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Assessment of Serum Acid Ceramidase Level and Gene Polymorphism in Iraqi Population with Acute Myocardial Infarction

A Thesis

Submitted to the Council of the College of Medicine / University of Babylon in Partial Fulfillment of the Requirements for the Degree of Doctor Philosophy in Science of Clinical Biochemistry

By
Rajaa Neama Amien Ali
M.Sc. Pharmacy / Clinical Laboratory Sciences
University of Baghdad / College of Pharmacy / 2014

Supervised By

Professor Dr. Moaed E. Al-Gazally
Dept. of Chemistry and Biochemistry

Professor Dr. Oday Jasim Alsalihi
Dept. of Internal Medicine and Clinical Cardiology

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

(قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا ۖ إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ)

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CERTIFICATE

We certify that this thesis entitled " **Assessment of Serum Acid Ceramidase Level and Gene Polymorphism in Iraqi Population with Acute Myocardial Infarction**" was carried under our supervision at the College of Medicine, University of Babylon, as a partial fulfillment for the requirement of degree of Doctor of Philosophy in Clinical Biochemistry.

Professor
Dr. Moaed E. Al-gazally
Supervisor

Professor
Dr. Oday Jasim Alsalihi
Supervisor

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

Professor
Dr. Abdulsamie Hassan Alta'ee
Head of Biochemistry Department
College of Medicine / University of Babylon

Examining Committee Certification

Decision of Examination Committee

We, the examining committee, certify that we have read the thesis entitled (**Assessment of Serum Acid Ceramidase Level and Gene Polymorphism in Iraqi Population with Acute Myocardial Infarction**).

We have examined the student (**Rajaa Neama Amien Al-Atrakchey**) in its contents at Department of Chemistry and Biochemistry-College of Medicine / University of Babylon), and that in our opinion it is accepted as a thesis for Degree of Ph.D. in Clinical Biochemistry with **Excellent** estimation.

Signature
Prof. Dr. Maysaa Jalal Majeed
College of Medicine / University of Baghdad
(Chairman)

Signature
Prof. Dr. Thana Mohammed Juda
College of Medicine / University of Babylon
(Member)

Signature
Prof. Dr. Hassan salim Aljumaily
College of Medicine / University of Babylon
(Member)

Signature
Prof. Dr. Suhayr Aesa Hussein
College of Medicine / University of Babylon
(Member)

Signature
Assist.Prof.Dr.Khawla A bed Alhamza Shemran
College of Medicine / University of Babylon
(Member)

Signature
Prof. Dr. Moaed E. Al-gazally
College of Medicine / University of Babylon
(Member and Supervisor)

Signature
Prof. Dr. Oday Jasim Alsalihi
College of Medicine / University of Babylon
(Member and Supervisor)

Approved for the college committee of graduate studies

Signature
Prof. Dr. Qahtan Hadi Hussein
Dean of College of Medicine / University of Babylon

Dedication

To …

- *The soul of My Kind Mother…and … my Father …who inspired me and gave me the potential power for success..*
- *All my family members … who shared my struggle..*
- *My beloved country & hero Iraqi army..*
- *All my friends..*
- *Syndicate of pharmacy..*
- *All My professors and colleagues at the College of Law.*
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List of Abbreviations

Abbreviation	Meaning
AC, aCDase	Acid Ceramidase
ACC	American College of Cardiology
ACE	angiotensin converting enzyme
ACS	Acute coronary syndrome
AHA	American Heart Association
AHD	Acute heart disease
AJs	adherens junctions
AMI	acute myocardial infarction
APC	the adenomatous polyposis coli protein
ASAH1	Human Acid ceramidase
AV	atrioventricular
BC	Beta-Catenin
BCL9	B-cell lymphoma-9
BH4	tetrahydrobiopterin
bp	base pair
BMI	body mass index
BSA	Bovine serum albumin
Ca^{+2}	calcium ion
CAD	coronary artery disease
cGMP	cyclic guanosine monophosphate
CDC	Centers for Disease Control and Prevention
CHD	coronary heart disease
CRP	C-Reactive Protein
CTNNβ1	Human Beta-Catenin

CVD	cardiovascular disease
DASH	Dietary Approaches to Stop Hypertension
ddH2O	double-distilled water
DM	Diabetes Mellitus
ECG	electrocardiogram
ECs	endothelial cells
EDTA	Ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtration rate
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ER-alpha	estrogen receptor alpha
ESC	European Society of Cardiology
F-actin	filamentous actin
FAD	flavin adenine dinucleotide
FATP	fatty acid transport proteins
FD	Farber disease
FFAs	Free fatty acids
FMN	flavin mononucleotide
GMP	guanosine monophosphate
GRACE	Global Registry of Acute Coronary Events
GSK3	glycogen synthase kinase 3
GTP	guanylyl triphosphate
GWA	genome-wide association
GWAS	genome-wide association studies
HGP	Human Genome Project
HF	Heart failure
HRP	Horseradish Peroxidase

hs-CRP	high sensitivity C-Reactive Protein
IHD	ischemic heart disease
iNOS	inducible Nitric Oxide synthase
LDL	Low Density Lipoprotein
LV	Left ventricular
MI	Myocardial infarction
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
nCDase	Neutral ceramidase
NCEP	National Cholesterol Education Program
nNOS	neuronal Nitric Oxide synthase
NO	nitric oxide
NOS	Nitric Oxide synthase
NRG1	neuregulin-1
NSTEMI	non-ST elevation myocardial infarction
OD	optical density
PAD	peripheral arterial disease
PCI	Percutaneous coronary intervention
PCR	Polymerase Chain Reaction
PYGO	protein Pygopus
rcf	relative centrifugal force
RFLP	Restriction Fragment Length Polymorphism
ROC	Receiver operating characteristic curve
SCORE	Systematic Coronary Risk Evaluation
sGC	soluble guanylyl cyclase
SNPs	single nucleotide polymorphisms
SPT	serine palmitoyl transferase

SSCP	Single-strand conformation polymorphism
STEMI	ST-elevation myocardial infarction
Tcf	T-cell factors
TIMI	Thrombolysis in Myocardial Infarction
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization

Summary

Myocardial infarction (MI) is one of the most frequently encountered reasons for hospital admission and it is commonly seen in all populations worldwide.

The aim of this study was to evaluate the changes of some biochemical parameters as predictors of myocardial infarction and associated with genetic polymorphism.

A total number of 120 participants, 60 patients and 60 apparently healthy were included in this study. The study was carried out on patients suffering from acute MI admitted to Imam Al-Hussein medical city and Karbala center for cardiac diseases and surgery, in Karbala City.

Blood and serum samples were obtained from all participant to measure the following parameters: Human high sensitivity C- reactive protein (hs-CRP), Human acid ceramidase (AC), Human nitric oxide synthase (NOs) and Beta-Catenin (BC) as well as determination acid ceramidase genetic polymorphism.

The result indicated that the most cases of diabetic patients with heart disease were at the age ≥ 50 years for both sexes, it was recorded (35%) and (50%) for male and female had diabetes mellitus in ≥ 50 years related with gender, respectively.

The findings of this study revealed that the patients above 50 years were 72% and below 50 years were 28%.

According to the results of the study, 78.3 % of the patients were suffering from high blood pressure and MI.

There were 46 patients lived in urban regions and diagnosed with heart disease, while the remaining 14 patients lived in rural areas.

Serum Acid ceramidase concentration was significantly decreased ($P < 0.05$) in all patients with MI compared to control group, and Serum Hs-CRP concentration was highly significantly increased ($P < 0.05$) in all patients with MI compared to control group. Additionally, Serum Beta-Catenin (BC) concentration was highly significantly decreased ($P < 0.05$) in all patients with MI compared to control group. On the other hand, when compared the patients with MI, the serum Nitric oxide synthase (NOS3) concentration was significantly higher in patients ($P< 0.05$) from that of the control group.

The distribution of genotype groups of acid ceramidase polymorphism "rs7844023" in patients with MI and controls revealed that 3.3% controls had TT genotype in their chromosomes, 50% had TC, and 46.7% had wild CC genotype frequencies, compared to 10% patients with MI who had TT genotypes, 23.3% patients with TC genotypes, and 66.7% patients with wild CC genotype frequencies.

On the other hand the distribution of genotype groups of acid ceramidase polymorphism "rs2427746" among patients with MI and controls found 33.4% patients with MI had GG genotype, 28.3% patients had AG, and 38.3 % patients had wild AA genotype frequency, while 13.3% controls had GG genotype, 23.3% controls had AG, and 63.4% had wild AA genotype frequency.

In conclusion: Genetic variation (rs2427746) of acid ceramidase gene is associated with myocardial infarction, otherwise there was no significant association with (rs7844023), on the other hand, Acid ceramidase and Beta Catenin may be used as diagnostic parameters for MI.

