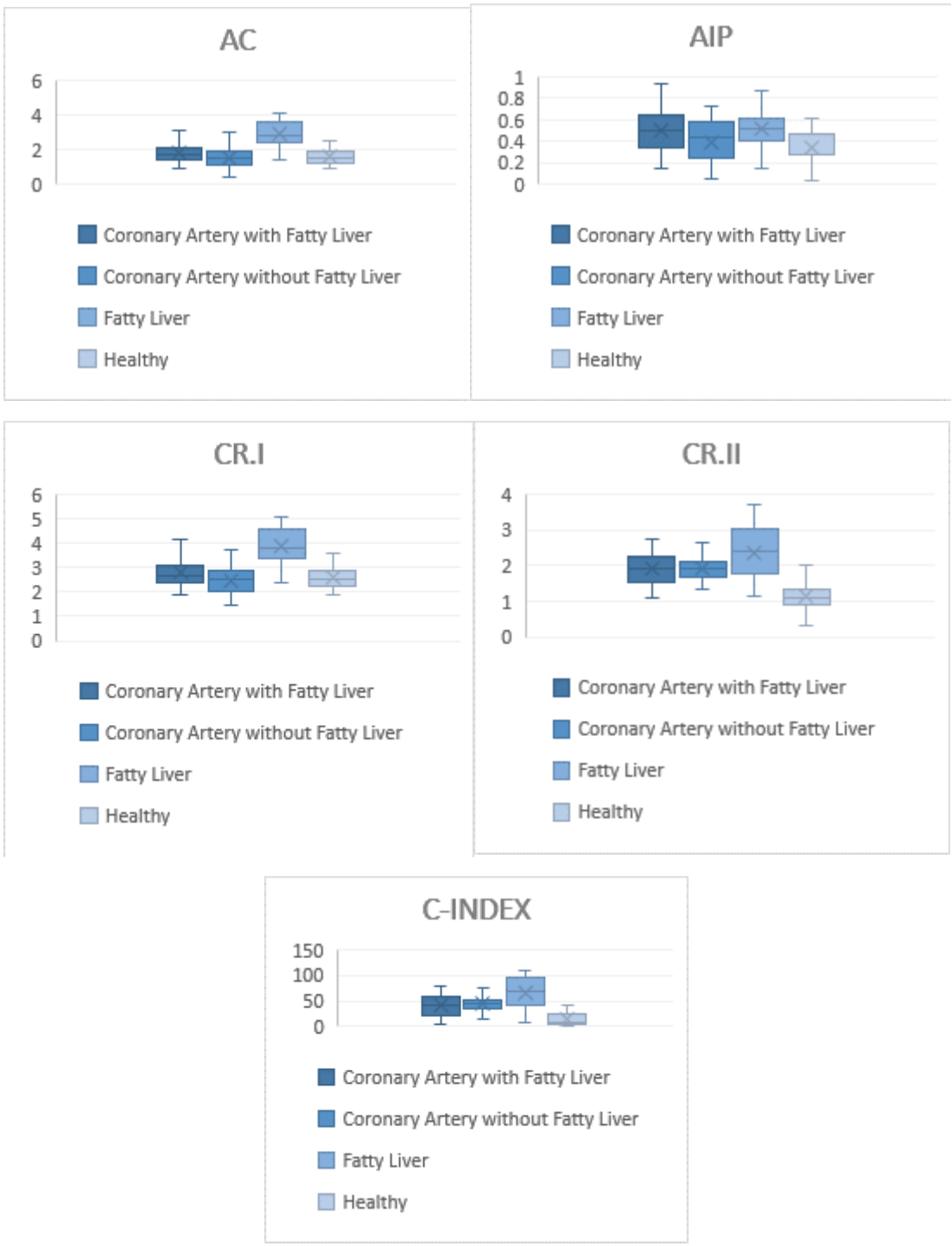


Figure (3-5): Boxplot of the Distribution of Atherogenic index in patients and Healthy groups.

3.2.4. Distribution of Liver Function Test

Table (3-5): The Comparison of Liver Function Test levels in patients and healthy groups.

Parameters	CAD+NAFLD N=45 Mean±SD	CAD N=45 Mean±SD	NAFLD N=45 Mean±SD	Healthy N=45 Mean±SD	P-value
Albumin(g/dl)	4.30 ± 0.22	4.35 ± 0.20	4.25± 0.19	4.17±0.13	**≤0.001[S]a**
AST(U/L)	30.14 ±13.74	23.71 ± 5.25	33.54± 6.97	27.35± 7.12	**≤0.001[S]b**
ALT(U/L)	45.39± 25.79	22.79±6.91	45.23± 10.0	27.59± 11.94	**≤0.001[S]c**
ALP(U/L)	164.73± 36.82	159.40±45.70	202.64±35.36	146.03±48.25	**≤0.001[S]d**
TB(mg/dl)	0.674 ± 0.388	0.713± 0.27	0.914 ±0.424	0.687± 0.28	**0.003[S]e**

ANOVA-test was *: significant at $p \leq 0.05$; N: number; SD: standard deviation; S: significant; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; TB: total bilirubin; by using Post Hoc test a: least significant difference between values in CAD and healthy; b: least significant difference between values in CAD and NAFLD; c: least significant difference between values in CAD+NAFLD and CAD; d: least significant difference between values in NAFLD and healthy; e: least significant difference between values in CAD+NAFLD and NAFLD.

The distribution of the main involved liver functions test parameters were examined as demonstrated in Table (3-5).

The levels of these markers in patients' subgroups were shown the following distribution: Albumin was (4.30), (4.35), and (4.25) respectively compared to control groups (4.17). The levels AST were (30.14), (23.71), (33.54) respectively, while in the control group was (27.35). And the levels of ALT were (45.39), (22.79), (45.23) compared to control levels (27.59).

On the other hand, ALP levels were shown an increase in the range levels in patients subgroups (164.73), (159.40); (202.64) compared to the control group (146.03).

Levels of total bilirubin were (0.674), (0.713), (0.914), and (0.687) respectively.

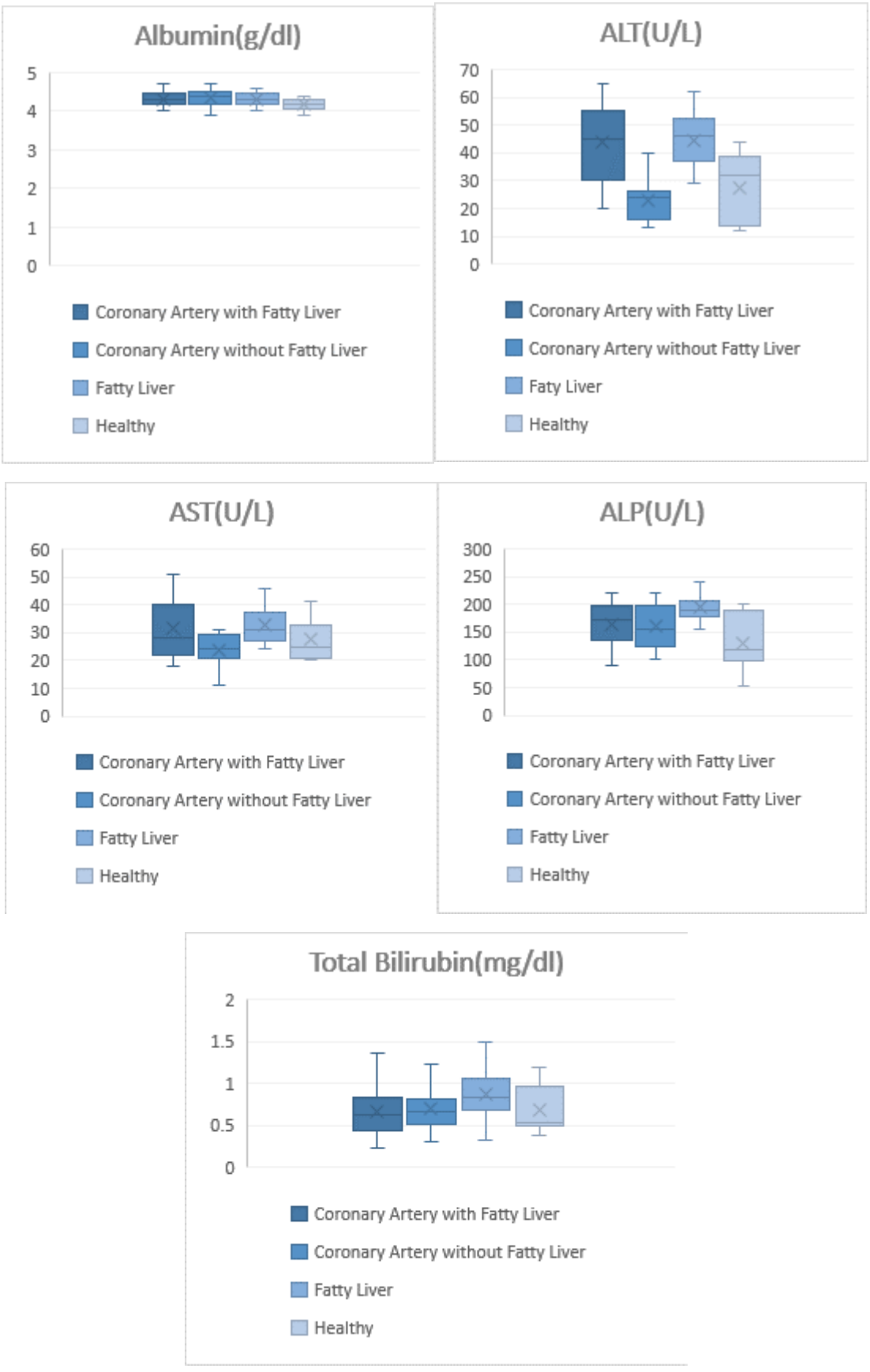


Figure (3-6): Boxplot of the distribution of serum Albumin, AST, ALT, ALP, and total bilirubin in patients and Healthy groups.

3.2.5. Distribution of GDF-15, osteocalcin, and zonulin.

Table (3-6): The Comparison of GDF-15, osteocalcin, and zonulin levels in patients and healthy groups.

Parameter	CAD+NAFLD N=45 Mean±SD	CAD N=45 Mean±SD	NAFLD N=45 Mean±SD	Healthy N=45 Mean±SD	P-value
GDF-15 (ng/L)	273.18±50.81	223.78±31.04	237.34±38.94	186.40±28.20	**≤0.001[S]a**
Osteocalcin (ng/ml)	7.88 ± 0.80	9.22±1.56	6.74±1.4	14.05±2.86	**≤0.001[S]b**
Zonulin (ng/ml)	94.80± 18.35	73.42±10.39	63.42±7.51	54.59 ± 5.13	**≤0.001[S]c**

ANOVA-test was *: significant at $p \leq 0.05$; N: number; SD: standard deviation; S: significant; GDF-15: Growth differentiation factor 15; by using Post Hoc test a: least significant difference between values in CAD+NAFLD and healthy; b: least significant difference between values in NAFLD and healthy; c: least significant difference between values in CAD+NAFLD and healthy.

As shown in Table (3-6) and Figure (3-7) which consists of the mean of GDF-15 for the study groups (Coronary Artery with non-alcoholic Fatty Liver, Coronary Artery without non-alcoholic Fatty Liver, Fatty Liver, and Healthy).

A significantly higher level of GDF-15 in patients groups (273.18), (223.78), and (237,34) respectively compared to the control group (186.40).

Figure (3-7): Boxplot of the distribution of serum GDF-15 in patients and Healthy groups.

As shown in Table (3-6) and Figure (3-8) which consists of the mean of osteocalcin for the study groups (Coronary Artery with non-alcoholic Fatty Liver, Coronary Artery without non-alcoholic Fatty Liver, Fatty Liver, and Healthy).

A significantly decreased level of osteocalcin in patients groups (7.88), (9.22), and (6.74) respectively compared to the control group (14.05).

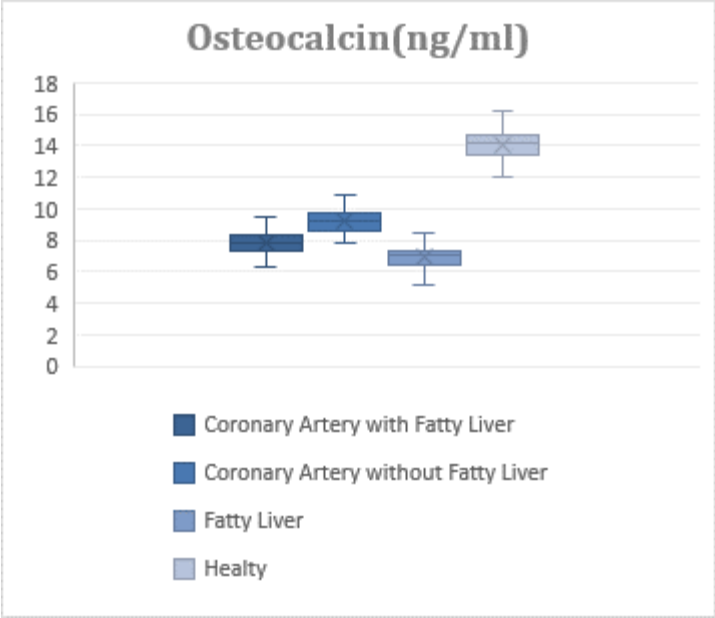


Figure (3-8): Boxplot of the distribution of serum osteocalcin in patients and Healthy groups.

In Table (3-6) and Figure (3-9), significantly higher levels of zonulin in patients groups (94.80), (73.42), and (63.42) respectively compared to the control group (54.59).

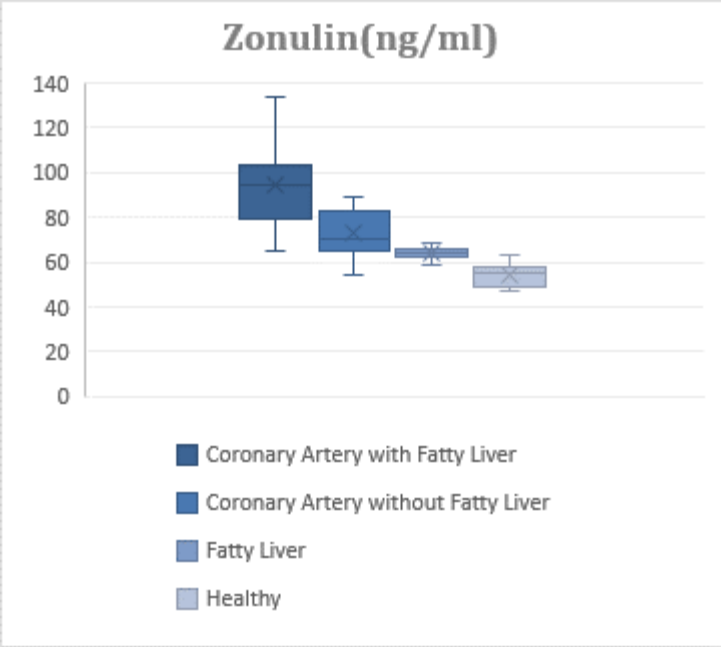


Figure (3-9): Boxplot of the distribution of serum zonulin in patients and healthy groups.