Chapter One

Introduction

And

Literature Review

Chapter One

Introduction & Literature Review

1.1. Definition of Acute Myocardial Infarction

Acute coronary syndrome, also known as ACS, is a general concept for coronary artery disease, which affects millions of people each years. These potentially fatal disorders arise when a blockage causes the blood flow to the heart to abruptly slow down or cease altogether (Ralapanawa, & Sivakanesan, 2021). Individuals with acute cardiac disease are susceptible to heart attack or unstable angina and myocardial infarction. The most common symptoms are shortness of breath (dyspnea) pressure (angina) or chest pain and vertigo (Dhanya *et al*., 2019).

Heart is consider the main part of the circulatory system in the body. Myocardial infarction (MI) is one of the most frequently encountered reasons for hospital admission and it is commonly seen in all populations worldwide (Pinaire Jessica., *et al*. 2019). Myocardial infarction means interruption of blood supply to a part of the heart, and it is almost always due to the formation of occlusive thrombus at the site of rupture or erosion of an atheromatous plague in coronary artery causing heart cell to die and without treatment, the infarct related artery remains permanently occluded in 30% of patients (Bloomfield., *et al.* 2006).

When there is a discrepancy between the oxygen available and the amount needed by the heart's muscles, a condition known as "ischemia" develops. This heart condition is caused by narrowing of the coronary arteries. (Rezende, *et al*., 2019).

Ischemic heart disease is typically driven on by atherosclerosis or the hardening of the arteries (also known as coronary artery disease), both of

which reduce the amount of blood that is able to circulate to the heart (Agbor *et al*., 2018).

Theoretical speaking, infarction of the myocardial muscle can be detected based on certain pathological features like coagulation necrosis causing loss of muscle cells (myocytes). When such a pathological change occurs, it triggers inflammation which subsequently leads to fibrosis and healing with a scar (Frangogiannis, 2015). The clinical approach to a patient suspected to have an acute MI consists of detailed history taking, electrocardiogram (ECG) changes, cardiac biochemistry evidence and imaging (Kumar and Christopher, 2009).

The reliability of each of these modalities as a diagnostic tool is dependent on multiple factors, the most significant being the window period between the time of infarction and the time the patient seeks medical attention (Neri Margherita., *et al.* 2017). The current approach to patients presenting with typical or atypical features of myocardial ischemia or infarction starts with making the provisional diagnosis of the acute coronary syndrome. Then, depending on the changes seen on the twelve lead ECG, the acute coronary syndrome is classified into ST-elevation MI (STEMI) or non-ST elevation MI (NSTEMI) (Levine Glenn N., *et al.* 2016). In spite of major leaps in the diagnostic tools and treatment of MI, ST-segment elevation MI persists as a leading cause for ill health in both the developed and developing world (Foth Christopher and Steven Mountfort. 2019).

The prevalence of coronary disease and infarction is on the rise in the developing countries, with further worsening of adverse cardiovascular events due to disadvantages like inadequate primary prevention policies and limited availability of medical help (Gaziano Thomas., *et al.* 2010).

The meteorically advances in acute coronary care and resuscitation since the twentieth century has led to considerable decline in mortality

and morbidity rates from STEMI. In the early parts of the twentieth century, therapy was centered around passive observation and monitoring than active intervention. Significant advances have opened the gates to the current reperfusion therapy, which along with intensive hemodynamic monitoring has improved the standards of acute coronary care and emergency management. The approach to ST-elevation MI is increasingly leaning towards practice guidelines and evidence-based medicine (Choudhury., *et al.* 2016).

In daily practice, even though the ECG remains an important test in diagnosis and detection of progression of the disease, there still remains ample potential for its role as a prognostic marker. The fundamental advantage that stands to be gained by realizing this potential is the time it saves for immediate intervention. In addition, it can serves as an instant and cost-effective method for risk assessment instead of waiting for biochemical and angiography results (Gibler., *et al.* 2018).

1.2. Epidemiology

Heart is the vital organ in the cardiovascular system, which includes different types of blood vessels, the coronary arteries are consider the important vessels, which taken oxygenated blood around the body, including the heart. The blood flow to heart can decrease or stopped completely when the arteries blocked or narrowed due to plaque (Macon *et al*., 2015).

In Iraq, the epidemiological data on the incidence and prevalence of coronary artery disease (CAD) as evidence of awareness are limited due to the unavailability of evidence-based national guidelines for the management of cardiovascular disease and surveillance studies as compared to other Eastern Mediterranean countries (Traina., *et al.* 2017). In a recent study in 2014, cardiovascular disease mortality was estimated

to account for 33% in Iraq (Mohammad *et al*., 2015). A better understanding of the burden of cardiovascular disease and associated risk factors in this region and increasing the public knowledge and awareness of CAD symptoms and its risk factors are highly imperative to control and prevent this disease. (Amen., *et al.* 2020).

The coronary artery disease (CAD) has become the most prevalent serious global burden of morbidity and mortality in industrialized countries and is a rapidly growing problem in developed countries. According to the Third Report by the World Health Organization (WHO), cardiovascular disease (CVD) is the leading cause of death worldwide; the World Health Organization estimates that globally 17.3 million people die from CVD each year, cardiovascular mortality on worldwide scale will likely surpass that of every major disease group, including infection, cancer, and trauma (Nascimento., *et al.* 2019; World Health Organization. 2020). Similar to many high-income countries during the 20th century, low and middle income countries are seeing an alarming increase in the rates of CVD, and this change is accelerating that is responsible for 80% of global deaths. Atherosclerosis is considered the main cause of acute myocardial infarction (AMI), in which 70% of fatal events among patients with AMI are caused by occlusion from atherosclerotic plaques (Bhatt., *et al.* 2006).

Despite a continuing decline in total cardiovascular mortality in most developed countries, the mortality rate from cardiovascular disease remains extremely high. Ischemic heart disease is one of the leading causes of death around the world, and its prevalence is continuously expanding (Mensah *et al*., 2017).

There were 7.3 million deaths caused by heart disease occur over the world, increase the incidence of heart disease more likely burden up on low- and – middle income people, In developing countries related rapid

team over of socio-economic aspects and increase individual needs, will increase socio problem which make the life complex, and make the person more liable to expose to the coronary heart disease (Gaziano, *et al*, 2010).

Some studies show that the risk of IHD or stroke varies by country of origin, destination, and length of stay, and indicated that most immigrant groups to Western Europe had equal or higher risk of IHD and stroke than the other population. Many immigrants from Eastern Europe, the Middle East, and South Asia were at risk, in terms of length of stay, most immigrants' risk of IHD seems to increase over time in urban areas. In Ontario, however, immigrants had a reduced risk of MI and stroke than long-term residents (Sohail, *et al*., 2015).

Clinical trials have demonstrated that the early detection and lowering the risk factors by aggressive treatment reduce cardiovascular risks. There are current worldwide variations in the global burden of ischemic heart disease. For instance, the prevalence of most cardiovascular risk factors has declined in the high-income nations such as in most European countries and the United States over the past 40 years. However, most Eastern Mediterranean countries have undergone the shift in the burden of CAD (Traina., *et al.* 2017). The main cause of SCD in adults is coronary heart disease (CHD) (80-85%), with more than 65% of cases associated with acute coronary circulation disorders, from 5 to 10% - with dilated cardiomyopathy and about 5-10% -with other heart diseases. Among young people, sudden death occurs in 20% of cases during sports, in 30% during sleep, in 50% under various circumstances during wakefulness (Abdurasulovich, 2022).

An estimated 65% of cases of acute heart disease in adults are caused by coronary circulation abnormalities, 10% are caused by dilated cardiomyopathy, and about 5-10% caused by other heart diseases.

Twenty percent of sudden death in young people occurs during athletic events, thirty percent during sleep, and fifty percent under a wide range of other circumstances while they are awake (Abdurasulovich, 2022).

Rates of death from ischemic heart disease (IHD) have been declined in most high-income countries, although cardiovascular disease still accounted for one in three of all deaths in the USA (Roger, *et al.,* 2011). In contrast, ischemic heart disease (IHD) is becoming a more common cause of death in the developing world (Gupta., *et al.,* 2008).

1.3. Pathogenesis of Acute Myocardial Infarction

Coronary artery disease or atherosclerosis is a disease process which is sometimes triggered by quite subtle physical or chemical insults to the endothelial cell layer of arteries. The "Response to Injury Theory" now has widespread disease in the world (Voskerician, 2018) and this theory suggests that the earliest event in atherogenesis is injury to the endothelium, which can be triggered by any number of insults, either alone or in combination. and these include (Mowery *et al*., 2008):

a) Physical injury or stress as a result of direct trauma or hypertension.
b) Turbulent blood flow, for example, where arteries branch.
c) Circulation of reactive oxygen species (oxidative stress).
d) Inflammation.
e) Hyperlipidemia (high blood concentrations of Low Density Lipoprotein (LDL) or Very Low Density Lipoprotein (VLDL).
f) Chronically elevated blood glucose concentrations.
g) Homocysteinaemia, in which an inherited metabolic defect leads to very high levels of the homocysteine, a metabolite of methionine, high concentrations of which are toxic to the endothelium (Verdoia *et al*., 2021).

In response to these insults, perturbation occurs where the endothelial cells secrete cytokines which then trigger and maintain an inflammatory response. The endothelial cells begin to produce cell surface adhesion molecules, causing monocytes and T-lymphocytes adhere to the endothelium and then migrate beneath it, by squeezing between the endothelial cells (Gegunde *et al*., 2021). Circulating monocytes and T-lymphocytes are attracted to the sites of injury by the cytokines. The endothelial cells also change the shape, causing the tight junctions between endothelial cells to loosen, increasing the permeability to fluid, lipids, and leucocytes. Lipoprotein particles, and especially Low-density lipoprotein (LDL), enter the artery wall and undergo oxidation. Oxidation of LDL in the artery wall occurs as a result of its exposure to nitric oxide, macrophages, and some enzymes such as lipoxygenase (Khosravi *et al*., 2019). Once they have migrated into the intima layer, monocytes differentiate into macrophages and begin to take up oxidized LDL that has entered the intima. Macrophages retain the lipid they take up, and as they become more lipid-laden, they are referred to as "foam cells." Eventually, the foam cells will undergo apoptosis and die, but the lipid will remain and accumulate in the intima. Fatty streaks are the first signs of atherosclerosis that are visible without magnification (Fioranelli *et al*., 2018). A fatty streak consists of lipid-containing foam cells in the artery wall just beneath the endothelium. It appears as a yellow discoloration in the artery's inner surface and occurs in the aorta and coronary arteries of most people by age 20. Over time, these fatty streaks can evolve into atherosclerotic plaques or they can remain stable or even regress (Mark *et al*., 2005).

Slowly growing plaques expand gradually due to the accumulation of lipid in foam cells and the migration and proliferation of smooth muscle cells. These plaques tend to stabilize and are not prone to rupture. The so-

called fibrin cap on the lesion matures. These plaques can build up and harden causing narrowing with reduction of blood flow to the heart. This can cause symptomatic (angina) or asymptomatic ischaemia. In contrast, other plaques grow more rapidly as a result of more rapid lipid deposition. These have thin fibrin caps that are prone to rupture. Once a plaque ruptures, it can trigger an acute thrombosis (clot) by activating platelets and the clotting cascade. This blood clot can cause partial or complete blockage (Odeberg., *et al.,* 2014).

Atherosclerosis is thought to begin fatty stripes of lipid which accumulates in the inner lumen of the arterial wall. These lesions commonly begin early in life, perhaps even in childhood, actually in the same time not all of fatty streak later on developed as lesions. Genetic and environment factors play as important factors which are involved in the progression of these lesions. Inflammatory restraint with injury to the vascular endothelium when the progression of these lesion. The injuries may be set due to smoking, hypertension or other factors. The multiple effects of inflammation may injure the inner wall of the arteries and the chemical substances can damage the endothelium where platelets may be accumulated and clots are released by active macrophages (Carreiro, 2006; Futterman and Lemberg, 2006).

Atherosclerosis is characterized by endothelial dysfunction, vascular inflammation, and the formation of atherosclerotic plaque. This buildup of atherosclerotic plaque causes an inadequate supply of oxygen to myocardial tissue, leading to myocardial hypoxia. Consequently, the plaque rupture and atherothrombosis cause further narrowing of coronary arteries and almost occluding the blood flow, leading to fatal acute coronary syndromes. The most evident manifestation of CAD is the AMI. For instance, the ruptured atherosclerotic plaques followed by thrombosis

and loss of blood flow in the coronary vessel cause the predominant signs and symptoms of AMI in the coronary arteries (Scheen, 2018).

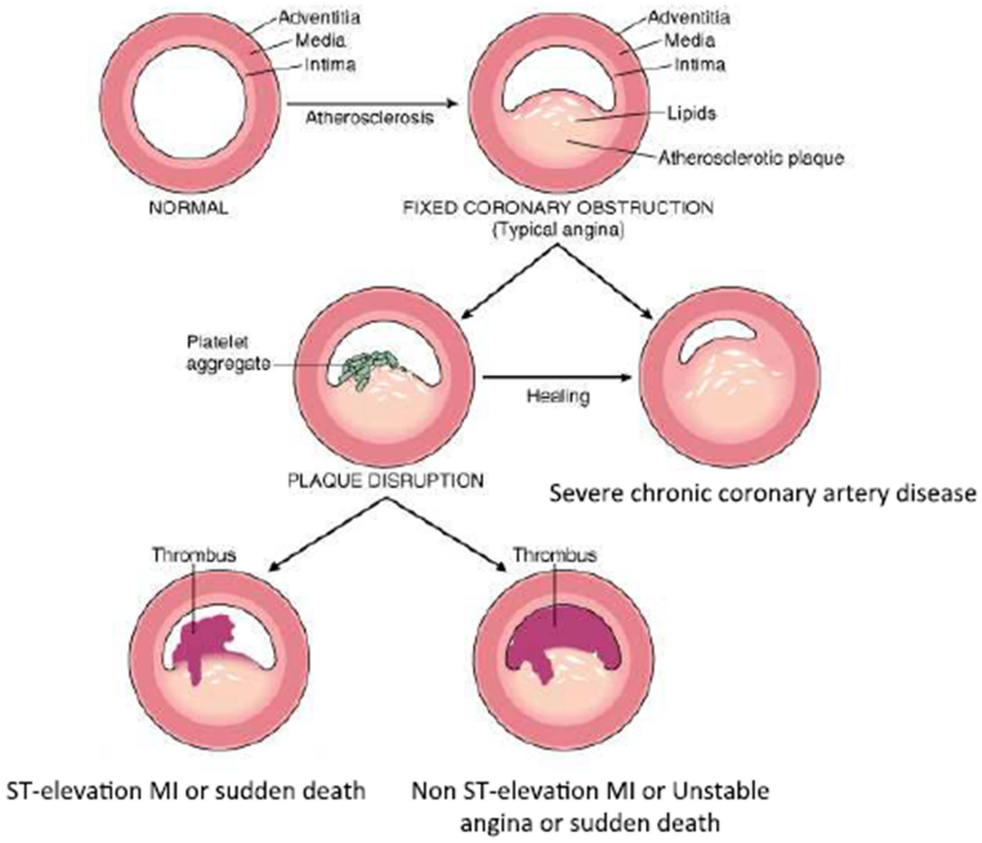


Figure (1-1): Coronary artery disease morphology (Kumar *et al*., 2015)

1.4. Risk Factors

There are several factors may increase the probability of a person to develop Acute heart disease (AHD). Persons with acute heart disease may be under a risk for expose to a cardiac event within 10 years later, peripheral arterial disease, abdominal aortic aneurysm, or carotid artery disorder. Some people do not have classic risk factors but elevated low-density Lipoprotein (LDL) cholesterol, also known as bad cholesterol, which is the primary goal of cholesterol-lowering therapy. (Porth and Matfin, 2009). And the most common risk factors for (AHD) can be

divided into modified and non-modified factors that increase the risk of heart disease (LeMone *et al*., 2014).

1.4.1. Non Modifiable Risk Factors:

1.4.1.1. Age

Age consider as a risk factors for Acute heart disease (AHD). In the developing countries people over 48 years old are more risky to get cardiovascular disease (Gorman, 2017). Nearly 4 to 5 case of deaths recorded due to heart disease occurs among old person within (65) years, old age may affect the hearts function, hearts may not work as well as young due to physiological changes such as the walls of the heart may be thickened and the arteries may be stiffened and become hard, which make the heart lose the ability to pump blood all over the body (Barnes, 2012).

1.4.1.2. Gender

Most of studies find out that men have a higher risk of cardiac disease than women due to the effect of testosterone hormone on lipid precipitation, with little difference after menopause. After age 65, the risk to expose to heart disease becomes parallel with men (Cevik *et al*., 2012).

Estrogen hormone provides natural protection for women during the fertile age to be away from cardiac events. Normally the protection decreases after menopause and the risk increases gradually (Haque *et al*., 2011).

1.4.1.3. Ethnicity

The genetics have important role in the cardiac disease, so that some ethnic groups have a higher risk than others of developing heart disease such as African – Americans, and South Asians (Oursler, *et al*., 2011).

1.4.1.4. Family History

Some forms of (CVD) like ischemic heart disease (IHD), are more common in persons who have positive family history (Barnes, 2012).

Several studies indicate that a family history is an independent risk factor for IHD (Patel and Ye, 2011; Sivapalaratnam *et al*., 2010; and Fischer *et al*., 2007).

1.4.1.5. Genetic Factor

Heart diseases are well known as the common cause of death nowadays. It affects the people suddenly without any previous alarm signs or symptoms. Using specific genetic testing which distinguishes the cardiovascular risk factors from chromosomal object, risk factors may stand by specific underlying disease process. So important to add a specific complete analysis in order to identify a complex monogenic disease such as cardiac diseases (Huma *et al*., 2012).

1.4.2. Modifiable risk factors:

1.4.2.1. Diabetes Mellitus

Diabetes Mellitus (DM) is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion. Insulin dependent (DM) is characterized by a near-total reliance on exogenous insulin for survival, non-insulin-dependent DM is characterized by relative insulin deficiency and/or insulin resistance, usually occurring in middle age, and most commonly in the obese (Kalofoutis *et al*., 2007).