3.3. Correlation between Biomarkers and studied parameters in patients groups.

3.3.1. Correlation between GDF-15 and parameters

Table (3-7): Correlations between GDF-15 and parameters in patients groups.

Parameters	GDF-15(ng/L)					
	Coronary Artery With Fatty Liver		Coronary Artery		Non-Alcoholic Fatty Liver	
	r	P	r	P	r	p
BMI(kg/m^2)	0.763**	**≤0.001[S]**	0.477**	0.001[S]	0.786**	**≤0.001[S]**
WC(cm)	0.397*	**0.016[S]**	0.294	0.162[NS]	0.502	**0.080[NS]**
TC(mg/dl)	0.108	0.414[NS]	0.033	0.831[NS]	-0.058	**0.707[NS]**
TG(mg/dl)	0.218	0.106[NS]	0.179	0.240[NS]	0.986**	**≤0.001[S]**
HDL(mg/dl)	-0.140	0.357[NS]	-0.074	0.629[NS]	-0.078	**0.609[NS]**
LDL(mg/dl)	0.037	0.807[NS]	-0.1	0.828[NS]	-0.067	**0.372[NS]**
AC	0.122	0.425[NS]	0.061	0.688[NS]	-0.004	**0.978[NS]**
AIP	0.278	0.064[NS]	0.085	0.581[NS]	0.965**	**≤0.001[S]**
CR-I	0.122	0.425[NS]	0.061	0.688[NS]	-0.004	**0.978[NS]**
CR-II	-0.331*	**0.027[S]**	-0.194	0.201[NS]	-0.128	**0.402[NS]**
C-index	-0.342*	**0.021[S]**	-0.249	0.099[NS]	-0.165	**0.278[NS]**
Albumin(g/dl)	0.041	0.788[NS]	-0.058	0.704[NS]	-0.067	**0.371[NS]**
AST(U/L)	0.369*	**0.013[S]**	0.210	0.165[NS]	0.339**	**≤0.001[S]**
ALT(U/L)	0.317*	**0.034[S]**	0.255	0.091[NS]	0.413**	**≤0.001[S]**
ALP(U/L)	-0.349	**0.019[S]**	0.140	0.789[NS]	0.089	**0.235[NS]**
TB(mg/dl)	0.030	0.844[NS]	-0.126	0.410[NS]	0.040	**0.594[NS]**
Osteocalcin(ng/ml)	-0.639**	**≤0.001[S]**	-0.171*	**0.022[S]**	-0.590**	**≤0.001[S]**
Zonulin(ng/ml)	0.616**	**≤0.001[S]**	0.657**	**≤0.001[S]**	0.701**	**≤0.001[S]**

**Correlation is significant at the 0.01 level, *Correlation is significant at the 0.05 level, - = negative; r: pearson correlation coefficients; P : P value; S: significant; NS: Non significant; BMI: body mass index; WC: waist circumference; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: verylow-density lipoprotein; AC: atherogenic coefficient; AIP: atherogenic index of plasma; CR-I, CR-II: castelli's risk indexes; C-index: cholesterol index ; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; TB: total bilirubin

GDF-15 correlations with biochemical parameters in patient groups are shown in Table (3-7).

The result showed that in a coronary artery with fatty liver group, there was a significant positive correlation between level of GDF-15 and BMI (P≤0.001), WC (P=0.016), AST (P=0.013), ALT (P=0.034), and zonulin (P≤0.001), and a significant negative correlation between GDF-15 level and CR-II (P=0.027), C-index (P=0.021), ALP (p=0.019) and osteocalcin (P≤0.001). TC, TG, LDL, Albumin, AC, AIP, CR-I, and TB have a non-significant positive correlation, while HDL has a non-significant negative correlation.

In the coronary artery without fatty liver group, there was a significant positive correlation between level of GDF-15 and BMI (P=0.001), zonulin(P≤0.001), and a significant negative correlation with osteocalcin (P=0.022). WC, TC, TG, AC, AIP, CR-I, AST, ALT, and ALP have a non-significant positive correlation, while HDL, LDL, CR-II, C-index, Albumin, and TB have a non-significant negative correlation.

In the fatty liver group, there was a significant positive correlation between level of GDF-15 and BMI, TG, AIP, AST, ALT, and zonulin (p≤0.001), and a significant negative correlation with osteocalcin (p≤0.001). WC, ALP, and TB have a non-significant positive correlation, while TC, HDL, LDL, AC, CR-I, CR-II, C-index, and Albumin have a non-significant negative correlation.

3.3.2 Correlation between osteocalcin and studied parameters.

Table (3-8): Correlations between osteocalcin and parameters in patients groups.

Parameters	Osteocalcin(ng/ml)					
	Coronary Artery With Fatty Liver		Coronary Artery		Non-Alcoholic Fatty Liver	
	r	p	r	p	r	p
BMI(kg/m^2)	-0.809**	**≤0.001[S]**	-0.537**	≤0.001[S]	-0.398**	**0.007[S]**
WC(cm)	-0.525**	**0.001[S]**	-0.571**	**0.004[S]**	-0.728**	**0.005[S]**
TC(mg/dl)	-0.049	0.749[NS]	0.016	0.917[NS]	0.049	0.748[NS]
TG(mg/dl)	-0.294*	**0.050[S]**	-0.338*	**0.023[S]**	-0.319*	**0.033[S]**
HDL(mg/dl)	0.241	0.111[NS]	-0.326*	**0.029[S]**	-0.149	0.328[NS]
LDL(mg/dl)	0.043	0.205[NS]	0.097	0.526[NS]	0.049	0.748[NS]
AC	-0.191	0.210[NS]	-0.239	0.119[NS]	0.087	0.570[NS]
AIP	-0.326*	**0.029[S]**	-0.344*	**0.021[S]**	-0.500	**≤0.001[S]**

CR-I	-0.191	0.210[NS]	-0.239	0.114[NS]	0.087	0.570[NS]
CR-II	0.154	0.312[NS]	-0.098	0.523[NS]	0.142	0.354[NS]
C-index	0.162	0.287[NS]	0.016	0.915[NS]	0.118	0.438[NS]
Albumin(g/dl)	-0.031	0.841[NS]	-0.218	0.150[NS]	0.217	0.153[NS]
AST(U/L)	-0.243	0.107[NS]	-0.116	0.449[NS]	-0.050	0.744[NS]
ALT(U/L)	-0.216	0.154[NS]	-0.214	0.157[NS]	-0.100	0.514[NS]
ALP(U/L)	0.306*	**0.041[S]**	0.165	0.279[NS]	0.217	0.153[NS]
TB(mg/dl)	-0.094	0.540[NS]	-0.312	0.077[NS]	0.037	0.809[NS]
GDF-15(ng/L)	-0.630**	**≤0.001[S]**	-0.178	0.243[NS]	-0.317*	**0.034[S]**
Zonulin(ng/ml)	-0.594**	**≤0.001[S]**	-0.318*	**0.033[S]**	-0.365*	**0.014[S]**

**Correlation is significant at the 0.01 level, *Correlation is significant at the 0.05 level, - = negative; r: pearson correlation coefficients; P : P value; S: significant; NS: Non significant; BMI: body mass index; WC: waist circumference; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: verylow-density lipoprotein; AC: atherogenic coefficient; AIP: atherogenic index of plasma; CR-I, CR-II: castelli's risk indexes; C-index: cholesterol index ; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; TB: total bilirubin

Osteocalcin correlations with biochemical parameters in patient groups are shown in Table (3-8).

The result showed that in the coronary artery with fatty liver group, there was a significant positive correlation between level of osteocalcin and ALP(P=0.041) and a significant negative correlation between osteocalcin level and BMI (≤0.001), WC (≤0.001), triglycerides (0.050), AIP (P=0.029), GDF-15 (≤0.001), and zonulin (≤0.001). HDL, LDL, CR-II, and C-index have a non-significant positive correlation, while total cholesterol, AC, CR-I, Albumin, AST, ALT, and total bilirubin have a non-significant negative correlation.

In the coronary artery without fatty liver group, there was a significant negative correlation between level of osteocalcin and BMI (P≤0.001), WC (P=0.004), TG (P=0.023), HDL (P=0.029), AIP (P=0.021), and zonulin (p=0.033). TC, LDL, C-index, and ALP have a non-significant positive correlation, while AC, CR.I, CR.II, Albumin, AST, ALT, TB, and GDF-15 have a non-significant negative correlation.

In the fatty liver group, there was significant negative correlation between level of osteocalcin and BMI (P=0.007), WC (P=0.005), TG (P=0.033), AIP (P≤0.001), GDF-15 (P=0.034), and zonulin (P=0.014). TC, LDL, AC, CR.I, CR.II, C-index, Albumin, ALP, and TB have a non-significant positive correlation, while HDL, AST, and ALT have a non-significant negative correlation.

3.3.3. Correlation between zonulin and studied parameters

Table (3-9): Correlations between zonulin and parameters in patients groups.

Parameters	Zonulin(ng/ml)					
	Coronary Artery With Fatty Liver		Coronary Artery		Non-Alcoholic Fatty Liver	
	r	P	r	p	r	p
BMI(kg/m^2)	0.758**	**<0.001[S]**	0.6**	**≤0.001[S]**	**0.540****	**≤0.001[S]**
WC(cm)	0.428**	**0.009[S]**	0.224	0.292[NS]	**0.588***	**0.035[S]**
TC(mg/dl)	0.232	0.126[NS]	0.1	0.699[NS]	0.156	**0.305[NS]**
TG(mg/dl)	0.007	0.962[NS]	-0.028	0.858[NS]	**0.503****	**≤0.001[S]**
HDL(mg/dl)	0.1	0.802[NS]	-0.132	0.782[NS]	-0.067	**0.660[NS]**
LDL(mg/dl)	0.1	0.145[NS]	0.1	0.838[NS]	0.114	**0.454[NS]**
AC	0.174	0.252[NS]	0.089	0.560[NS]	0.158	**0.300[NS]**
AIP	0.068	0.655[NS]	0.017	0.913[NS]	0.360*	**0.015[S]**
CR-I	0.174	0.252[NS]	0.089	0.560[NS]	0.158	**0.300[NS]**
CR-II	-0.053	0.729[NS]	-0.257	0.088[NS]	0.151	**0.321[NS]**
C-index	-0.049	0.750[NS]	-0.316*	**0.033[S]**	0.087	**0.571[NS]**
Albumin(g/dl)	0.123	0.474[NS]	-0.252	0.095[NS]	-0.349*	**0.019[S]**
AST(U/L)	-0.1	0.606[NS]	0.1	0.608[NS]	0.107	**0.485[NS]**
ALT(U/L)	-0.127	0.407[NS]	0.258	0.087[NS]	-0.053	**0.730[NS]**
ALP(U/L)	-0.234	0.123[NS]	-0.171	0.261[NS]	-0.341*	**0.022[S]**
TB(mg/dl)	0.1	0.661[NS]	0.090	0.557[NS]	-0.010	**0.947[NS]**
GDF-15(ng/L)	0.616**	**≤0.001[S]**	0.652**	**≤0.001[S]**	0.440*	**0.003[S]**
Osteocalcin(ng/ml)	-0.594**	**≤0.001[S]**	-0.318*	**0.033[S]**	-0.365*	**0.014[S]**

**Correlation is significant at the 0.01 level, *Correlation is significant at the 0.05 level, - = negative; r: pearson correlation coefficients; P : P value; S: significant; NS= Non significant; BMI: body mass index; WC: waist circumference; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very low-density lipoprotein; AC: atherogenic coefficient; AIP: atherogenic index of plasma; CR-I, CR-II: castelli's risk indexes; C-index: cholesterol index ; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; TB: total bilirubin.

Zonulin correlations with biochemical parameters in patient groups are showed in Table (3-9).

The result showed that in a coronary artery with fatty liver group, there was a significant positive correlation between level of zonulin and BMI (P≤0.001), WC (P=0.009), and GDF-15 (P≤0.001), and a significant negative correlation between zonulin level and osteocalcin (≤0.001).TC, TG, HDL, LDL, AC, AIP, CR-I, Albumin, and TB have a non-significant positive correlation, while CR-II, C-index, AST, ALT, and ALP have a non-significant negative correlation.

In coronary artery without fatty liver group, there was significant positive correlation between level of zonulin and BMI (P≤0.001, and a significant negative correlation between zonulin and C-index (P=0.033), osteocalcin (P=0.033). WC, TC, LDL, AC, AIP, CR. I, AST, ALT, and TB have a non-significant positive correlation, while TG, HDL, CR-II, Albumin, and ALP have a non-significant negative correlation.

In a fatty liver group, there was a significant positive correlation between level of zonulin and BMI (P≤0.001), WC (P=0.035), TG (P≤0.001), AIP (P-0.015), and GDF-15 (P=0.003), and a significant negative correlation between zonulin level and Albumin (P=0.019), ALP (P=0.022), and osteocalcin (P=0.014). TC, LDL, AC, CR. I, CR.II, C-index, and AST have a non-significant positive correlation, while HDL, ALT, and TB have a non-significant negative correlation.

3.4. Receiver operating characteristic (ROC)

ROC curve and AUC analysis for the GDF-15, Osteocalcin, and Zonulin for coronary artery with non-alcoholic fatty liver group. Results of the receiver operating curve (ROC) curve and AUC analysis as a diagnostic parameter was shown that GDF-15 and Zonulin have a good performance , data are presented in Table (3-12).

Table (3-10): AUC, optimal threshold, sensitivity, and specificity of GDF-15, osteocalcin, and zonulin obtained by ROC curve in CAD+NAFLD group.