Diabetes acts as a strong effecting factor for premature CVD mortality (Smith *et al*., 2007). Cardiac events and death may be increased when they are associated with DM (Emberson *et al*., 2005).

Heart disease and other forms of CVD are the major causes of mortality in type-2 DM, and are major contributors to morbidity and depreciation in

quality of life of people with diabetes (Yang *et al*., 2007; Ding *et al*., 2005).

1.4.2.2. Hypertension

High blood pressure known as a major modifiable factor which increases the occurrence of CVD, also can contributes as a cause of IHD, as elevated blood pressure can be harmful to the heart and lead in time to heart damages and IHD (Abboud, *et al*., 2012).

High blood pressure increases the pressure in the blood vessels, when the heart acts against this resistance the myocardium needs to work harder. With time, the heart muscle become thick, the muscle needs more power to pump blood, elevated blood pressure can cause IHD because heart muscle needs an increased supply of oxygen. High blood pressure can contributes to change of the blood vessel walls. This may increase cholesterol deposits in the blood vessels (Victor *et al*., 2008).

1.4.2.3. Smoking

Smoking considers as a risk factor for CVD which can be controlled. More than sixteen thousands of death caused by CVD. One year cessation of smoking may decreases the chance of expose to cardiac attack than those who smoke, and if the heart attack occurs, the chance of death is greater for the smoker person than non-smoker, so the programs of smoking cessation is effective for persons who have coronary artery diseases by using available community resources (Niznick, 2011**)**.

Effects of smoking on the heart:

- Elevates blood pressure causing hypertension.
- Disturbs the cholesterol balances which increase the hardening of the arteries.

• Increases the level of carbon monoxide with decrease oxygen in blood.

• Elevates the blood consistency which produce clots.

• Increases the chance of sudden death.

• Acts as a factor to increase incidence of strokes.

• Increases the risk to develop peripheral disease and abdominal aneurysms.

• It plays an important factor in developing lung disease such as emphysema and bronchitis.

• Smoking contributes actively to expose to lung cancer **(**Niznick, 2011**)**.

1.4.2.4. Obesity

When body mass index (BMI) reach 30 kg/m^2 or greater obesity, such a person has higher rates of hypertension, diabetes and hyperlipidemia. Obese men over 50 had twice incidence of heart disease than persons with normal weight. Waist to hip ratio greater than 0.8 in (women) or 0.9 in (men) increases the risk of heart disease (LeMone *et al.,* 2014).

1.4.2.5. Sedentary Behavior

Continue on daily exercise decrease the risk for exposed to cardiac disease. Several studies find out that playing video games, using computer and even watching television for long hours associated with gaining weight. This sedentary behavior increase the risk to expose to cardiac disease (Shiroma and Lee, 2010). A regular daily program of physical activities are useful to decrease the development of cardiac disease. The benefits of regular exercise is to increase oxygen supply to the heart muscle and improve the myocardial function and electrical stability. Also regular exercise plays a positive role in decreasing blood pressure and blood lipids, decreases insulin resistance, platelet

aggregation and keeping weight within normal range (Oursler, *et al*., 2011).

1.4.2.6. Psychosocial Stress

Many clinical studies find out that psychosocial problem contributes independently to increase the occurrence of heart diseases. Chronic psychosocial stressors such as unsolved problems at work or unstable family relationship, lack of social support, negative emotions including depression and hostility, the burden of family care-giving, and low socio-economic status have been shown to be independent factors predictive of heart disease. Psychosocial factors show a tendency to cluster in the same individuals and groups, such as those with low socioeconomic status. Stress may affect heart diseases directly through neuroendocrine and platelet activation, or indirectly through higher frequency of adverse health behaviors such as smoking, poor diet, and sedentary lifestyle, which increase the risk of heart diseases. Acute psychological stress increases the incidence of heart disease, and it has been reported that intense grief in the days after death of a significant person may trigger the onset of (MI). The pathophysiological mechanism of acute emotional stress remains unclear, but it is assumed to be related to hemodynamic stress in the coronary arteries and rupture of an atherosclerotic plaque, with consequent thrombosis (Vujcic *et al*., 2016).

1.4.2.7. Hyperlipidemia

Elevated cholesterol in the blood over time builds up a fat-like substance in the walls of arteries causing arteries to become narrow and the blood flow to the heart is slowed or blocked. Many factors that increase the level of cholesterol in the blood such as, diet, weight, age, gender, sedentary behaviors and heredity high cholesterol level can be controlled and managed by lifestyle changes, improving physical

activities, healthy diet, and loss of weight, because increasing the level of cholesterol plays a great risk factor to increase the developing of heart disease (Aubeidia, 2006).

1.4.2.8. Excessive Alcohol Intake

Alcohol considers one of the risk factors which increase the risk of cardiac diseases. An average moderate intake of alcohol for men is about one to two drinks per day while for women one drink per day. More than this amount cause heart problems such as irregular heartbeats, elevated blood pressure, stroke and cardiomyopathy. Calories which are release from alcohol may produce fat to the body which may elevate the occurrence of cardiac diseases (Barnes, 2012).

Alcohol directly and indirectly impacts numerous aspects of hepatic lipid flux that ultimately leads to lipid accumulation. The simplest example is that alcohol metabolism itself directly causes steatosis. In the process of metabolizing ethanol to acetate, 2 equivalents of reduced NADH are generated per equivalent of ethanol oxidized. This metabolism robustly increases the ratio of NADH:NAD+ within the cell, which then favors inhibition of FA β-oxidation in the liver. The net effect is to favors triglyceride accumulation in the hepatocytes (Aune *et al*., 2019; You & Arteel, 2019).

1.4.2.9. Diet

Un healthy diet associated commonly with the risk of the cardiac disease, while healthy eating behaviors such as high fruits, vegetables, whole grains and using unsaturated fatty acids have a protective effect, in addition to consuming nutrients as antioxidants, folic acid, and vitamin B, with omega-3 (Bhupathiraju & Tucker, 2011; Hayman *et al*., 2009).

Dietary Approach to Stop Hypertension (DASH) diet decreases blood pressure and significantly lowers coronary heart disease risk. The DASH diet improves endothelial function in hypertensive and obese hypertensive patients (Taghavi *et al*., 2019).

1.5. C-Reactive Protein (CRP)

A number of numerous investigations have found that CRP levels routinely rise during myocardial necrosis and coronary ischemia. Despite these early findings, it was not use until the 1990s that renewed interest in the link between CRP and cardiovascular disease (CVD) was seen. It was discovered in the mid-1990s that high serum CRP concentrations predict future cardiovascular disease (CVD) using immunoassays for high sensitivity CRP (hs-CRP), which were more sensitive than those previously used routinely (Kaur *et al*., 2017 and Avan *et al*., 2018).

Early risk screening has been made possible by the use of risk scores that have demonstrated high prognostic ability, such as the Thrombolysis in Myocardial Infarction (TIMI) and Global Registry of Acute Coronary Events (GRACE) scores (Ribeiro *et al*., 2014). Multiple studies have demonstrated that it can be used to predict bad occurrences during hospitalization as well as in the long term after recovery for patient have coronary artery disease (Al Aseri *et al*., 2019).

It is well recognized that inflammation is a key player in the pathophysiology of atherosclerosis, and there has been an increase in interest in the examination of inflammatory biomarkers in coronary artery disease (CAD) during the last decade (Liu *et al*., 2020). The presence of inflammation has been shown to be connected with the destabilization of chronic arterial plaques, which results in acute coronary syndromes (Polyakova, *et al*., 2020).

Several studies on the predictive value of hs-CRP have been done to overcome AMI, the C-reactive protein (CRP) is a plasma protein that belongs to the pentraxins family of proteins, CRP is frequently employed as a general inflammatory marker because it is an acute phase protein created by hepatocytes in response to proinflammatory cytokines, particularly interleukin-6, and because it is an acute phase protein, synthesized by neutrophils (Ristagno *et al*., 2019).

The American Heart Association and the Centers for Disease Control and Prevention (CDC) of the United States categorize people into cardiovascular risk groups based on their hs-CRP levels as follows: Low risk: is defined as less than 1.0 mg/L; average risk: is defined as between 1.0 and 3.0 mg/L; and high risk: is defined as greater than 3.0 mg/L (Musunuru *et al*., 2008).

An interesting finding is that a greater level of hs-CRP in patients with acute coronary syndromes has been demonstrated to be a predictor of in-hospital cardiac events, regardless of the GRACE risk score, whereas those with lower values have a decreased risk of cardiovascular disease (Ahmed *et al*., 2012; Magoon *et al*., 2020).

Some of studies refer to individuals with high-normal hs-CRP have a 1.5 to 4 times greater chance of having a heart attack than those with low-normal hs-CRP. It is necessary appears that inflammatory indicators can be used to supplement the scoring system's prognostic information (Wang *et al.,* 2019). Myocardial infraction is linked to a significant amount of myocardial inflammation, which triggers a systemic inflammatory response (Milano *et al*., 2018).