Test Variable	Cut-off points	Sensitivity	Specificity	AUC	CI (95%)	p-value
GDF-15 (ng/L)	250.605	0.79	0.91	0.816	0.749-0.884	≤0.001
Osteocalcin (ng/ml)	8.732	0.88	0.60	0.694	0.621-0.768	≤0.001
Zonulin (ng/ml)	69.79	0.93	0.83	0.941	0.907-0.975	≤0.001

In CAD+NAFLD group, GDF-15, and zonulin had the highest AUC, which was 0.816 [95% CI (confidence interval) =0.749 – 0.884, Sensitivity = 0.79, Specificity= 0.91, Cut-off point = 250.605] and 0.941[95% CI (confidence interval) = 0.907-0.975, Sensitivity = 0.93, Specificity% = 0.83, Cut-off point = 69.79] respectively. Osteocalcin had AUC which was 0.694 [95%CI (confidence interval) = 0.621-0.768, sensitiveity= 0.88, Specificity = 0.60, Cut-off point = 8.732].

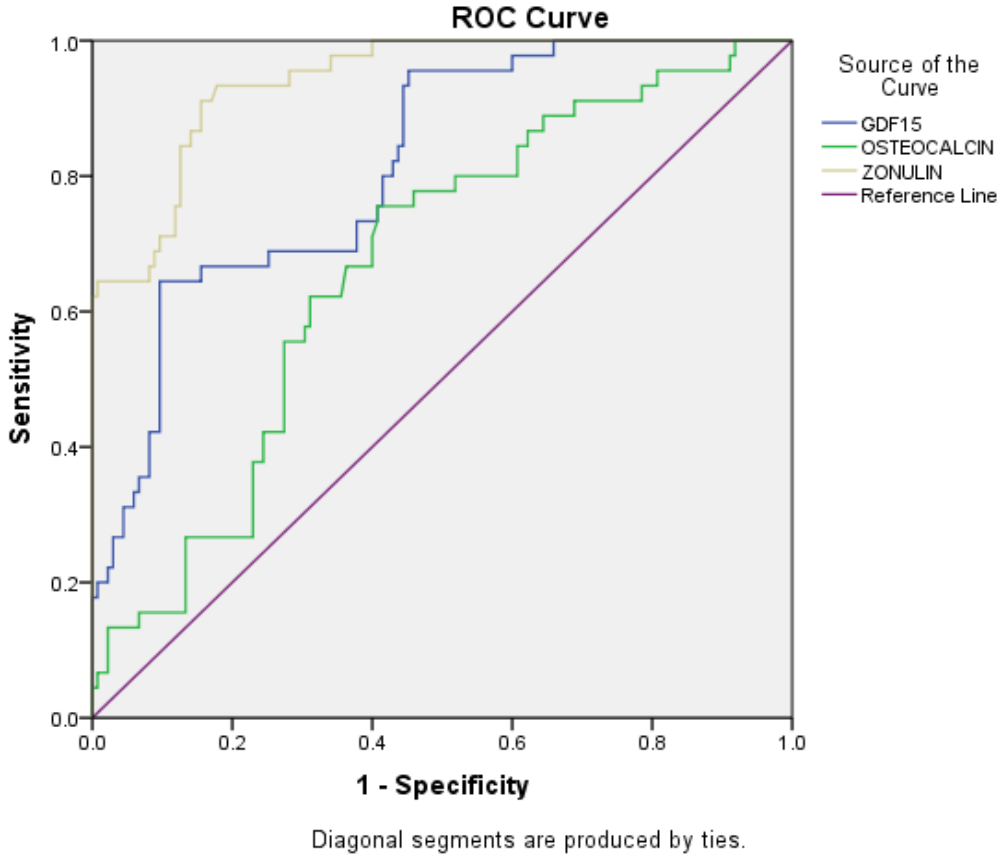


Figure (3-10): Receiver Operativ Characteristic (ROC) curve of serum GDF-15, osteocalcin, and zonulin levels as discriminators of CAD+NAFLD cases among patients.

Table (3-11): AUC, optimal threshold, sensitivity, and specificity of GDF-15, osteocalcin, and zonulin obtained by the ROC curve for CAD patients.

Test Variable	Cut-off points	Sensitivity	Specificity	AUC	CI (95%)	p-value
GDF-15 (ng/L)	220.920	0.53	0.60	0.487	0.396-0.578	0.795
Osteocalcin (ng/ml)	11.484	0.52	0.34	0.398	0.318-0.520	0.041
Zonulin (ng/ml)	65.395	0.75	0.60	0.636	0.554-0.719	0.006

In CAD group, the AUC of GDF-15 was 0.487 [95% Cl (Confidence interval) =0.396-0.578, Sensitivity= 0.53, Specificity = 0.60 Cut-off point = 220.920], osteocalcin 0.398 [95% Cl (Confidence interval) =0.318-0.520, Sensitivity = 0.52, Specificity= 0.34 Cut-off point = 11.484], and for zonulin was 0.636 [95% Cl (Confidence interval) =0.554-0.719, Sensitivity= 0.75, Specificity = 0.6 Cut-off point = 65.395],

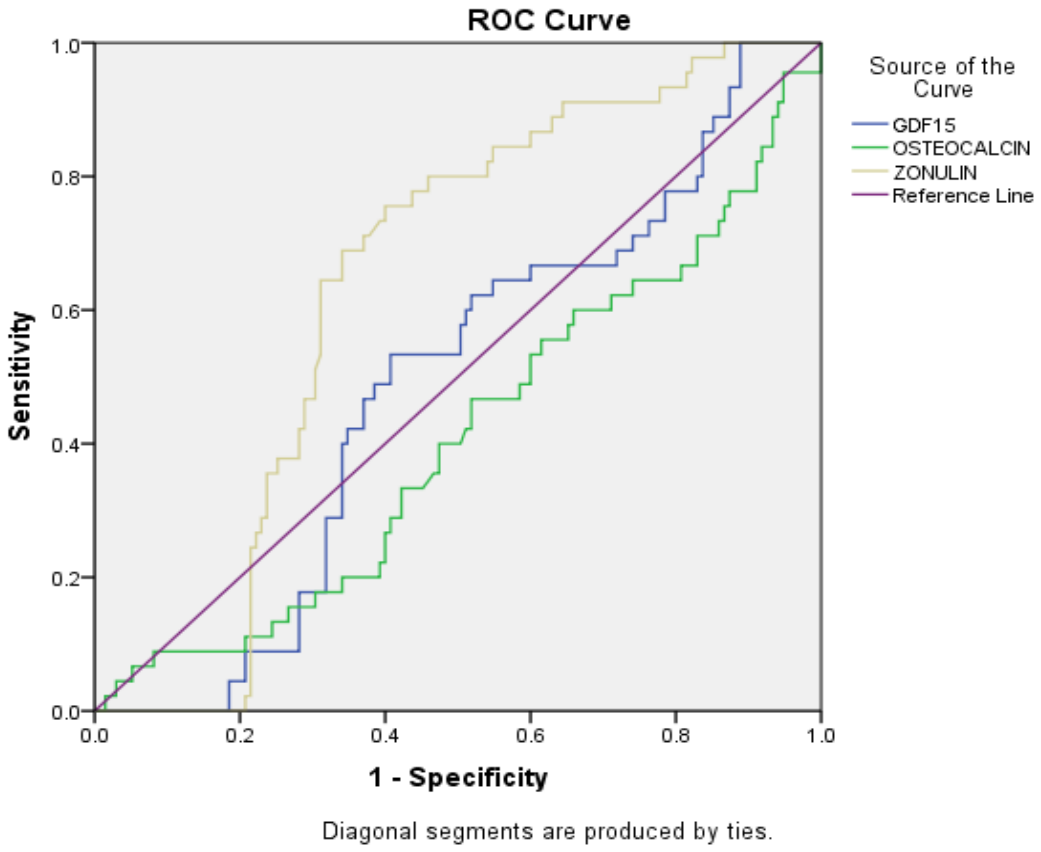


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Figure (3-11): Receiver operating characteristic (ROC) curve of GDF-15, osteocalcin, and zonulin in CAD patients.

Table (3-12): AUC, optimal threshold, sensitivity, and specificity of GDF-15 and osteocalcin obtained by the ROC curve for NAFLD patients.

Test Variable	Cut-off points	Sensitivity	Specificity	AUC	CI (95%)	p-value
GDF-15 (ng/L)	210.278	0.71	0.87	0.863	0.790-0.935	≤0.001
Osteocalcin (ng/ml)	7.365	0.84	0.91	0.908	0.861-0.954	≤0.001
Zonulin (ng/ml)	57.313	0.57	0.3	0.392	0.314-0.501	0.031

In NAFLD group, GDF-15 had the AUC, which was 0.863 [95% Cl (Confidence interval) =0.790-0.935, Sensitivity = 0.71, Specificity% = 0.87, Cut-off point = 210.278], osteocalcin 0.908 [95% Cl (Confidence interval) =0.0.861-0.954, Sensitivity = 0.84, Specificity = 0.91, Cut-off point = 7.365], and zonulin 0.392 [95% Cl (Confidence interval) =0.314-0.501, Sensitivity = 0.57, Specificity= 0.3, Cut-off point = 57.313].

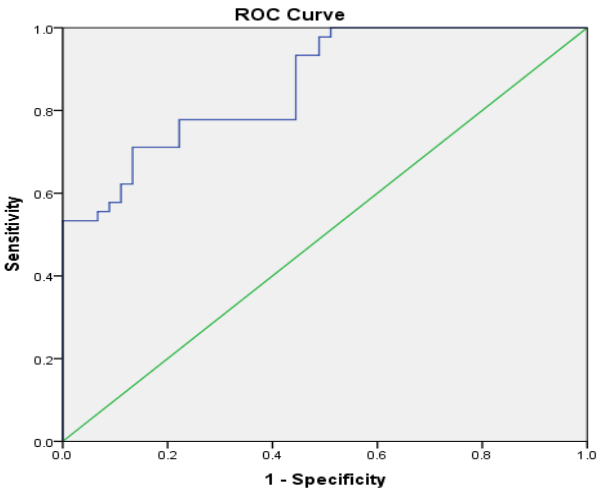

Figure (3-12): Receiver operating characteristic (ROC) curve of GDF-15 in NAFLD patients.

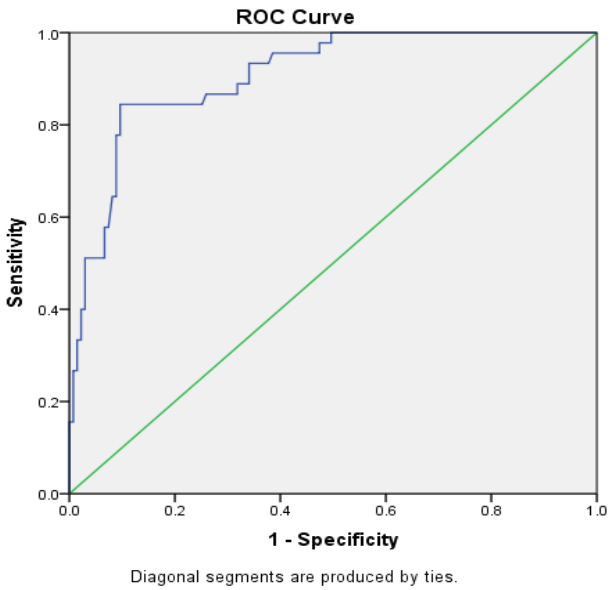

Figure (3-13): Receiver operating characteristic (ROC) curve of osteocalcin in NAFLD patients.

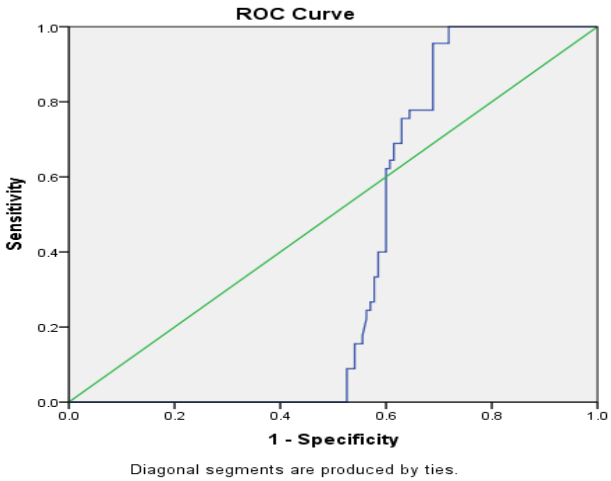

Figure (3-14): Receiver operating characteristic (ROC) curve of zonulin in NAFLD patients.

Chapter Four

Discussion

4. Discussion

4.1. The liver-heart axis

The heart-liver axis is related to the MS and acts as a direct connection between the white adipose tissue, the liver and the heart by a systemic signaling led by organic cytokines such as adipokines, hepatokines, and cardiomyokines, predicting the NAFLD-related CVD risk **(Baars *et al*., 2022)**.

Compared to healthy individuals, patients with NAFLD, obesity and diabetes display an increased intestinal permeability and increased bacterial growth in the small intestine (endotoxemia**) (Caturano *et al*., 2021)**. Metabolic endotoxemia can occur in the form of lipopolysaccharides (LPS) entering portal circulation and impairing the immune response by binding to toll-like receptor 4 (TLR) and activating the inflammatory cascade **(Caturano *et al*., 2021; Marušić *et al*., 2021)** . This process acts on insulin signaling, and favors hepatic steatosis and progression to NASH; on the other hand, it promotes endothelial dysfunction, LDL oxidation, and thrombogenesis, destabilizing the atherosclerotic plaques **(Sanduzzi Zamparelli *et al*., 2016)**

It was found that patients with CAD and NAFLD both have gut dysbiosis. The intestinal microbiota might be different in patients with NAFLD and CAD than in those who only have CAD **(Zhang *et al*., 2019).**

Despite the development of atherosclerosis can be accelerated and promoted by NAFLD, the exact pathophysiology remains poorly understood. The potential mechanisms for accelerating atherosclerotic CVD in NAFLD patients were complex and might be related to lipid disturbances, IR, chronic inflammation, and endothelial dysfunction. Patients with NAFLD had a pro-atherosclerotic lipid profile characterized by high triglyceride, elevated very low-density lipoprotein (VLDL), and low levels of HDL, which lead to an increased CVD risk **(Zhang *et al*., 2020).**

4.2. Demographic and anthropometric characteristics

In the present study, it had been noticed that most patients were in the age group 51-61 (43.3%) and age group ≥62 (35.6%). A research by Benjamin in 2019 showed that when compared to other populations, older persons have a significantly greater prevalence of the majority of CVDs **(Benjamin *et al*., 2019)**. Age is a significant independent risk factor for CVD due to the increased likelihood of having any additional cardiac risk factors, such as diabetes and obesity. Numerous variables, including elevated oxidative stress, inflammation, apoptosis, and general cardiac degradation and degeneration, have been linked to the high frequency of CVD in this population. Additionally, age is linked to a higher risk of diabetes, obesity, and frailty **(Rodgers *et al*., 2019).**

The American Hospital Association (AHA) reports that elderly men are more likely than older women to have coronary heart disease (CHD) **(Benjamin *et al*., 2019).**

In the current study, the male percentage was 70.4% and the female was 29.6% as shown in the Table (3-1). According to numerous research, there are sex differences in CAD patients as a result of male patients' greater rates of infection. These sex differences are mostly caused by social behavior and biological causes. Men are thought to smoke more frequently than women for a variety of social reasons and the biology of humans. Prior to menopause, women are typically well protected against cardiovascular illness; nevertheless, following menopause, the risk for heart disease in women significantly rises. With the start of advanced age, CVD has been proven to be significantly increased by the loss of sex hormones. When compared to age-matched males, premenopausal women have a generally lower incidence of CVD, which is commonly attributed to the cardioprotective effects of estrogen. Males have also been demonstrated to benefit from the cardioprotective effects of estrogen. According to one study, the progressive drop

in estrogen levels after puberty makes men 10-15 years more likely than women to suffer heart disease **(Rodgers *et al*., 2019).**

In previous study by Baker in 2003 stated that because estrogen levels gradually fall after puberty, men are prone to experience heart disease 10-15 years earlier than women **(Baker *et al*., 2003)**.

Men are more likely than women during the reproductive years to have NAFLD, both in terms of prevalence and severity. NAFLD does however happen more frequently in women after menopause, indicating that estrogen may be protective. There are sex disparities for the main NAFLD risk factors as well. Male individuals had a higher prevalence of hepatic tumors than female subjects did, and animal models of NAFLD generally replicate the sex differences seen in patients, including more severe steatosis and steatohepatitis, more proinflammatory/profibrotic cytokines, and more severe steatosis **(Lonardo *et al*., 2019).**

Through this study, it had been noticed that a large percentage of patients were overweight (37.03%) and obese (44.4%). As known obesity is one of the main risk factors for both coronary artery and non-alcoholic fatty liver **(Koliaki *et al*., 2019).** Also (57.7%) of patients have type 2 diabetes, (53.3%) have hypertension. Type 2 diabetes mellitus (DM) and hypertension(HTN) are established risk factors for cardiovascular disease (CVD). Moreover, patients with DM and HTN have an increased risk of cardiovascular mortality compared with patients who have either condition alone. Further, DM and HTN are common conditions that frequently present together. One study reported that up to 75% of adults with DM also have HTN and patients with HTN alone often show evidence of insulin resistance **(AL-Azzam *et al*., 2021).**

Two-thirds of patients had a sedentary lifestyle, which is considered one of the risk factors that developing coronary artery and non-alcoholic fatty liver disease.

The percentage of patients with coronary artery who have a family history of the disease was (46.6%).

4.3. Lipid Profile and Some Liver Function Test.

In this study, the results indicated that the levels of lipid profiles were normal or nearly normal range in patient groups (CAD+NAFLD and CAD) as shown in Table (3-3) and Fig. (3-4), this is due to drugs used in the hospital.

The gold standard for treating dyslipidemia is statin medication. Statins are prescribed as the first-line pharmacological therapy for the reduction of cardiovascular **(Niedzielski *et al*., 2020; Musunuru, 2021)**. The mechanism by which statins act to reduce liver cholesterol production is based on the competitive inhibition of rate-controlling enzymes in cholesterol synthesis 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase). This enzyme catalyzes the conversion of HMG-CoA to a mevalonic acid, a necessary step in the biosynthesis of cholesterol.

Statin works by inhibiting HMG-CoA reductase, causing upregulation of the LDL-C receptors on the surface of the liver cell and increasing the removal of LDL-C from the blood. In addition, statins lower serum triglycerides concentrate and modify endothelial function, inflammatory responses, plaque stability, and thrombus formation. As a result, statin appears to play a major role in decreasing the risk of coronary heart disease (CHD) and all-cause mortality **(Trentman *et al*., 2016; Habte, 2020)**.

In the fatty liver group, there is little elevation in lipid profile level. Dyslipidemia is known as a risk factor for NAFLD. Individuals in the NAFLD group had a higher TC, TG, LDL, and lower HDL as compared to the healthy group (**Santhoshakumari and Radhika, 2017)**.

As described in Table (3-5), fig. (3-6), and fig. (3-7) showed a slight elevation in AST, ALT, and ALP concentrations. The mean levels of hepatic enzymes were a little high in the non-alcoholic fatty liver group (**Mansour-Ghanaei *et al*., 2019)**. NAFLD patients have LFTs that are normal or very close to normal. Transaminases may be somewhat elevated in patients (ALT > AST) **(Sharma and Arora, 2020)**.

4.4. Growth differentiation factor-15

The results showed an increased level of GDF-15 in patient groups as described in Table (3-6) and Fig. (3-8). Several human research on GDF-15 concentrations in cardiovascular disorders have been conducted.

Potential mechanisms have been suggested for the association of GDF-15 with adverse outcomes in atherosclerosis, including worse baseline cardiac disease severity, inflammation, ischemia, volume overload, and adipokines. Elevated GDF-15 has been shown to promote inflammation and angiogenesis, implying that GDF-15 may play an important role in the pathogenesis of atherosclerosis. While GDF-15 is a cardiovascular risk factor, whether GDF-15 contributes directly to atherosclerosis development has not been established and the precise relationships between GDF-15 and atherosclerosis are incompletely understood. GDF-15 was originally identified as a factor overexpressed in activated macrophages to regulate inflammation, which is involved in all stages of atherosclerosis, from its initiation and progression to its thrombotic complications, demonstrating that leukocyte deficiency of GDF-15 improves atherosclerotic plaque stability by impairing macrophage migration and promoting collagen deposition **(Wang *et al*., 2019a)**.

GDF-15 increases during tissue injury and inflammatory states and is associated with cardiometabolic risk. Increased GDF-15 levels are associated with cardiovascular diseases such as hypertrophy, heart failure, atherosclerosis, endothelial dysfunction, obesity, insulin resistance, diabetes, and chronic kidney

diseases in diabetes. Increased GDF-15 level is linked with the progression and prognosis of the disease condition. Age, smoking, and environmental factors are other risk factors that may increase GDF-15 levels. Most of the scientific studies reported that GDF-15 plays a protective role in different tissues **(Schopfer *et al*., 2014; Adela, 2015)**

Schlittenhardt *et al* shown that GDF-15 is inducible in human macrophages by ox-LDL and its mediators *in vitro* and is supposed to contribute to oxidative stress-dependent consequences in arteriosclerotic plaques modulating apoptosis and inflammatory processes in activated macrophages **(Schlittenhardt *et al*., 2004)**. Atherosclerosis is initiated by lipoprotein accumulation in the intimal layer of the arterial vessel wall, which recruits circulating monocytes **(Hansson and Hermansson, 2011)**

Once enter the arterial intima, the monocytes differentiate into macrophages; then the latter ingests lipoproteins, in particular oxidized lipoproteins, and finally converts into foam cells, which promotes atherosclerosis progression **(Braunersreuther *et al*., 2007)**. Huang in 2021 showed that GDF-15 may suppress atherosclerosis and plaque formation by inhibiting lipoprotein accumulation and inflammation activation **(Huang *et al*., 2021)**.

In the present study, it observed an association between plasma concentrations of GDF-15 and NAFLD. Galuppo in 2022 observed that the degree of change in GDF15 paralleled the degree of change in intrahepatic fat content over time corroborating the hypothesis that GDF15 synthesis might increase with an increase in lipid synthesis in the liver **(Galuppo *et al*., 2022)**. A Study by Luan showed that GDF15 concentrations are increased with increased production of lipids in the liver of a mouse model. In the study, the authors demonstrated that GDF15 may provide a protective effect against the inflammatory insult induced

by viruses and bacteria and concluded that GDF15 attenuates the inflammatory response by modulating lipid metabolism **(Luan *et al*., 2019)**.

4.5. Osteocalcin

Table (3-7) that illustrated the osteocalcin levels, observed low levels of serum osteocalcin in coronary patients. According to research, there is a negative correlation between arterial calcification (carotid or coronary atherosclerosis) and osteocalcin levels **(Magni *et al*., 2016)**. The majority of studies reported inverse, independent, and significant correlations between total osteocalcin and outcomes such as aortic calcification, and coronary calcification or atherosclerosis. Circulating total osteocalcin was significantly lower in patients with cardiovascular conditions. The mechanistic evidence linking osteocalcin with atherosclerosis CVD is unclear compared with metabolic conditions such as type 2 diabetes, several pathways have been proposed. Conditions such as insulin resistance, metabolic syndrome, non-alcoholic fatty liver disease, and type 2 diabetes, which are closely linked to circulating osteocalcin, accelerate the progression of atherosclerotic lesions and are strongly linked to the development of atherosclerosis. Osteocalcin may be involved in the calcification process at arterial and valvular sites, leading to reduced elasticity and compliance of the vasculature **(Seidu *et al*., 2019).**

Also, the levels of osteocalcin in non-alcoholic fatty liver individuals were decreased.

In human investigations, circulating osteocalcin levels, whether they were carboxylated or undercarboxylated, were generally inversely related to the incidence of type 2 diabetes **(Booth *et al*., 2013; Kunutsor *et al*., 2015)**, central obesity, and metabolic syndrome **(Oosterwerff *et al*., 2013)**.

According to studies, osteocalcin was found to be adversely correlated with NAFLD **(Du *et al*., 2015; Yang *et al*., 2016)**. There hasn't been much research

done on the mechanism through which osteocalcin influences the onset of NAFLD. According to recent investigations, either enhanced lipid absorption or improved glucose metabolism may be secondary effects of osteocalcin's positive effects on NAFLD. **(Xia *et al.*, 2021)**.

Osteocalcin levels in patients with T2DM and NAFLD may decrease due to disorders of glucose and lipid metabolism **(El Amrousy and El-Afify, 2020)**

Previous study showed that in subjects with NAFLD, IR was significantly associated with gut permeability by circulating zonulin. Furthermore, the increased prevalence of T2DM in patients with higher zonulin levels suggests that zonulin could be investigated as a potential risk factor for the development of both T2DM as well as other clinical conditions such as cardiovascular disease **(Rosso *et al*., 2021)**.

4.6. Zonulin

Table (3-8) and Fig. (3-10) showed the levels of zonulin in patients groups, indicated that zonulin levels were high in patients groups.

New researches show that type 1 diabetes mellitus, obesity, or hypertension are at increased risk for cardiovascular disease (CVD) when there is gut dysbiosis **(Ascher and Reinhardt, 2018; Ghosh *et al*., 2021; Han *et al*., 2021)**. Atherosclerosis and arterial thrombosis have been linked to the gut microbiota in recent years **(Lässiger-Herfurth *et al*., 2019).**

A growing body of evidence suggests that dysbiosis, a state of microbiota imbalance in the gut, contributes critically to the development of vascular inflammation.

The atherogenic inflammatory state can be initiated by an unhealthy gut microbiota and promoted by subsequent dysregulation of local and systemic immune responses. The macrophages residing in the intestinal lamina propria and

intima layer of the artery can be activated through the reception of several types of signals from gut microbiota, including translocated live bacteria, and structural components of dead bacteria (e.g., lipopolysaccharides (LPS). These microbially-derived small molecules can be transmitted from the gut lumen into the body through transcellular or paracellular routes while larger molecules such as LPS may be translocated through a disrupted gut barrier. The immune cells react to these bacterial signals locally and systemically and produce various cytokines and proteins to direct a defensive response or an immune tolerance to the gut microbiota **(Tilg *et al*., 2020)**.

Furthermore, immune cells primed in the intestine by the gut microbiota may migrate to distal vessels and organs to induce immunopathogenesis. Atherosclerosis is primarily impacted by microbiota via two main mechanisms. First, bacterial translocation and bacterial structural components that trigger the immune system cause an inflammatory reaction. Bacteria from the gut or oral cavity directly penetrate the atherosclerotic plaque or trigger a proatherogenic response by translocating MAMPs like lipopolysaccharide (LPS), which are molecules associated with microbes. This would intensify atherosclerosis by increasing the production of pro-inflammatory cytokines and chemokines.

Second, certain microbial processes that involve dietary components can result in the synthesis of both advantageous and detrimental small molecules. These metabolites might promote the onset of atherosclerotic plaques or worsen the condition.

Zonulin is a paracrine protein, with a molecular mass of 47-kDa, released by several cell lines, including intestinal cells, after exposure to gliadin-specific peptides or bacteria **(Wood *et al*., 2020)**. Studies on type 2 diabetes, obesity or acute or chronic CVD patients revealed an increase in serum levels of zonulin

coincidentally with elevated levels of LPS **(Aasbrenn *et al*., 2020; Carnevale *et al*., 2020)**.

The interaction between the gut and the liver, the so-called 'gut-liver axis', is considered to play a critical role in the development and progression of NAFLD in both children and adults. Crosstalk between the gut and liver is facilitated through the intestinal barrier. This intestinal barrier consists of structural elements (mucus and closely linked epithelial cells sealed by tight junctions), immune cells, and soluble mediators (e.g. IgA, antimicrobial peptides). An intact intestinal barrier is able to restrict translocation of bacterial products while allowing active transport of nutrients across the tight junctions **(Albillos *et al*., 2020)**. Increased IP can lead to the translocation of microbial products from the gut to the liver through the portal system. An efficient immunological barrier limits this process, promoting a local immune response in activated mesenteric lymph nodes. When this primary firewall fails, microbes and microbial compounds reach the liver, where they activate Kupffer cells by binding Toll-like receptors. Kupffer cells orchestrate several processes, such as the release of inflammatory cytokines and reactive oxygen species, the recruitment of innate immune cells, the activation of hepatic stellate cells. The uncontrolled perpetuation of this pathogenic mechanism results in liver inflammation and damage, fibrogenesis and systemic inflammation **(Nicoletti *et al*., 2019)**. Known factors that contribute to an increased IP include consumption of a Western diet (i.e. high fat intake), gut microbiome perturbations, pro-inflammatory cytokines, alcohol, and use of antibiotics **(Leech *et al*.,2019)**.