In the early stages of AMI, high-sensitivity CRP (hs-CRP) may be a reasonable indicator of the extent of the inflammatory response to myocardial infarction (Milano *et al*., 2018).

With exception of heart necrosis and infarct size, various types of tissue damage, such as atherosclerotic mass, underlying inflammatory process, and circulating proinflammatory cytokines, may result in an elevation of hs-CRP in STEMI patients (Avan *et al*., 2018). Hs-CRP has been demonstrated to be related with a variety of hospital outcomes, including death, MI, and unstable angina. That this biomarker is important in the risk assessment of individuals with acute coronary syndromes, is underscored by this fact (Polyakova, *et al*., 2020).

The activation of the complement system and platelets, suppression of fibrinolysis, promotion of the proliferation of smooth muscle cells, microphage polarization, and lipid deposition have all been demonstrated in experimental studies to be mechanisms by which CRP can influence the progression of CAD (Wanga *et al*., 2019). Some of studies have demonstrated in vivo that CRP is involved in the promotion of inflammation, platelet aggregation, and thrombosis in transgenic mice (Grad *et al*., 2012).

Hs-CRP levels in patients with AMI may correlate with the extent of coronary artery disease, the size of myocardial necrosis, the risk of recurrent acute coronary syndrome, the risk of new-onset atrial fibrillation, ventricular tachycardia, heart failure decompensation or development, and the risk of death. It should be recognized that CRP is a marker of inflammation and that it is a non-specific indicator for many acute and chronic conditions that may coexist in individuals with AMI, including heart failure (Zhang, *et al*., 2021).

1.6. Acid Ceramidase (EC:3.5.1.23)

Acid ceramidase (aCDase) is a N-acylsphingosine deacylase with a pH optimum in the acidic range. The enzyme is found in the lysosomal and endosomal compartments of the cell. The enzyme is largely involved in the breakdown of ceramide. In humans, a hereditary lack of lysosomal aCDase causes 'Farber disease,' a lyosomal lipid storage condition (Beck *et al*., 2020).

The human acid ceramidase gene spans about 30 kb in length and contains 14 exons ranging in size from 46 to 1201 bp. The exon/intron junctions were determined and found to follow the GT-AG rule. The putative promoter region had a GC content over 60%, lacked a TATA box, and contained several sequences matching transcription factor binding sites, including nine SP-1 sites. The human AC gene was mapped to the chromosomal region 8p21.3–p22 by in situ hybridization and FISH analyses. (Li, *et al*., 1999).

Acid ceramidase (aCDase) regulates several cellular activities by hydrolyzing lysosomal membrane ceramide substrate into sphingosine, the backbone of all sphingolipids (Gebai *et al*., 2018).

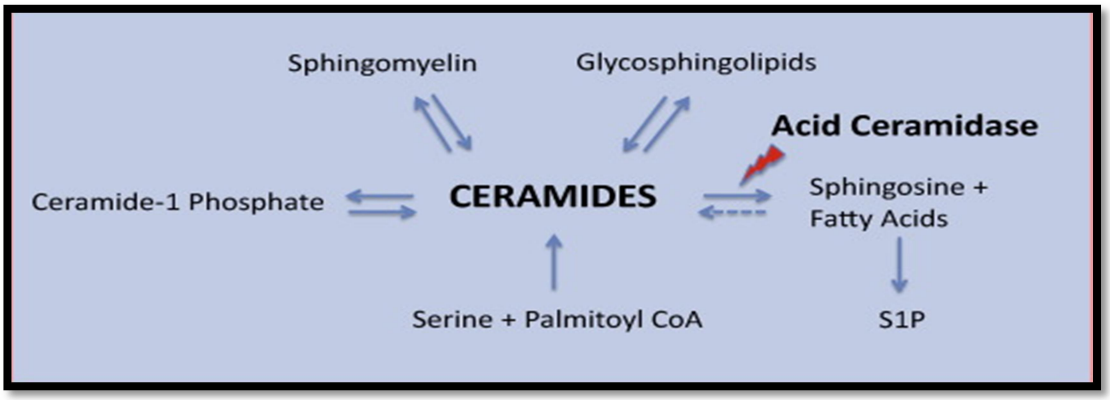


Figure (1-2): Coronary Acid ceramidase pathways (Schuchman, 2016)

Acid ceramidase is a heterodimeric glycoprotein with a molecular weight with around 50 kDa, also it was made up of two parts.: The alpha-subunit has a molecular weight of around 13 kDa. The beta-unit (molecular weight: 40 kDa) has 5 or 6 N-linked oligosaccharide units, but the alpha-unit is not (Fabian *et al*., 2018). The alpha is made up of a globular helical domain formed by five-helices and preceded by a 36-residue linker region, whereas the Beta-subunit is made up of two central anti-parallel sheets flanked on either side by a total of six–helices, (Figure 1-3).

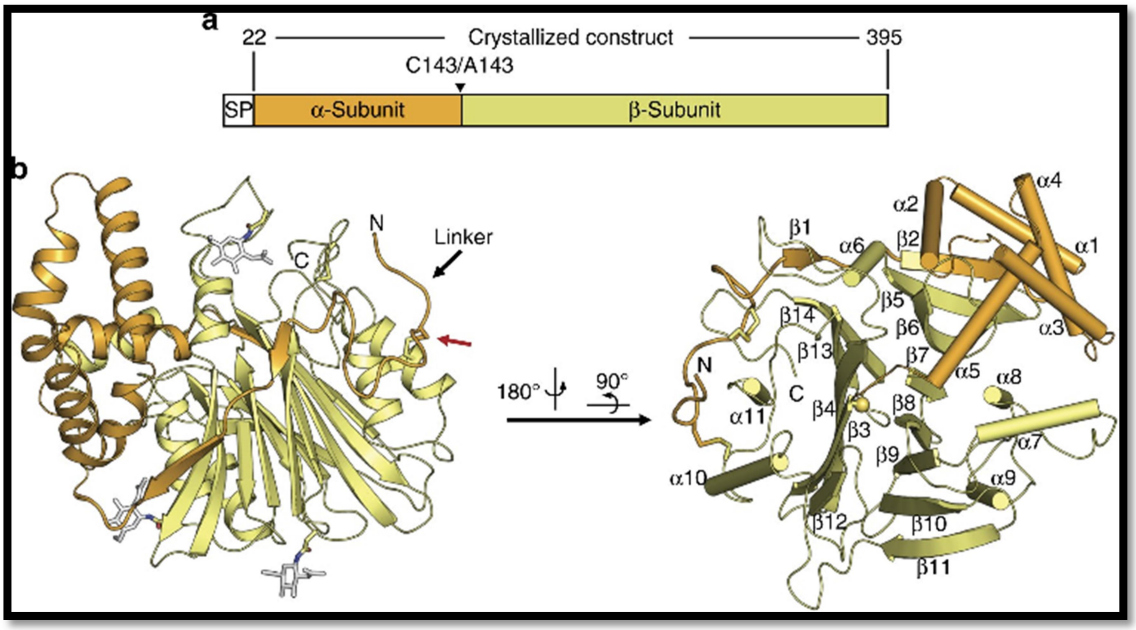


Figure (1-3): Structural basis for the activation of acid ceramidase (Gebai *et al*., 2018)

Ceramidases are classified according to their pH optimum (alkaline, neutral, or acidic), cellular localization, main structure, and function in humans (Mao & Obeid, 2008). Neutral ceramidase (nCDase) is involved in the digestion and catabolism of dietary sphingolipids, as well as the regulation of sphingolipid metabolites in the intestine (Coant, & Hannun, 2019). Cell differentiation, DNA damage-induced cell death, and cell proliferation are all regulated by alkaline ceramidases. The best-studied

member of the family, acid ceramidase (aCDase), is known for its role in Farber disease (FD), a human genetic condition (Xu *et al*., 2021).

Within 12 months of the original hospitalization, up to 10% of patients admitted for acute myocardial infarction (AMI) would have another episode of AMI. Some lipid groups, such as ceramides, sphingomyelin, phosphatidylcholines, and cholesterol esters, have been shown to play a physiological role in atherosclerosis and AMI in recent investigations (Chaudhry *et al*., 2014).

Ceramides are one of the most bioactive membrane lipids, controlling signal transduction pathways that determine whether a cell lives or dies (Stancevic, 2010). Ceramides accumulate in atheromatous plaques in the coronary arteries (Choi, *et al.,* 2021) and their glycosylated forms, glucosylceramides and lactosylceramides, are more numerous in arterial tissue with apparent plaque formation (Chatterjee, *et al*., 2021). Ceramides are produced by the myocardium in response to ischemia and reperfusion, and these ceramides induce mitochondrial autophagy and apoptosis, It is now possible to investigate the link between ceramides and cardiovascular death in stable and unstable coronary artery disease (CAD) cohorts since plasma ceramides are now easily measurable. (Laaksonen *et al*., 2016).

Ceramides have been linked to a number of fundamental processes in the development of atherosclerosis, including lipoprotein absorption, inflammation, and apoptosis. Six fatty acyl selective ceramide synthases create ceramide species, and it is becoming evident that different ceramide species have different physiological activities (Bismuth *et al*., 2008). Park *et al*., (2006) believe that monitoring ceramide species ratios can provide insight into the metabolic regulation of atherosclerosis.

Ceramides are extensively implicated in mitochondrial-induced apoptotic cell death, which is a critical event triggering cell death in ischemia-reperfusion injury, the insult that leads to numerous long-term sequelae in AMI (Vaena *et al*., 2021).

Heart ceramide levels are raised in models of cardiac lipotoxicity, which is caused by the overexpression of long-chain acyl CoA synthase and fatty acid transport proteins (FATP) in the heart muscle cells (Chiu *et al*., 2005). Ceramide is created by two pathways: 1) condensation of palmitoyl CoA with serine by serine palmitoyl transferase (SPT), and 2) hydrolysis of sphingomyelin by sphingomyelinase (sphingomyelinase hydrolysis) (Hayter H., & Hannun 2020).

1.7. Endothelial Nitric Oxide Synthase (EC:1.14.13.39)

Endothelial Nitric Oxide synthase (eNOS) produces nitric oxide (NO), which is important for coronary blood flow modulation vascular resistance reduction, and platelet aggregation and adhesion inhibition, all of which help to prevent coronary circulatory failure, thrombosis, and atherosclerosis (Förstermann *et al*., 2017; Janssens *et al*., 1992).

Several pathologic factors affect endothelial function, including smoking, chronic alcohol consumption, hypercholesterolemia, obesity, hyperglycemia, and hypertension. Reduced nitric oxide synthase (NOS) expression and activity, decreased NO bioavailability, and increased generation of oxygen radicals, and endogenous NOS inhibitors are among the causes leading to endothelial dysfunction. Endothelial dysfunction appears to be linked to atrial fibrillation. In humans, endothelial dysfunction is a significant predictor of coronary artery disease (CAD). Ischemic heart disease is thought to be highly predictive of penile erectile

dysfunction, which is linked to reduced bioavailability of NO generated by eNOS and neuronal NOS (nNOS) (Toda *et al*., 2011).

NO is created when L-arginine is converted to L-citrulline in the presence of oxygen and cofactors such as calmodulin, tetrahydrobiopterin (BH4), nicotinamide adenine dinucleotide phosphate (NADPH), heme, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) by the enzyme nitric oxide synthase (NOS), figure (1-4). The activation of nNOS (NOS I) and eNOS (NOS III) requires calcium ion (Ca^{+2}), but not inducible NOS (iNOS, NOS II). iNOS is not expressed by itself; it is primarily activated in macrophages by bacterial lipopolysaccharide and cytokines (Garhöfer G., & Schmetterer, 2019 ; Manda-Handzlik *et al*., 2020 ; Alkaitis, & Crabtree, 2012).

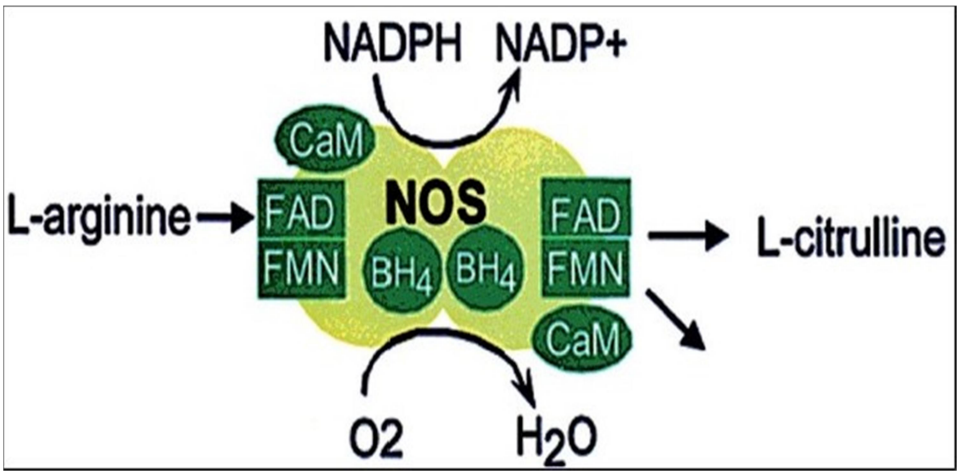


Figure (1-4): Nitric oxide synthase pathway (Andrew & Mayer, 1999)

Nitric oxide (NO) from the endothelium, which is produced by the oxidation of L-arginine to L-citruline by the action of endothelial nitric oxide synthase (eNOS), is thought to be a key atheroprotective component in the cardiovascular system (Jaramillo *et al*., 2010).

Endothelial NO acts to prevent atherosclerosis by causing vasodilation, higher blood flow, decreased blood pressure, platelet aggregation and

adhesion inhibition, leukocyte adhesion inhibition, and reduced smooth muscle proliferation (Severino *et al*., 2019). NO is mediated by cyclic guanosine monophosphate (cyclic GMP), which is generated from guanylyl triphosphate (GTP) by soluble guanylyl cyclase, NO produced by nNOS has a critical role as a neurotransmitter from the peripheral efferent nerves in the blood vessel, mediating non-adrenergic non-cholinergic inhibitory responses to parasympathetic nerve stimulation (Liu *et al*., 2019).

Endothelial NO inhibits smooth muscle contraction and platelet aggregation, as well as contributing to angiogenesis and cytoprotection in the heart, making it an essential modulator of coronary blood flow. The

Endothelial nitric oxide synthase (eNOS) is one of three nitric oxide synthase isoforms that have sequence and function homology. In 1993, the NOS3 gene was cloned and placed on chromosome 7q35-36. The gene spans 4.4 kb of genomic DNA and contains 26 exons that code for a 135-kD protein with 1,203 amino acids. Upstream promoter sequences containing transcription factor-binding sites that facilitate regulation by stress and estrogens. The eNOS protein produces nitric oxide in a constitutive manner, which involves the transfer of five electrons provided by nicotinamide adenine dinucleotide phosphate during the conversion of L-arginine to L-citrulline (Papi *et al*., 2019).

Nitric oxide (NO) is a short-lived gas molecule that has a variety of physiological and biochemical impacts on the body. Many biological processes use NO as a 2nd messenger molecule. The majority of NO's actions are mediated by circular guanosine monophosphate production. The enzyme nitric oxide synthases (NOS) in the body produces NO (Änggård, 1994).

Nitric oxide produced by the endothelium is a key atheroprotective mediator, and acquired deficiencies in nitric oxide production are related with an increase in cardiovascular risk factors. When compared to age-matched individuals without a family history of, young, healthy individuals with a first-degree relative who died of coronary heart disease before the age of 55 years have impaired endothelium-dependent, flow-mediated dilatation of the brachial artery (a largely nitric oxide–dependent response) (Dong *et al*., 2018).

In endothelial cells, increasing intracellular calcium in response to vasodilator agonists like acetylcholine and bradykinin activates eNOS and increases NO generation. NO diffuses into vascular smooth muscle cells, activating soluble guanylyl cyclase (sGC), which produces cyclic guanosine monophosphate (cGMP), which causes vasorelaxation (figure 1-5).