Zonulin secretion is followed by an increase in gut permeability secondary to the disassembly of the zonula occludens-1 from the tight junctional complex **(Olivieri *et al.*, 2022)**. Activation of the zonulin pathway may represent a defensive mechanism that "flushes out" microorganisms, contributing to the host's innate immune response against changes in the microbiome system (**(Fasano, 2020)**. The increased intestinal permeability leads to the translocation of bacterial

substances, such as LPS, and products of their metabolism, as short-chain fatty acids, in the systemic circulation, eventually determining a low-grade inflammatory status, also called endotoxemia that leads to metabolic dysregulation **(Olivieri *et al.*, 2022)**

The impairment of intestinal barrier, due to alterations in gut microbiota, has been implicated in the onset and progression of non-alcoholic fatty liver disease (NAFLD) (**Rasso et al., 2021)**. Recently, several studies have focusing on the role of zonulin as non-invasive biomarker of intestinal permeability.

Serum zonulin increased proportionally to waist circumference and BMI. Excess weight is associated with increased intestinal permeability **(Cortez *et al.*, 2021).** The serum zonulin assay has been considered an easy-to-perform surrogate marker of the dysfunctional intestinal barrier **(Cortez *et al.*, 2021)**. Intestinal dysbiosis, characteristic of subjects who consume a high-fat and low-fiber diet **(Martina, 2019).**

In line with the previous investigation **(Yakut *et al*., 2021)**, the results reported substantial positive associations between BMI and GDF-15 levels in each group in this current study as shown in Table(3-9).

Table (3-10), illustrated that Serum osteocalcin concentration was significantly and negatively correlated with BMI, waist circumstance, and triglycerides in both coronary arteries and non-alcoholic fatty liver

According to Luo, Serum osteocalcin levels were negatively correlated with cardiovascular risk factors, such as obesity, diabetes, dyslipidemia, and nonalcoholic fatty liver disease **(Luo *et al*., 2015b)**.

Another study by Dumitru in 2019 showed a negative correlation between osteocalcin levels and triglycerides. In human, numerous studies have shown that

the levels of the hormone osteocalcin in the blood and BMI are inversely related **(Dumitru *et al*., 2019)**.

In table (3-11), that illustrated the correlations of zonulin. We indicated that there were positive significant correlations between zonulin and BMI and waist circumference in patients groups supporting a previous study by (**Erkan *et al*., 2023)**.

Chapter Five

Conclusion

and

Recommendation

5. Conclusions and Recommendations

5.1. Conclusions

According to the observed data, we can conclude the following:

1. The GDF-15 in NAFLD and CAD could function as an early predictor marker for cardiovascular risk, non-alcoholic fatty liver, and appears to be a direct participant in the atherosclerotic process and metabolic changes.
2. The decreased level of osteocalcin in the NAFLD and CAD patients groups might be returned to the potential role for improve endothelial function and reducing the pathological mechanisms that promote the development of atherosclerosis in patients with NAFLD. Further, the effect of osteocalcin on vascular calcification is unclear. In most cases, calcification was associated with the presence of osteocalcin, which increased relative to the degree of calcification.
3. These results indicate that the increased level of zonulin is associated with inflammatory responses in liver tissue and the development of liver diseases.

5.2. Recommendations

1. Additional studies can be done on different cardiovascular diseases such as a cute coronary syndrome to determine its correlation with the markers.
2. Assessment the prevalence of NAFLD in the higher risk population such as obese, hypertensive, and diabetics.
3. Study the diagnostic and therapeutic role of zonulin in other liver diseases like (hepatitis, cirrhosis, and hepatocellular carcinoma (HCC))

References

References

Aasbrenn, M., Lydersen, S., and Farup, P. G. **(2020)**. Changes in serum zonulin in individuals with morbid obesity after weight-loss interventions: a prospective cohort study. BMC endocrine disorders, 20(1), 1-9.

Abeysekera, K. W., Fernandes, G. S., Hammerton, G., Portal, A. J., Gordon, F. H., Heron, J., *et al*. **(2020)**. Prevalence of steatosis and fibrosis in young adults in the UK: a population-based study. The lancet Gastroenterology & hepatology, 5(3), 29

Adela R, Banerjee SK. GDF-15 as a Target and Biomarker for Diabetes and Cardiovascular Diseases: A Translational Prospective. J Diabetes Res. **(2015)**; 26273671, 2015:490842. doi: 10.1155/2015/490842. Epub 2015 Jul 27. PMID:; PMC4530250., PMCID:

Ahmadi, A. R., Sadeghian, M., Alipour, M., Taheri, S. A., Rahmani, S., and Abbasnezhad, A. **(2020)**. The effects of probiotic/synbiotic on serum level of zonulin as a biomarker of intestinal permeability: A systematic review and meta-analysis. Iranian Journal of Public Health, 49(7), 1222.

Al-Azzam, N., Al-Azzam, S., Elsalem, L., and Karasneh, R. **(2021)**. Hypertension prevalence and associated factors among patients with diabetes: A retrospective cross-sectional study from Jordan. Annals of medicine and surgery, 61, 126-131.

Albillos, A., De Gottardi, A., and Rescigno, M. **(2020)**. The gut-liver axis in liver disease: Pathophysiological basis for therapy. Journal of hepatology, 72(3), 558-577.

Alizadehsani, R., Khosravi, A., Roshanzamir, M., Abdar, M., Sarrafzadegan, N., Shafie, D., *et al*. **(2021**). Coronary artery disease detection using artificial intelligence techniques: A survey of trends, geographical

differences and diagnostic features 1991-2020. Computers in Biology and Medicine, 128, 104095.

Alloubani, A., Nimer, R., and Samara, R. **(2021)**. Relationship between hyperlipidemia, cardiovascular disease and stroke: a systematic review. Current Cardiology Reviews, 17(6), 52-66.

Arnett, D. K., Blumenthal, R. S., Albert, M. A., Buroker, A. B., Goldberger, Z. D., Hahn, E. J., *et al*. **(2019)**. 2019 ACC/AHA guideline on the primary prevention of cardiovascular disease: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. Circulation, 140(11), e596-e646.

Ascher, S., and Reinhardt, C. **(2018)**. The gut microbiota: an emerging risk factor for cardiovascular and cerebrovascular disease. European Journal of Immunology, 48(4), 564-575.

Asrani, S. K., Devarbhavi, H., Eaton, J., andKamath, P. S. **(2019)**. Burden of liver diseases in the world. Journal of hepatology, 70(1), 151-171.

Baars, T., Gieseler, R. K., Patsalis, P. C., and Canbay, A. **(2022)**. Towards harnessing the value of organokine crosstalk to predict the risk for cardiovascular disease in non-alcoholic fatty liver disease. Metabolism, 155179.

Badimon, L., Padró, T., and Vilahur, G. **(2012)**. Atherosclerosis, platelets and thrombosis in acute ischaemic heart disease. European Heart Journal: Acute Cardiovascular Care, 1(1), 60-74.

Baek, S. J., and Eling, T. **(2019)**. Growth differentiation factor 15 (GDF15): A survival protein with therapeutic potential in metabolic diseases. Pharmacology & therapeutics, 198, 46-58.

Bahit, M. C., Kochar, A., and Granger, C. B. **(2018)**. Post-myocardial infarction heart failure. JACC: Heart failure, 6(3), 179-186.

Bajelan, M., Roodi, N. E., Daloee, M. H., Farhangnia, M., and Kuchaksaraei, A. S. **(2019)**. The effect of low testosterone and estrogen levels on progressive coronary artery disease in men. Reports of Biochemistry & Molecular Biology, 8(2), 168.

Baker, L., Meldrum, K. K., Wang, M., Sankula, R., Vanam, R., Raiesdana, A., *et al*. **(2003)**. The role of estrogen in cardiovascular disease. Journal of surgical research, 115(2), 325-344.

Barbaro, M. R., Cremon, C., Morselli-Labate, A. M., Di Sabatino, A., Giuffrida, P., Corazza, G. R., *et al*. **(2020)**. Serum zonulin and its diagnostic performance in non-coeliac gluten sensitivity. Gut, 69(11), 1966-1974.

Bayrak, M. **(2020)**. Non-invasive diagnosis of nonalcoholic fatty liver disease: impact of age and other risk factors. The Aging Male, 23(5), 1275-1282.

Bechthold, A., Boeing, H., Schwedhelm, C., Hoffmann, G., Knüppel, S., Iqbal, K., *et al*. **(2019)**. Food groups and risk of coronary heart disease, stroke and heart failure: a systematic review and dose-response meta-analysis of prospective studies. Critical reviews in food science and nutrition, 59(7), 1071-1090.

Benjamin, E. J., Muntner, P., Alonso, A., Bittencourt, M. S., Callaway, C. W., Carson, A. P., *et al*. **(2019).** Heart disease and stroke statistics—2019 update: a report from the American Heart Association. *Circulation*, *139*(10), e56-e528.

Benjamin, E. J., Virani, S. S., Callaway, C. W., Chamberlain, A. M., Chang, A. R., Cheng, S., *et al*. **(2018)**. Heart disease and stroke statistics—2018

update: a report from the American Heart Association. *Circulation*, *137*(12), e67-e492.

Beverly, J. K., and Budoff, M. J. **(2020)**. Atherosclerosis: Pathophysiology of insulin resistance, hyperglycemia, hyperlipidemia, and inflammation. Journal of diabetes, 12(2), 102-104.

Bhardwaj, S., Bhattacharjee, J., Bhatnagar, M.K. and Tyagi, S. **(2013**). Atherogenic Index of Plasma, Castelli Risk Index and Atherogenic CoefficientNew parameters in assessing cardiovascular risk. International Journal of Pharmacy and Biological Science, 3:359-364.

Bishop, M. L. **(2020)**. Clinical Chemistry: Principles, Techniques, and Correlations, Jones and Bartlett Learning.

Björkegren, J. L., and Lusis, A. J. **(2022)**. Atherosclerosis: recent developments. Cell.

Booth, S. L., Centi, A., Smith, S. R., and Gundberg, C. **(2013)**. The role of osteocalcin in human glucose metabolism: marker or mediator?. Nature Reviews Endocrinology, 9(1), 43-55.

Braunersreuther, V., Zernecke, A., Arnaud, C., Liehn, E. A., Steffens, S., Shagdarsuren, E., *et al*. **(2007)**. Ccr5 but not Ccr1 deficiency reduces development of diet-induced atherosclerosis in mice. Arteriosclerosis, thrombosis, and vascular biology, *27*(2), 373-379.

Brown, J. C., Gerhardt, T. E., and Kwon, E. **(2020)**. Risk factors for coronary artery disease.

Carnevale, R., Sciarretta, S., Valenti, V., di Nonno, F., Calvieri, C., Nocella, C., *et al*. **(2020)**. Low-grade endotoxaemia enhances artery thrombus growth via Toll-like receptor 4: implication for myocardial infarction. European heart journal, 41(33), 3156-3165.

Castelli, W.P., Abbott, R.D. and McNamara, P.M. **(1983**). Summary estimates of cholesterol are used to predict coronary heart disease. Circulation, 67:730- 734.

Castillo, V., Figueroa, F., González-Pizarro, K., Jopia, P., and Ibacache-Quiroga, C. **(2021)**. Probiotics and prebiotics as a strategy for non-alcoholic fatty liver disease, a narrative review. Foods, 10(8), 1719.

Caturano, A., Acierno, C., Nevola, R., Pafundi, P. C., Galiero, R., Rinaldi, L., *et al*. **(2021)**. Non-alcoholic fatty liver disease: From pathogenesis to clinical impact. Processes, 9(1), 135.

Cazac, G. D., Lăcătușu, C. M., Mihai, C., Grigorescu, E. D., Onofriescu, A., and Mihai, B. M. **(2022)**. New Insights into Non-Alcoholic Fatty Liver Disease and Coronary Artery Disease: The Liver-Heart Axis. Life, 12(8), 1189.

Chalasani, N., Younossi, Z., Lavine, J. E., Charlton, M., Cusi, K., Rinella, M., *et al*. **(2018)**. The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases.

Chan, W. K., Tan, A. T. B., Vethakkan, S. R., Tah, P. C., Vijayananthan, A., and Goh, K. L. **(2013)**. Non-alcoholic fatty liver disease in diabetics–prevalence and predictive factors in a multiracial hospital clinic population in Malaysia. Journal of gastro

Ciccia, F., Guggino, G., Rizzo, A., Alessandro, R., Luchetti, M. M., Milling, S., *et al*. **(2017)**. Dysbiosis and zonulin upregulation alter gut epithelial and vascular barriers in patients with ankylosing spondylitis. Annals of the rheumatic disease, *76*(6), 1123-1132.

Cortez, A. P. B., Fisberg, M., & de Morais, M. B. **(2021)**. Intestinal permeability and small intestine bacterial overgrowth in excess weight adolescents. Pediatric Obesity, 16(5), e12741.

Cui, Y., Wang, Q., Chang, R., Zhou, X., and Xu, C. **(2019)**. Intestinal barrier function–non-alcoholic fatty liver disease interactions and possible role of gut microbiota. Journal of agricultural and food chemistry, 67(10), 2754-2762.

Davison, B. A., Harrison, S. A., Cotter, G., Alkhouri, N., Sanyal, A., Edwards, C., *et al*. **(2020)**. Suboptimal reliability of liver biopsy evaluation has implications for randomized clinical trials. Journal of hepatology, 73(6), 1322-1332.

Desa, U. N. **(2019**). World population prospects 2019: Highlights. New York (US): United Nations Department for Economic and Social Affairs, 11(1), 125.

Dirckx, N., Moorer, M. C., Clemens, T. L., and Riddle, R. C. **(2019)**. The role of osteoblasts in energy homeostasis. Nature Reviews Endocrinology, 15(11), 651-665.

Du, J., Pan, X., Lu, Z., Gao, M., Hu, X., Zhang, X., *et al*. **(2015)**. Serum osteocalcin levels are inversely associated with the presence of nonalcoholic fatty liver disease in patients with coronary artery disease. International Journal of Clinical

Dumitrascu, D. L., and Neuman, M. G. **(2018)**. Non-alcoholic fatty liver disease: an update on diagnosis. Clujul medical, 91(2), 147.

DUMITRU, N., CARSOTE, M., COCOLOS, A., PETROVA, E., OLARU, M., DUMITRACHE, C., *et al*. **(2019)**. The link between bone osteocalcin and

energy metabolism in a group of postmenopausal women. Current health sciences journal, 45(1), 47.

El Amrousy, D., and El-Afify, D. **(2020)**. Osteocalcin and osteoprotegerin levels and their relationship with adipokines and proinflammatory cytokines in children with nonalcoholic fatty liver disease. Cytokine, 135, 155215.

Erkan, G. C., Sumnu, A., Ertugrul, G., Ulfer, G., Cakici, C., Aydin, H. C., *et al*. **(2023)**. The Relationship between Zonulin and Metabolic Syndrome in Renal Transplant Patients. Clinical Laboratory, 69(1).

Estes, C., Razavi, H., Loomba, R., Younossi, Z., and Sanyal, A. J. **(2018)**. Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease. Hepatology, 67(1), 123-133.

Fasano, A. **(2012)**. Intestinal permeability and its regulation by zonulin: diagnostic and therapeutic implications. Clinical Gastroenterology and Hepatology, 10(10), 1096-1100.

Fasano, A. **(2020)**. All disease begins in the (leaky) gut: role of zonulin-mediated gut permeability in the pathogenesis of some chronic inflammatory diseases. F1000Research, 9.

Fossati, P. and Prencipe, L. **(1982)**. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clinical chemistry, 28: 2077-2080.

Fraile, J. M., Palliyil, S., Barelle, C., Porter, A. J., and Kovaleva, M. **(2021)**. Non-alcoholic steatohepatitis (NASH)–A review of a crowded clinical landscape, driven by a complex disease. Drug design, development and therapy, 3997-4009.

Francula-Zaninovic, S., and Nola, I. A. **(2018**). Management of measurable variable cardiovascular disease'risk factors. Current cardiology reviews, 14(3), 153-163.

Friedewald, W. T., Levy, R. I., and Fredrickson, D. S. **(1972).** Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clinical chemistry, 18(6), 499-502.

Galuppo, B., Agazzi, C., Pierpont, B., Chick, J., Li, Z., Caprio, S., *et al*. **(2022)**. Growth differentiation factor 15 (GDF15) is associated with non-alcoholic fatty liver disease (NAFLD) in youth with overweight or obesity. Nutrition & Diabetes, 12(1), 9.

Ghosh, S. S., Wang, J., Yannie, P. J., Cooper, R. C., Sandhu, Y. K., Kakiyama, G., *et al*. **(2021)**. Over-expression of intestinal alkaline phosphatase attenuates atherosclerosis. Circulation research, 128(11), 1646-1659.

Gómez-Álvarez, E., Verdejo, J., Ocampo, S., Ponte-Negretti, C. I., Ruíz, E., and Ríos, M. M. **(2020)**. The CNIC-polypill improves atherogenic dyslipidemia markers in patients at high risk or with cardiovascular disease: results from a real-world setting in Mexico. *IJC Heart & Vasculature*, *29*, 100545.

Gu, J., Liu, S., Du, S., Zhang, Q., Xiao, J., Dong, Q., *et al*. **(2019)**. Diagnostic value of MRI-PDFF for hepatic steatosis in patients with non-alcoholic fatty liver disease: a meta-analysis. European radiology, 29, 3564-3573.

Habte, M. L., Melka, D. S., Degef, M., Menon, M. K. C., Yifter, H., and Feyisa, T. O. **(2020)**. Comparison of lipid profile, liver enzymes, creatine kinase, and lactate dehydrogenase among type II diabetes mellitus patients on statin therapy. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy, 13, 763.

Haldar, D., Kern, B., Hodson, J., Armstrong, M. J., Adam, R., Berlakovich, G., *et al*. **(2019)**. Outcomes of liver transplantation for non-alcoholic steatohepatitis: A European Liver Transplant Registry study. Journal of hepatology, 71(2), 313-322.

Han, Y. H., Onufer, E. J., Huang, L. H., Sprung, R. W., Davidson, W. S., Czepielewski, R. S., *et al*. **(2021)**. Enterically derived high-density lipoprotein restrains liver injury through the portal vein. Science, 373(6553), eabe6729.

Hansson, G. K., and Hermansson, A. **(2011)**. The immune system in atherosclerosis. Nature Immunologyy, 12(3), 204-212.

Hay, S. I., Abajobir, A. A., Abate, K. H., Abbafati, C., Abbas, K. M., Abd-Allah, F., *et al*. **(2017)**. Global, regional, and national disability-adjusted life-years (DALYs) for 333 diseases and injuries and healthy life expectancy (HALE) for 1 95 countries and territories, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet*, *390*(10100), 1260-1344.

He, X., Su, J., Ma, X., Lu, W., Zhu, W., Wang, Y., *et al*. **(2020)**. The association between serum growth differentiation factor 15 levels and lower extremity atherosclerotic disease is independent of body mass index in type 2 diabetes. *Cardiovascular Diabetology*, *19*, 1-8.

Helvaci, M. R., Tonyali, O., Abyad, A., and Pocock, L. **(2019**). The safest value of plasma triglycerides. World Family Med, 17(7), 22-27.

Hong, S., Zhou, Y., Shang, J., Xiao, C., and Sun, J. **(2020)**. Opportunities and challenges of deep learning methods for electrocardiogram data: A systematic review. Computers in biology and medicine, 122, 103801.

Huang, H., Chen, Z., Li, Y., Gong, K., Xiao, L., Fu, H., *et al*. **(2021)**. GDF-15 Suppresses Atherosclerosis by Inhibiting oxLDL-Induced Lipid Accumulation and Inflammation in Macrophages. Evidence-Based Complementary and Alternative Medicine, 2021.

Hwang, Y. C., Jee, J. H., Jeong, I. K., Ahn, K. J., Chung, H. Y., and Lee, M. K. **(2012)**. Circulating osteocalcin level is not associated with incident type 2 diabetes in middle-aged male subjects: mean 8.4-year retrospective follow-up study. Diabetes care, *35*(9), 1919-1924.

Irimia, J. M., Meyer, C. M., Segvich, D. M., Surendran, S., DePaoli-Roach, A. A., Morral, N., *et al*. **(2017)**. Lack of liver glycogen causes hepatic insulin resistance and steatosis in mice. Journal of Biological Chemistry, 292(25), 10455-10464.

Ismaiel, A., and Dumitraşcu, D. L. **(2019)**. Cardiovascular risk in fatty liver disease: the liver-heart axis—literature review. Frontiers in Medicine, 6, 202., D. L. (2019). Cardiovascular risk in fatty liver disease: the liver-heart axis—literature review. Frontiers in Medicine, 6, 202.

Jabbour, R. J., and Curzen, N. **(2023)**. National Institute for Health and Care Excellence guidelines on myocardial revascularisation. Heart.

Kamishima, K., Ogawa, H., Jujo, K., Yamaguchi, J., and Hagiwara, N. **(2019).** Relationships between blood pressure lowering therapy and cardiovascular events in hypertensive patients with coronary artery disease and type 2 diabetes mellitus: the HIJ-CREATE sub-study. Diabetes research and clinical practice, 149, 69-77.

Katz, D., and Gavin, M. C. **(2019)**. Stable Ischemic Heart Disease. Annals of Internal Medicine, 171(3), ITC17-ITC32.

Khan, M. U., Aziz, S., Naqvi, S. Z. H., and Rehman, A. **(2020, March)**. Classification of coronary artery diseases using electrocardiogram signals. In 2020 International Conference on Emerging Trends in Smart Technologies (ICETST) (pp. 1-5). IEEE.

Khatana, C., Saini, N. K., Chakrabarti, S., Saini, V., Sharma, A., Saini, R. V., *et al*. **(2020)**. Mechanistic insights into the oxidized low-density lipoprotein-induced atherosclerosis. Oxidative medicine and cellular longevity, 2020.

Knuuti, J., and Revenco, V. **(2020)**. 2019 ESC Guidelines for the diagnosis and management of chronic coronary syndromes. European heart journal, 41(5), 407-477.

Koliaki, C., Liatis, S., and Kokkinos, A. **(2019)**. Obesity and cardiovascular disease: revisiting an old relationship. Metabolism, 92, 98-107.

Kunutsor, S. K., Apekey, T. A., and Laukkanen, J. A. **(2015)**. Association of serum total osteocalcin with type 2 diabetes and intermediate metabolic phenotypes: systematic review and meta-analysis of observational evidence. European journal of epidemiology, *30*, 599-614.

Lässiger-Herfurth, A., Pontarollo, G., Grill, A., and Reinhardt, C. **(2019)**. The gut microbiota in cardiovascular disease and arterial thrombosis. Microorganisms, 7(12), 691.

Leech, B., McIntyre, E., Steel, A., and Sibbritt, D. **(2019)**. Risk factors associated with intestinal permeability in an adult population: A systematic review. International Journal of Clinical Practice, 73(10), e13385.

Legese, N., and Tadiwos, Y. **(2020)**. Epidemiology of hypertension in Ethiopia: a systematic review. Integrated blood pressure control, 135-143.

Li, Z., Xue, J., Chen, P., Chen, L., Yan, S., and Liu, L. **(2014)**. Prevalence of nonalcoholic fatty liver disease in mainland of China: A meta-analysis of published studies. Journal of gastroenterology and hepatology, 29(1), 42-51.

Lin, X., Brennan-Speranza, T. C., Levinger, I., and Yeap, B. B. **(2018)**. Undercarboxylated osteocalcin: experimental and human evidence for a role in glucose homeostasis and muscle regulation of insulin sensitivity. Nutrients, 10(7), 847.

Lin, X., Onda, D. A., Yang, C. H., Lewis, J. R., Levinger, I., and Loh, K. **(2020)**. Roles of bone-derived hormones in type 2 diabetes and cardiovascular pathophysiology. Molecular metabolism, 40, 101040.

Lin, X., Onda, D. A., Yang, C. H., Lewis, J. R., Levinger, I., and Loh, K. **(2020)**. Roles of bone-derived hormones in type 2 diabetes and cardiovascular pathophysiology. Molecular metabolism, 40, 101040.

Liu, H., and Lu, H. Y. **(2014).** Nonalcoholic fatty liver disease and cardiovascular disease. World journal of gastroenterology: WJG, 20(26), 8407.

Liu, M., Jiang, Y., Wedow, R., Li, Y., Brazel, D. M., Chen, F., *et al*. **(2019)**. Association studies of up to 1.2 million individuals yield new insights into the genetic etiology of tobacco and alcohol use. Nature Genetics 51(2), 237-244.

Lloyd-Jones, D. M., and Levy, D. **(2012)**. Epidemiology of hypertension. In Hypertension: A Companion to Braunwald's Heart Disease: Second Edition (pp. 1-11). Elsevier Inc.

Lockhart, S. M., Saudek, V., and O'Rahilly, S. **(2020)**. GDF15: a hormone conveying somatic distress to the brain. Endocrine reviews, 41(4), bnaa007.

Loffredo, L., Ivanov, V., Ciobanu, N., Deseatnicova, E., Gutu, E., Mudrea, L., *et al*. **(2020)**. Is there an association between atherosclerotic burden, oxidative stress, and gut-derived lipopolysaccharides?.

Lonardo, A., Nascimbeni, F., Ballestri, S., Fairweather, D., Win, S., Than, T. A., *et al*. **(2019).** Sex differences in nonalcoholic fatty liver disease: state of the art and identification of research gaps. Hepatology, 70(4), 1457-1469.

Loomba, R., and Adams, L. A. **(2019)**. The 20% rule of NASH progression: the natural history of advanced fibrosis and cirrhosis caused by NASH. Hepatology (Baltimore, Md.), 70(6), 1885.

Loomba, R., Friedman, S. L., and Shulman, G. I. **(2021)**. Mechanisms and disease consequences of nonalcoholic fatty liver disease. Cell, 184(10), 2537-2564.

Lopez, E. O., Ballard, B. D., and Jan, A. **(2022**). Cardiovascular disease. In StatPearls [Internet]. StatPearls Publishing.

Luan, H. H., Wang, A., Hilliard, B. K., Carvalho, F., Rosen, C. E., Ahasic, A. M., *et al*. **(2019)**. GDF15 is an inflammation-induced central mediator of tissue tolerance. Cell, 178(5), 1231-1244.

Luo, Y. Q., Ma, X. J., Hao, Y. P., Pan, X. P., Xu, Y. T., Xiong, Q., ... & Jia, W. P. **(2015)**. Inverse relationship between serum osteocalcin levels and nonalcoholic fatty liver disease in postmenopausal Chinese women with normal blood glucose levels. Acta

Magni, P., Macchi, C., Sirtori, C. R., and Corsi Romanelli, M. M. **(2016)**. Osteocalcin as a potential risk biomarker for cardiovascular and metabolic diseases. Clinical Chemistry and Laboratory Medicine (CCLM), 54(10), 1579-1587.

Malekmohammad, K., Bezsonov, E. E., and Rafieian-Kopaei, M. **(2021)**. Role of lipid accumulation and inflammation in atherosclerosis: focus on molecular and cellular mechanisms. Frontiers in Cardiovascular Medicine, 8, 707529.

Malekmohammad, K., Sewell, R. D., and Rafieian-Kopaei, M. **(2019).** Antioxidants and atherosclerosis: mechanistic aspects. Biomolecules, 9(8), 301.

Mansour-Ghanaei, R., Mansour-Ghanaei, F., Naghipour, M., and Joukar, F. **(2019)**. Biochemical markers and lipid profile in nonalcoholic fatty liver disease patients in the PERSIAN Guilan cohort study (PGCS), Iran. Journal of Family Medicine and Primary Care, *8*(3), 923.

Martina, A., Felis, G. E., Corradi, M., Maffeis, C., Torriani, S., and Venema, K. **(2019)**. Effects of functional pasta ingredients on different gut microbiota as revealed by TIM-2 in vitro model of the proximal colon. Beneficial microbes, 10(3), 301-313.

Martín-Domínguez, V., González-Casas, R., Mendoza-Jiménez-Ridruejo, J., García-Buey, L., and Moreno-Otero, R. **(2013)**. Pathogenesis, diagnosis and treatment of non-alcoholic fatty liver disease. Rev Esp Enferm Dig, 105(7), 409-20.

Marušić, M., Paić, M., Knobloch, M., and Liberati Pršo, A. M. **(2021)**. NAFLD, insulin resistance, and diabetes mellitus type 2. Canadian Journal of Gastroenterology and Hepatology, 2021.