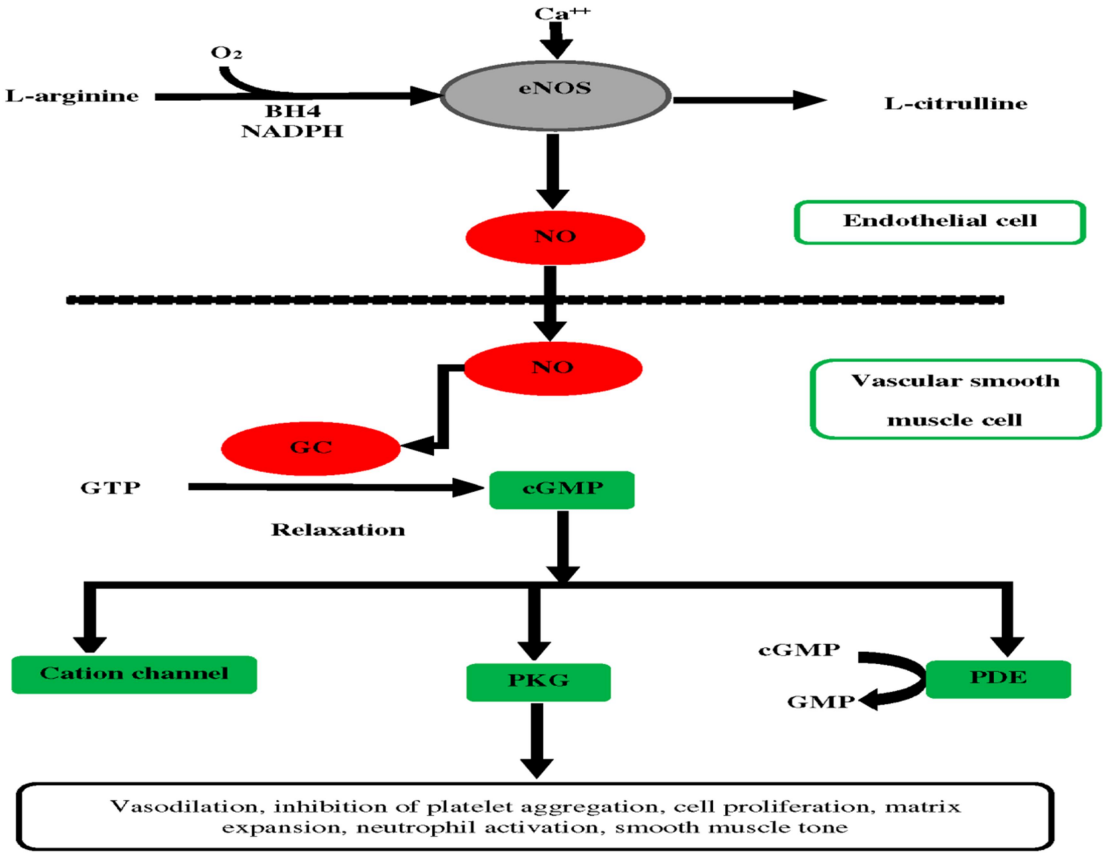


Figure (1-5): Synthesis of nitric oxide from precursor L-arginine in endothelial cells and mechanism of vasodilation in vascular smooth muscle (Ahmad *et al.*, 2018), BH4: tetrahydrobiopterin; NADPH: nicotinamide adenine dinucleotide phosphate; GTP: guanosine triphosphate; cGMP: Cyclic guanosine monophosphate; PKG: protein kinase G; PDE: Phosphodiesterase; NO: nitic oxide; GC: generator cells.

1.8. Beta-Catenin (BC)

Catenins are a family of proteins that co localize with the animal cell adhesion molecule cadherin. The first two catenins identified (Peyrieras *et al.*, 1985) were given the names α-catenin and β-catenin. β-Catenin can interact with α-catenin and can also interact with filamentous actin (F-actin). (Serebryannyy *et al.*, 2017). Catenin interacts with the cytoplasmic tail of traditional cadherins directly. The word "catenin" was chosen ('catena' means 'chain' in Latin) because it was hypothesized that catenins would act as a link between cadherins and the cytoskeleton (Mège, & Ishiyama, 2017).

Simple epithelia in higher species require cell-cell adhesion complexes to maintain form, function, and polarity. These complexes, which aid in cell growth regulation as well as the formation and maintenance of epithelial layers, are referred to as adherens junctions and normally contain cadherin, α-catenin, and β-catenin (Chiarella *et al*., 2018). Catenins were involved in cellular structure and polarity even before Wnt signaling pathways and cadherins were developed and integrated (Barcelona *et al*., 2021).

Catenins are primarily involved in the mechanical connection of cadherins to actin filaments, as shown in the adhesion junctions of epithelial cells and other structures (Gehren *et al*., 2015). WNT/β-catenin is a developmental signal system that has remained basically constant during evolution and is required for embryonic development, damage repair, and tissue homeostasis (Nusse & Clevers, 2017).

Catenin beta-1, commonly called beta-catenin (β-catenin), is a protein encoded by the CTNNB1 gene in humans, beta-catenin is a protein with two functions: it regulates and coordinates cell–cell adhesion as well as gene transcription (MacDonald *et al*., 2009). The CTNNB1 gene encodes β-catenin is a subunit of the cadherin protein complex and acts as an intracellular signal transducer in the Wnt signaling pathway, it is a member of the catenin protein family and homologous to γ-catenin. Beta-catenin is widely expressed in many tissues. (Grainger & Willert, 2018). It belongs to the catenin protein family, which is also known as plakoglobin. Beta-catenin is found in a variety of tissues (van Amerongen, 2020). Beta-catenin is found in adherens junctions in intercalated disc structures in cardiac muscle, which are important for electrical and mechanical interaction between neighboring cardiomyocytes (Bastakoty D., & Young, 2016).

WNTs (translated products of the WNT gene) are cysteine-rich glycoproteins released into the extracellular matrix by cells that trigger receptor-mediated signaling in cells neighboring (Niehrs, 2012). Several cancers, such as hepatocellular carcinoma, colorectal carcinoma, lung cancer, malignant breast cancers, ovarian and endometrial carcinoma, have been linked to mutations or overexpression of the β-catenin gene (Shamsian *et al*., 2020).

Several types of heart disease, including dilated cardiomyopathy, have been linked to changes in the beta-catenin protein's localization and levels of expression (Lin *et al*., 2017).

Complexes of cell–cell adhesion are required for the creation of complex animal tissues. Catenin is a component of a protein complex that contributes to the formation of adherens junctions (Sousa *et al*., 2019). These complexes of cell–cell adhesion are required for the establishment and maintenance of epithelial cell layers and barriers. As a component of the complex, β-catenin has the ability to govern cell proliferation and cell adhesion. Additionally, it may be responsible for conveying the contact inhibition signal that instructs cells to cease dividing once the epithelial sheet has been completed (Bush *et al*., 2019). The complex of E-cadherin, β-catenin, and α-catenin is only weakly linked with actin filaments. To connect to the actin cytoskeleton, adherens junctions require considerable protein dynamics, facilitating mechanotransduction (Sousa *et al*., 2019).

In cardiac muscle, beta-catenin forms a complex with N-cadherin at adherens junctions within intercalated disc structures, which are responsible for the electrical and mechanical coupling of adjacent cardiac cells. Researchers have discovered that the presence and distribution of beta-catenin is spatiotemporally regulated during the redifferentiation of adult rat ventricular cardiomyocytes in culture, and that this regulation

occurs during the differentiation of these cells in vitro (Marinou *et al*., 2012).

In humans, altered beta-catenin expression profiles have been linked to dilated cardiomyopathy. The expression of beta-catenin is generally upregulated in patients with dilated cardiomyopathy (Cantù *et al*., 2018). Patients with end-stage dilated cardiomyopathy had nearly doubled estrogen receptor alpha (ER-alpha), mRNA and protein levels, and the ER-alpha/beta-catenin interaction, which was present at intercalated discs of control, non-diseased human hearts, was lost, suggesting that the loss of this interaction at the intercalated disc may plays a role in the progression of heart failure (Mahmoodzadeh *et al*., 2006). Beta-catenin coordinates different elements of hearing development with the B-cell lymphoma-9 (BCL9) and protein Pygopus (PYGO) proteins, and mutations in BCL9 or PYGO in model species like the mouse and zebrafish induce symptoms that are very comparable to human congenital cardiac diseases (Cantù *et al*., 2018).

1.9. Diagnosis of Acute Myocardial Infarction

The first diagnosis is to evaluate the symptoms and signs in order to identify patients who have unstable angina or other Acute coronary syndrome (ACS) (Baumgartner & De Backer, 2020). The cornerstone of angina diagnosis is a thorough medical history. Although a diagnosis based solely on history can be made with a high degree of accuracy, physical examination and objective tests are frequently required to confirm the diagnosis, rule out other diagnosis, and determine the severity of the underlying disease (Albus *et al*., 2017). Any signs or symptoms of cardiovascular disease (CVD) as well as risk factors should be included in the history (i.e. family history of CVD, dyslipidaemia, diabetes, hypertension, smoking, and other lifestyle factors).

Location, nature, length, and relationship to effort, as well as other exacerbating or alleviating factors, are the four categories of discomfort associated with myocardial ischaemia (angina pectoris). Myocardial ischaemia causes pain in the chest, near the sternum, but it can also be felt anywhere in the body, from the epigastrium to the lower jaw or teeth, between the shoulder blades, or in either arm to the wrist and fingers (Prachanukool *et al*., 2016). The discomfort is usually brief— 10 minutes or less in the majority of instances, and more frequently a few minutes or less—and chest pain lasting seconds is unlikely to be caused by CAD (Albus *et al*., 2017).

The second diagnosis which include comorbidities and other factors that contribute to symptoms. Before any testing is undertaken, the patient's overall health, comorbidities, and quality of life must be assessed. There have been significant technical and technological advances in Percutaneous coronary intervention (PCI) over recent years, and this is now the preferred revascularization modality in patients with single-vessel or low-risk multi vessel disease, which may include a trial of anti-anginal medication even if a diagnosis of CAD has not been established. If there is a need to confirm the diagnosis, non-invasive functional imaging for ischaemia may be an alternative (Baumgartner & De Backer, 2020).

Other diagnostic testing may be needed to rule out gastrointestinal, pulmonary, or musculoskeletal causes of chest discomfort if the symptoms is obviously non-anginal. These patients should, however, undergo risk-factor modification based on guidelines and routinely used risk charts such as Systematic Coronary Risk Evaluation (SCORE) (Graham *et al*., 2021). There are more steps for testing in patients with suspected CAD includes electrocardiograms (ECGs), exercise stress tests, and X-rays, they are some of the diagnostic tools that can be used to

determine whether or not a patient has a heart-related condition. Such testing can be done on an outpatient basis (Feldman *et al*., 2021).