Massera, D., Biggs, M. L., Walker, M. D., Mukamal, K. J., Ix, J. H., Djousse, L., *et al*. **(2018)**. Biochemical markers of bone turnover and risk of incident diabetes in older women: the cardiovascular health study. Diabetes Care, 41(9), 1901-1908.

Mendoza, D., Newman, R. C., Albala, D., Cohen, M. S., Tewari, A., Lingeman, J., *et al*. **(1996).** Laparoscopic complications in markedly obese urologic patients (a multi-institutional review). Urology, 48(4), 562-567.

Shahjehan, R. D., and Bhutta, B. S. **(2021).** Coronary artery disease. In StatPearls [Internet]. StatPearls Publishing.

Targher, G., Tilg, H., and Byrne, C. D. (2021). Non-alcoholic fatty liver disease: a multisystem disease requiring a multidisciplinary and holistic approach. The lancet Gastroenterology & hepatology, 6(7), 578-588.

Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L., and Wymer, M. (2016). Global epidemiology of nonalcoholic fatty liver disease—meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology, 64(1), 73-84.

Wijarnpreecha, K., Aby, E. S., Ahmed, A., and Kim, D. (2021). Evaluation and management of extrahepatic manifestations of nonalcoholic fatty liver disease. Clinical and molecular hepatology, 27(2), 221.

Mirmiran, P., Bahadoran, Z., Gaeini, Z., Moslehi, N., and Azizi, F. **(2019)**. Effects of Ramadan intermittent fasting on lipid and lipoprotein parameters: An updated meta-analysis. Nutrition, Metabolism and Cardiovascular Diseases, 29(9), 906-915.

Mitchell, G. F., and Powell, J. T. **(2020**). Arteriosclerosis: a primer for "In Focus" reviews on arterial stiffness. Arteriosclerosis, thrombosis, and vascular biology, 40(5), 1025-1027.

Mizokami, A., Kawakubo-Yasukochi, T., and Hirata, M. **(2017)**. Osteocalcin and its endocrine functions. Biochemical pharmacology, 132, 1-8.

Moolla, A., de Boer, J., Pavlov, D., Amin, A., Taylor, A., Gilligan, L., *et al*. **(2020)**. Accurate non-invasive diagnosis and staging of non-alcoholic fatty liver disease using the urinary steroid metabolome. Alimentary pharmacology & therappy

Moran, A. E., Forouzanfar, M. H., Roth, G. A., Mensah, G. A., Ezzati, M., Murray, C. J., *et al*. **(2014)**. Temporal trends in ischemic heart disease mortality in 21 world regions, 1980 to 2010: the Global Burden of Disease 2010 study. Circulation, 129(14), 1483-1492.

Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., Cushman, M., *et al*. **(2016)**. Heart disease and stroke statistics—2016 update: a report from the American Heart Association. circulation, 133(4), e38-e360.

Mundi, M. S., Velapati, S., Patel, J., Kellogg, T. A., Abu Dayyeh, B. K., and Hurt, R. T. **(2020)**. Evolution of NAFLD and its management. Nutrition in Clinical Practice, 35(1), 72-84.

Musunuru, K. **(2021).** Treating coronary artery disease: beyond statins, ezetimibe, and PCSK9 inhibition. Annual Review of Medicine, 72, 447-458.

National Center for Biotechnology Information **(2023)**. PubChem Compound Summary for CID 102601936, Osteocalcin(7-19)(human). Retrieved September 23, 2023.

Nicoletti, A., Ponziani, F. R., Biolato, M., Valenza, V., Marrone, G., Sganga, G., *et al*. **(2019)**. Intestinal permeability in the pathogenesis of liver

damage: From non-alcoholic fatty liver disease to liver transplantation. World journal of *Gastroenterology*, *25*(33), 4814.

Niedzielski, M., Broncel, M., Gorzelak-Pabiś, P., and Woźniak, E. **(2020)**. New possible pharmacological targets for statins and ezetimibe. Biomedicine & Pharmacotherapy, 129, 110388.

Norris C.M., Yip C.Y.Y., and Nerenberg K.A. **(2020)**. "State of the science in women's cardiovascular disease: a Canadian perspective on the influence of sex and gender". J Am Heart Assoc 2020; e015634., 9:4.

Olamoyegun, M. A., Oluyombo, R., and Asaolu, S. O. **(2016)**. Evaluation of dyslipidemia, lipid ratios, and atherogenic index as cardiovascular risk factors among semi-urban dwellers in Nigeria. Annals of African medicine, 15(4), 194.

Olivieri, F., Maguolo, A., Corradi, M., Zusi, C., Huber, V., Fornari, E., *et al*. **(2022)**. Serum zonulin as an index of glucose dysregulation in children and adolescents with overweight and obesity. Pediatric Obesity, 17(10), e12946.

Oosterwerff, M. M., van Schoor, N. M., Lips, P., and Eekhoff, E. M. **(2013)**. Osteocalcin as a predictor of the metabolic syndrome in older persons: a population-based study. Clinical Endocrinology, 78(2), 242-247.

Pagidipati, N. J., and Gaziano, T. A. **(2013)**. Estimating deaths from cardiovascular disease: a review of global methodologies of mortality measurement. Circulation, 127(6), 749-756.

Petersen, M. C., and Shulman, G. I. **(2018)**. Mechanisms of insulin action and insulin resistance. Physiological reviews, 98(4), 2133-2223.

Portillo-Sanchez, P., Bril, F., Maximos, M., Lomonaco, R., Biernacki, D., Orsak, B., *et al*. **(2015)**. High prevalence of nonalcoholic fatty liver disease

in patients with type 2 diabetes mellitus and normal plasma aminotransferase levels. *The journal of clinical endocrinology & metabolism*, *100*(6), 2231-2238.

Puchner, S. B., Lu, M. T., Mayrhofer, T., Liu, T., Pursnani, A., Ghoshhajra, B. B., *et al*. **(2015).** High-risk coronary plaque at coronary CT angiography is associated with nonalcoholic fatty liver disease, independent of coronary plaque and stenosis burden: results from the ROMICAT II trial. Radiology, 274(3), 693-701.

Rahman, M. T., Ghosh, C., Hossain, M., Linfield, D., Rezaee, F., Janigro, D., *et al*. **(2018)**. IFN-γ, IL-17A, or zonulin rapidly increase the permeability of the blood-brain and small intestinal epithelial barriers: Relevance for neuro-inflammatory diseases. *Biochemical and biophysical research communications*, *507*(1-4), 274-279.

Reynolds, H. R., Shaw, L. J., Min, J. K., Spertus, J. A., Chaitman, B. R., Berman, D. S., *et al*. **(2020)**. Association of sex with severity of coronary artery disease, ischemia, and symptom burden in patients with moderate or severe ischemia: secondary analysis of the ISCHEMIA randomized clinical trial. JAMA cardiology, 5(7), 773-786.

Qammar, N., Zain, M., and Javeed, H. M. R. **(2020)**. Interpretation of Coronary Artery Disease through Environmental/Genetic Risk Factors and Contributing Genes: A Comprehensive Review. Acta Psychopathol, 12(6), 38.

Rives, C., Fougerat, A., Ellero-Simatos, S., Loiseau, N., Guillou, H., Gamet-Payrastre, L., *et al*. **(2020)**. Oxidative stress in NAFLD: Role of nutrients and food contaminants. Biomolecules, 10(12), 1702.

Rehman, S., Khan, A., and Rehman, A. **(2021).** Physiology, coronary circulation. In StatPearls [Internet]. StatPearls Publishing.

Rodgers, J. L., Jones, J., Bolleddu, S. I., Vanthenapalli, S., Rodgers, L. E., Shah, K., *et al*. **(2019)**. Cardiovascular risks associated with gender and aging. Journal of cardiovascular development and disease, 6(2), 19.

Rosato, V., Masarone, M., Dallio, M., Federico, A., Aglitti, A., and Persico, M. **(2019)**. NAFLD and extra-hepatic c]]omorbidities: current evidence on a multi-organ metabolic syndrome. International journal of environmental research and public health, 16(18), 3415.

Rossi, M., Battafarano, G., Pepe, J., Minisola, S., and Del Fattore, A. **(2019)**. The endocrine function of osteocalcin regulated by bone resorption: A lesson from reduced and increased bone mass diseases. International journal of molecular sciences, 20(18), 4502.

Rosso, C., Caviglia, G. P., Armandi, A., Ribaldone, D. G., and Bugianesi, E. **(2021)**. Association between gut permeability and insulin resistance: Any role for zonulin in patients with non-alcoholic fatty liver disease?. *Clinics and Research in Hepatology and Gastroenterology*, *45*(2), 101611-101613.

Roth, G. A., Abate, D., Abate, K. H., Abay, S. M., Abbafati, C., Abbasi, N., *et al*. **(2018).** Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. The Lancet, 392(10159), 1736-1788.

Roth, G. A., Johnson, C., Abajobir, A., Abd-Allah, F., Abera, S. F., Abyu, G., *et al*. **(2017)**. Global, regional, and national burden of cardiovascular

diseases for 10 causes, 1990 to 2015. Journal of the American college of cardiology, 70(1), 1-25.

Said, M. A., van de Vegte, Y. J., Zafar, M. M., van der Ende, M. Y., Raja, G. K., Verweij, N., *et al*. **(2019)**. Contributions of interactions between lifestyle and genetics on coronary artery disease risk. Current cardiology reports, 21, 1-8.

Salem, A. M., Davis, J., Gopalan, D., Rudd, J. H., Clarke, S. C., Schofield, P. M., *et al.* **(2023)**. Characteristics of conventional high-risk coronary plaques and a novel CT defined thin-cap fibroatheroma in patients undergoing CCTA with stable chest pain. Clinical imaging

Samuel, V. T., and Shulman, G. I. **(2018)**. Nonalcoholic fatty liver disease as a nexus of metabolic and hepatic diseases. Cell metabolism, 27(1), 22-41.

Santhoshakumari TMJ, and Radhika G, Kanagavalli P. **(2017)**. A study of anthropometric and lipid profile parameters in non-alcoholic fatty liver disease patients attending a tertiary care hospital at puducherry. *IOSR J Dent Med Sci (IOSR-JDMS)* 2017;16:33–7.

Schlittenhardt, D., Schober, A., Strelau, J., Bonaterra, G. A., Schmiedt, W., Unsicker, K., *et al*. **(2004)**. Involvement of growth differentiation factor-15/macrophage inhibitory cytokine-1 (GDF-15/MIC-1) in oxLDL-induced apoptosis of human macrophages in vitro and in arteriosclerotic lesions. *Cell and tissue research*, *318*, 325-333.

Schopfer, D. W., Ku, I. A., Regan, M., and Whooley, M. A. **(2014)**. Growth differentiation factor 15 and cardiovascular events in patients with stable ischemic heart disease (The Heart and Soul Study). American heart journal, 167(2), 186-192.

Seidu, S., Kunutsor, S. K., and Khunti, K. **(2019)**. Association of circulating osteocalcin with cardiovascular disease and intermediate cardiovascular phenotypes: systematic review and meta-analysis. Scandinavian Cardiovascular Journal, 53(6), 286-295.

Shahjehan, R. D., and Bhutta, B. S. **(2022)**. Coronary artery disease. In StatPearls [Internet]. StatPearls Publishing.

Shang, L., Feng, M., Zhou, X., and Tang, B. **(2019)**. Growth differentiation factor 15: a promising prognostic stratification biomarker in patients with acute coronary syndrome. International Journal of Cardiology, 297, 16.

Shao, C., Wang, J., Tian, J., and Tang, Y. D. **(2020)**. Coronary artery disease: from mechanism to clinical practice. Coronary Artery Disease: Therapeutics and Drug Discovery, 1-36.

Sharma, M., and Acharya, U. R. **(2019**). A new method to identify coronary artery disease with ECG signals and time-Frequency concentrated antisymmetric biorthogonal wavelet filter bank. Pattern Recognition Letters, 125, 235-240.

Sharma, P., and Arora, A. **(2020)**. Clinical presentation of alcoholic liver disease and non-alcoholic fatty liver disease: spectrum and diagnosis. Translational gastroenterology and hepatology, 5.

Shu, H., Pei, Y., Chen, K., and Lu, J. **(2016)**. Significant inverse association between serum osteocalcin and incident type 2 diabetes in a middle-aged cohort. Diabetes/Metabolism Research and Reviews, 32(8), 867-874.

Sicari R, Cortigiani L. The clinical use of stress echocardiography in ischemic heart disease. Cardiovasc Ultrasound. (**2017)** Mar 21; 28327159, 15(1):7. doi: 10.1186/s12947-017-0099-2. PMID:; PMC5361820., PMCID:

Singh, P., Silvester, J., Chen, X., Xu, H., Sawhney, V., Rangan, V., *et al*. **(2019)**. Serum zonulin is elevated in IBS and correlates with stool frequency in IBS-D. United European gastroenterology journal, 7(5), 709-715.

Somani, S., Russak, A. J., Richter, F., Zhao, S., Vaid, A., Chaudhry, F., *et al*. **(2021)**. Deep learning and the electrocardiogram: review of the current state-of-the-art. EP Europace, 23(8), 1179-1191.

Spiers, J., Brindley, J. H., Li, W., and Alazawi, W. **(2022)**. What's new in non-alcoholic fatty liver disease?. Frontline Gastroenterology, 13(e1), e102-e108.

Stefan, N., Häring, H. U., and Cusi, K. **(2019)**. Non-alcoholic fatty liver disease: causes, diagnosis, cardiometabolic consequences, and treatment strategies. The lancet Diabetes & endocrinology, 7(4), 313-324.

Suchitra, M. R., Vadivel, S., Balasubramanian, M., and Parthasarathy, S. **(2018).** A study of awareness about" Nutrition in Hypertension" among college teachers in a semi urban town of South India. Asian Journal of Pharmaceutical Research and Health Care, 30-35.

Tacey, A., Hayes, A., Zulli, A., and Levinger, I. **(2021)**. Osteocalcin and vascular function: is there a cross-talk?. Molecular metabolism, 49, 101205.

Tacke, F., and Weiskirchen, R. **(2021)**. Non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH)-related liver fibrosis: mechanisms, treatment and prevention. Annals of Translational Medicine, 9(8).

Tajik, N., Frech, M., Schulz, O., Schälter, F., Lucas, S., Azizov, V., *et al*. **(2020)**. Targeting zonulin and intestinal epithelial barrier function to prevent onset of arthritis. Nature communications, 11(1), 1995.

Tang, D., Geng, F., Yu, C., and Zhang, R. **(2021**). Recent application of zebrafish models in atherosclerosis research. Frontiers in Cell and Developmental Biology, 9, 643697.

Temple, N. J. **(2018)**. Fat, sugar, whole grains and heart disease: 50 years of confusion. Nutrients, 10(1), 39.

Tilg, H., Zmora, N., Adolph, T. E., and Elinav, E. **(2020)**. The intestinal microbiota fuelling metabolic inflammation. Nature Reviews Immunology, 20(1), 40-54.

Ting, D. S., Peng, L., Varadarajan, A. V., Keane, P. A., Burlina, P. M., Chiang, M. F., *et al*. **(2019)**. Deep learning in ophthalmology: the technical and clinical considerations. Progress in retinal and eye research, 72, 100759.

Trentman, T. L., Avey, S. G., and Ramakrishna, H. **(2016)**. Current and emerging treatments for hypercholesterolemia: A focus on statins and proprotein convertase subtilisin/kexin Type 9 inhibitors for perioperative clinicians. Journal of anaesthesiology, clinical pharmacology, 32(4), 440.

Trinder, P. **(1969)**. Enzymatic methods for glucose determination. Ann Clin Biochem, 6: 24-26.

Trost, J., Ferraro, R. A., Sharma, G., Hays, A. G., Boden, W. E., Blumenthal, R. S., *et al*. **(2022)**. CCTA Should Be the New Diagnostic Gateway for Evaluating Intermediate-Risk Stable Angina Patients. JACC: Advances, 1(4), 1-4.

Ullah, M., Wahab, A., Khan, S. U., Zaman, U., ur Rehman, K., Hamayun, S., *et al*. **(2022)**. Stent as a Novel Technology for Coronary Artery Disease and their Clinical Manifestation. Current Problems in Cardiology, 101415.

Ullah, R., Rauf, N., Nabi, G., Ullah, H., Shen, Y., Zhou, Y. D., *et al*. **(2019)**. Role of nutrition in the pathogenesis and prevention of non-alcoholic fatty

liver disease: recent updates. International journal of biological sciences, 15(2), 265.

Ulusoy, Ş. **(2013)**. Assessment of cardiovascular risk in hypertensive patients: a comparison of commonly used risk scoring programs. Kidney international supplements, 3(4), 340-342.

Urano, T., Shiraki, M., Kuroda, T., Tanaka, S., Urano, F., Uenishi, K., *et al*. **(2018)**. Low serum osteocalcin concentration is associated with incident type 2 diabetes mellitus in Japanese women. Journal of Bone and Mineral Metabolism, 36, 470-477.

Wang, J., He, W., Tsai, P. J., Chen, P. H., Ye, M., Guo, J., *et al*. **(2020)**. Mutual interaction between endoplasmic reticulum and mitochondria in nonalcoholic fatty liver disease. Lipids in Health and Disease, 19, 1-19.

Wang, J., Wei, L., Yang, X., and Zhong, J. **(2019a)**. Roles of growth differentiation factor 15 in atherosclerosis and coronary artery disease. Journal of the American Heart Association, 8(17), e012826.

Wang, Y., Zhen, C., Wang, R., and Wang, G. **(2019b)**. Growth-differentiation factor-15 predicts adverse cardiac events in patients with acute coronary syndrome: A meta-analysis. The American Journal of Emergency Medicine, 37(7), 1346-1352.

Weiskirchen, R., Weiskirchen, S., and Tacke, F. **(2019)**. Organ and tissue fibrosis: Molecular signals, cellular mechanisms and translational implications. Molecular aspects of medicine, 65, 2-15.

WHO. Iraq Health Profile. Cairo; **2017**. (Accessed March 20, 2019, at http://apps.who.int/ bookorders).

Wong, N. D., Budoff, M. J., Ferdinand, K., Graham, I. M., Michos, E. D., Reddy, T., *et al*. **(2022**). Atherosclerotic cardiovascular disease risk

assessment: An American Society for Preventive Cardiology clinical practice statement. American Journal of preventive, 10, 100335.

Wood Heickman, L. K., DeBoer, M. D., and Fasano, A. **(2020)**. Zonulin as a potential putative biomarker of risk for shared type 1 diabetes and celiac disease autoimmunity. Diabetes/metabolism research and reviews, 36(5), e3309.

Woodward, M. **(2019)**. Cardiovascular disease and the female disadvantage. International journal of environmental research and public health, 16(7), 1165.

Xia, M., Rong, S., Zhu, X., Yan, H., Chang, X., Sun, X., *et al*. **(2021)**. Osteocalcin and non-alcoholic fatty liver disease: lessons from two population-based cohorts and animal models. Journal of Bone and Mineral Research, 36(4), 712-728.

Yakut, K., Öcal, D. F., Öztürk, F. H., Öztürk, M., Oğuz, Y., Sınacı, S., *et al*. **(2021)**. Is GDF-15 level associated with gestational diabetes mellitus and adverse perinatal outcomes?. Taiwanese Journal of Obstetrics and Gynecology, 60(2), 221-224.

Yamazaki, H., Tsuboya, T., Tsuji, K., Dohke, M., and Maguchi, H. **(2015)**. Independent association between improvement of nonalcoholic fatty liver disease and reduced incidence of type 2 diabetes. Diabetes care, 38(9), 1673-1679.

Yan, Y., Song, D., Wu, J., and Wang, J. **(2020)**. Long non-coding RNAs link oxidized low-density lipoprotein with the inflammatory response of macrophages in atherogenesis. Frontiers in Immunology, 11, 24.

Yang, H. J., Shim, S. G., Ma, B. O., and Kwak, J. Y. **(2016)**. Association of nonalcoholic fatty liver disease with bone mineral density and serum

osteocalcin levels in Korean men. European journal of gastroenterology & hepatology, 28(3), 338.

Yildirim, O., Baloglu, U. B., Tan, R. S., Ciaccio, E. J., and Acharya, U. R. **(2019**). A new approach for arrhythmia classification using deep coded features and LSTM networks. Computer methods and programs in biomedicine, 176, 121-133.

Younossi, Z., Tacke, F., Arrese, M., Chander Sharma, B., Mostafa, I., Bugianesi, E., *et al*. **(2019)**. Global perspectives on nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Hepatology, 69(6), 2672-2682.

Żak-Gołąb, A., Kocełak, P., Aptekorz, M., Zientara, M., Juszczyk, Ł., Martirosian, G., *et al*. **(2013)**. Gut microbiota, microinflammation, metabolic profile, and zonulin concentration in obese and normal weight subjects. International journal of endocrinology, 2013.

Zhang, L., She, Z. G., Li, H., and Zhang, X. J. **(2020)**. Non-alcoholic fatty liver disease: a metabolic burden promoting atherosclerosis. Clinical Science, 134(13), 1775-1799.

Zhang, X., and Xu, H. **(2023)**. Prediction of Angina Pectoris Events in Middle-Aged and Elderly People Using RR Interval Time Series in the Resting State: A Cohort Study Based on SHHS. International Journal of Computational Intelligence Systems, 16(1), 10.

Zhang, Y., Xu, J., Wang, X., Ren, X., and Liu, Y. **(2019)**. Changes of intestinal bacterial microbiota in coronary heart disease complicated with nonalcoholic fatty liver disease. BMC genomics, 20(1), 1-12.

Zwakenberg, S. R., Gundberg, C. M., Spijkerman, A. M., van der A, D. L., Van Der Schouw, Y. T., and Beulens, J. W. **(2015)**. Osteocalcin is not

associated with the risk of type 2 diabetes: findings from the EPIC-NL study. PLoS One, 10(9), e0138693.

Appendixes

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Department of Chemistry and Biochemistry

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Dr. Ahmed Qasim Al-Haidari – Kerbala Heart Center

((Experimental Data))

Association Between Growth Differentiation Factor-15 and Osteocalcin in Cardiovascular Diseases with/without Non-Alcoholic Fatty Liver Disease of 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:
Sex:
Family History:

Diabetes Mellitus	Yes	No
Hypertension	Yes	No
Smoking	Yes	No
Sedentary Life Style	Yes	No
Ultra Sound:		
Growth Differentiation Factor-15:		
Osteocalcin:		
Zonulin:		
Lipid Profile		
Total Cholesterol:		
HDL-Cholesterol:		
Triglycerides:		
LDL-Cholesterol:		
VLDL-Cholesterol:		
Liver Function Tests		
Albumin:	ALT:	
TSB:	AST:	
ALP:		
Height:		
Weight:		
Waist:		
BMI:		
Medication:		

الخلاصـــة

يعد مرض الشريان التاجي (CAD) من امراض القلب والأوعية الدموية الأكثر شيوعًا (CVD)، ، وهو أحد الأسباب الرئيسية للوفاة في جميع أنحاء العالم وقد زاد مؤخرًا انتشاره في البلدان المنخفضة والمتوسطة الدخل. يحدث بسبب تصلب الشرايين، مما يؤدي إلى انخفاض تدفق الدم إلى عضلة القلب البعيدة عن طريق تضييق تجويف الشرايين.

مرض الكبد الدهني غير الكحولي NAFLDهو مرض الكبد الأكثر شيوعًا وهو المظهر الكبدي لمتلازمة التمثيل الغذائي. يرتبط NAFLD بكل من المراضة المرتبطة بالكبد وخارج الكبد. من بين الامراض خارج الكبد، أمراض القلب والأوعية الدموية هي السبب الرئيسي للوفاة في مرضى NAFLD . تظهر الدراسات الوبائية وجود علاقة بين CAD و NAFLD.

هدفت هذه الدراسة إلى دراسة دور العلامات البيوكيميائية؛ عامل تمايز النمو 15، الأوستيوكالسين، والزونولين كعلامات تشخيصية مبكرة لـ CAD وNAFLD.

تم إجراء منهج دراسة المرضى-الاصحاء على 180 شخصًا تتراوح أعمارهم بين (40-70) عامًا، وشملت 135 مريضًا و45 شخصًا يتمتعون بصحة جيدة في محافظة كربلاء. تم جمع العينات طوال الفترة من أكتوبر 2022 إلى يناير 2023. تم تقسيم المرضى إلى ثلاث مجموعات، (45) مرضى شريان تاجي مع كبد دهني غير كحولي، (45) مرضى شريان تاجي، و (45) مرضى كبد دهني غير كحولي. تم إخضاع مرضى CAD وNAFLD للفحص الطبي بحثًا عن العلامات والأعراض من قبل طبيب متخصص. تم قياس مستويات العلامات الحيوية في الدم للمعايير التالية: تم قياس مستويات GDF-15، الأوستيوكالسين، والزونولين باستخدام تقنية ELISA .تم إجراء قياس مستوى الدهون ومستويات وظائف الكبد في مصل الدم باستخدام كيمياء التحليل الذاتي. تم تقييم العلاقة بين العلامات البيوكيميائية وشدة المرض. تم تقييم كفاءة القيمة التنبؤية باستخدام منحنى ROC.

أشارت النتائج إلى أن مستويات GDF-15 أظهرت مستويات متزايدة بشكل ملحوظ في CAD مع حالات NAFLD مقارنة بحالات CAD و NAFLD بشكل منفصل. وكانت المستويات (273.18 نانوجرام/لتر), (223.78 نانوغرام/لتر) ,و(237.34 نانوغرام/لتر) على التوالي. كما أظهر الزونولين مستويات أعلى في مجموعة CAD+NAFLD، حيث بلغت (94.80 نانوجرام/مل), (73.42 نانوجرام/مل) ,و (63.42 نانوجرام/مل) على التوالي. من ناحية أخرى، أظهر المرضى انخفاضًا ملحوظًا في مستويات الأوستيوكالسين مقارنة بمجموعة الاصحاء. أوضحت النتائج أيضًا منحنى ROC وتحليل AUC للعلامات كمعلمات تشخيصية محتملة. أظهر مستوى الزونولين أداءً تشخيصيًا جيدًا لمرضى CAD+NAFLD المتوقعين مقارنةً

بمجموعة الاصحاء. كانت نقاط التشخيص المثالية للتنبؤ بـ CAD+NAFLD بواسطة) GDF-15الحساسية = 79%، النوعية = 91%) عند مستوى = 250.605، والأوستيوكالسين (الحساسية = 88%، النوعية = 60%) عند المستوى = 8.732 والزونولين. (الحساسية = 93%، النوعية = 83%) عند المستوى = 69.79.

الاستنتاج ، زيادة مستوى مصل GDF-15 في NAFLD وCAD يعمل كعلامة تنبؤ مبكر لمخاطر القلب والأوعية الدموية، والكبد الدهني غير الكحولي، ويبدو أنه مشارك مباشر في عملية تصلب الشرايين والتغيرات الأيضية. قد يعود انخفاض مستوى الأوستيوكالسين في مجموعات مرضى NAFLD وCAD إلى الدور المحتمل لتحسين وظيفة بطانة الأوعية الدموية وتقليل الآليات المرضية التي تعزز تطور تصلب الشرايين لدى المرضى الذين يعانون من NAFLD. غالبًا ما يكون هذا التأثير نتيجة لنتائج التمثيل الغذائي ومع ذلك، ما إذا كان هناك تفاعل مباشر بين الأوستيوكالسين والأوعية الدموية لم يتم توضيحه بالكامل بعد. كشفت النتائج الحالية أن زيادة مستويات الزونولين في المصل في مجموعات مرضى NAFLD ومرض الشريان التاجي تشير إلى الدور المحتمل في التسبب في حدوث NAFLD وتطوره.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء ـ كلية الطب
فرع الكيمياء والكيمياء الحياتية

الارتباط بين عامل تمايز النمو 15 ,الزونولين,و أوستيوكالسين في أمراض القلب والأوعية الدموية مع / بدون مرض الكبد الدهني غير الكحولي للمرضى العراقيين

رسالة ماجستير

مقدمة إلى مجلس كلية الطب/ فرع الكيمياء والكيمياء الحياتية / جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في (الكيمياء السريرية)

من قبل

علياء احمد جاسم

بكالوريوس تحليلات مرضية-كلية العلوم الطبية التطبيقية – جامعة كربلاء/ (2017 – 2018)

بإشراف كل من

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كلية العلوم – جامعة الكوفة

أيلول 2023 ميلادية

صفر 1445 هجرية