In individuals with suspected Heart failure (HF), the echocardiography and electrocardiogram (ECG) are the most relevant diagnostics. The echocardiography can tell us about chamber volumes, ventricular systolic and diastolic function, wall thickness, and valve function right away, this knowledge is critical in selecting the best course of treatment (e.g. an Angiotensin converting enzyme (ACE) inhibitor and beta-blocker for systolic dysfunction or surgery for aortic stenosis) (Bauersachs *et al*., 2019). The ECG displays the heart's rhythm and electrical conduction, indicating whether there is sinoatrial illness, atrioventricular (AV) block, or aberrant intraventricular conduction (Henning, 2020). The ECG may also show signs of Left ventricular (LV) hypertrophy or Q waves (showing a loss of viable myocardium), which could provide insight into the cause of HF (Finocchiaro *et al*., 2020).

Many patients with suspected HF who are referred for echocardiography are not found to have a significant cardiac abnormality because the signs and symptoms of HF are so non-specific. When echocardiography is unavailable, measuring the blood concentration of a natriuretic peptide, a family of hormones secreted in increased amounts when the heart is diseased (Zhang *et al*., 2020).

In addition to routine biochemical [sodium, potassium, creatinine/estimated glomerular filtration rate (eGFR)] and haematological testing (haemoglobin, haematocrit, ferritin, leucocytes, and platelets), thyroid disease can mimic or aggravate coronary heart disease. Undiagnosed diabetes is frequent in people with HF, hence blood glucose should be checked. In HF, liver enzymes may be abnormal (essential for both amiodarone and warfarin) (Hamilton *et al*., 2020 ; Mujović *et al*., 2020).

1.10.Treatment and Prevention of Myocardial Infarction

The main objective of treatment for coronary artery disease in patients is improvement in both the short- and long-term prognosis. Therefore, it is important to estimate the risk of adverse events when planning treatment strategies. Usually, an early invasive strategy (within 24 h) is chosen when meeting high-risk criteria and a late invasive strategy (within 72 h) in the case of moderate risk criteria (Arora *et al*. 2018). On the basis of coronary angiography results, the heart team should discuss and decide which treatment strategy to be taken, including conservative treatment, because it is sometimes face multi-vessel disease with unclear ischemic culprit lesions or multiple lesions that are all supposed to be culprit lesions. Because emergency coronary angiography is not always followed by coronary revascularization, they remain uncertainties regarding the optimal potency and timing of antiplatelet therapy (Bainey *et al*. 2017).

Treatment of heart disease varies depending on the condition and severity of the disease. Coronary artery disease, can be managed with lifestyle changes or medication, whereas a serious heart rhythm problem may necessitate the use of an implantable device such as a pacemaker (Burri *et al*., 2021).

A percutaneous coronary intervention (PCI) is a minimally invasive procedure to open blocked coronary arteries (Bergman, *et al*., 2020).

Nitrates undergo complex metabolic changes, reduce intracellular calcium levels, leads to vasodilation, which is the main cardiovascular effect of nitrate (Liu, *et al*., 2020).

Anticoagulants, or blood thinners that reduce blood clotting, are used to treat certain blood vessel, heart, and heart rhythm conditions. These

medications aid in the prevention of harmful blood clots from forming in the blood vessels or heart, as well as the prevention of clots from growing larger and causing more serious problems (Volpe & Gallo, 2020).

The American College of Cardiology (ACC) and the American Heart Association (AHA) guidelines as well as those of European Society of Cardiology (ESC) recommend, for prevention of stent thrombosis after stent deployment, aspirin plus minimum of 6 months of clopidogrel for stable CAD patients with low bleeding risk and aspirin plus 1–3 months of clopidogrel for those with high bleeding risk (Naidu *et al*., 2022).

Cholesterol-lowering medications, such as statins, reduce LDL (the "bad") cholesterol levels in the blood (Byrne, 2019).

Increased blood flow across the body is made possible by angiotensin-converting enzyme (ACE) inhibitors, which work by decreasing levels of hormones that regulate blood pressure. This allows blood to flow more easily throughout the body (Maghiar *et al*., 2020).

Beta-blockers reduce the effects of adrenaline on the heart by reducing the heart rate and lowering blood pressure. This lowers blood pressure, requiring less effort from the heart (Kotecha *et al*., 2017).

Calcium channel blockers are medications that prevent calcium from entering the cells of the blood arteries and the heart. This medicine has the potential to reduce the heart's pumping strength while also relaxing the blood vessels (Cholack *et al*., 2021).

Diuretics, often known as water pills, are medications that help to rid the body of excess fluids and sodium through urination, thereby reducing the burden on the heart. These pills also help to reduce the accumulation of

fluid in the lungs as well as other regions of the body, such as the ankles and legs (Faris *et al*., 2012).

1.11. Genetics and Myocardial Infarction

Genomic medicine is having an impact in several medical fields, including oncology, pharmacology, and cardiology. Genomic information is increasingly being used as part of individual clinical care, and genomic medicine is having an impact in several medical fields, especially in rare and undiagnosed diseases. The Human Genome Project (HGP) has made it possible to use genomic information to enhance health, but translating new discoveries into patient treatment can take years. Since the completion of the HGP, researchers have concentrated on determining how variations in an individual's DNA affect disease and health, elucidating disease etiologies and prognoses, identifying disease susceptibility variants, and improving the efficacy and safety of pharmacological treatments (Khera, *et al*., 2019).

All of these investigations assume that proteins involved in the pathophysiology of atherosclerosis have mutations that make them a possible contributing cause of myocardial infarction. Although there have been over 5000 papers in this subject to far, only a handful of them have found a consistent link between genes involved in lipid metabolism and an increased risk of myocardial infarction (Škrlec, *et al*., 2019).

Single Nucleotide polymorphism that are linked to disease phenotypes are becoming increasingly sought after by researchers. As a final objective, many of these association studies hope to shed light on how diseases work in the body, which in turn will help develop more effective preventative and treatment methods. Discovering disease susceptibility loci in the human genome include the process of scanning markers all

over the genomes of a large number of people (Population) in order to discover genetic variants related with disease by genome-wide association studies (GWAS), it is a non-prescriptive method that uses a large number of markers to identify genetic variations that are associated with a disease phenotype in a large number of samples (Pers *et al*., 2015).

The most important genes increasing the risk for AMI are lymphotoxin-a gene (LTA), LGALS2, LDLR, and APOA5. A deeper understanding of the underlying functional genomic circuits may present new opportunities for research in the future (Tirdea *et al.,* 2022).

A positive family history for myocardial infarction is known to be a major cardiovascular risk factor. As a result, current European guidelines propose that siblings and children of people who have had a MI receive increased primary prevention. Although the genes underpinning the heritable component of MI were previously unknown, the introduction of modern molecular genetic approaches, particularly genome-wide association (GWA) research, has led to the discovery of multiple genetic variations linked to an increased risk of MI (Erdmann *et al*., 2010). Years of experience in conducting wide-genomic association studies (GWAS) have demonstrated that polygenic inheritance of common genetic variants with small effect is a significant part of the risk of developing multifactorial diseases. Based on GWAS data, a polygenic scale has been developed that allows the risk of developing, early MI (Goncharova *et al*., 2020).

With the heritability of CAD and MI estimated at approximately 50% to 60%, understanding the genetic architecture of CAD and MI has proven to be difficult and costly due to the heterogeneity of clinical CAD and the

underlying multi-decade complex pathophysiological processes that involve both genetic and environmental interactions (Dai, 2016).

1.12. Aims of the Study:

1. Study the gene polymorphism of acid ceramidase genes: (rs7844023 and rs2427746) and their role in acute myocardial infarction in Iraqi population.
2. Study of some biochemical parameters (Human acid ceramidase, Human nitric oxide synthase, and Human beta-catenin) in acute myocardial infarction patients and compared to controls, in order to know the benefit of them as a diagnostic parameters.

Chapter Two

Materials and Methods

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Materials

The specific chemicals, kits and instruments used in this study with their suppliers and manufacture origin were listed in tables (2-1) and (2-2).

2.1.1. Chemicals and kits

All the chemicals and the standard kits used in this study were listed in table (2-1).

Table (2-1): Chemicals and kits.

No.	Chemicals	Company/Country
1	Agarose	BDH/ England
2	DNA ladder (100bp)	Bioneer/ Korea
3	Human nitric oxide synthase ELISA Kit	Elabscience/ USA
4	Human beta-catenin ELISA Kit	Elabscience/ USA
5	Human acid ceramidase ELISA Kit	BT-laboratory/ China
6	High sensitivity C-reactive protein ELISA Kit	Elabscience/ USA
7	BFaI Restriction Enzyme	Promega/ USA
8	Bovine Serum Albumin	Promega/USA
9	Buffer C 10X	Promega/USA
10	Ethanol 95%	Systerm/ Malaysia
11	Ethidium Bromide	Sigma / USA
12	G-spin™ Total DNA Extraction Mini Kit	Intron/ Korea
13	Nuclease Free Water	Promega/USA
14	PCR Premix	Bioneer/ Korea

No.	Chemicals	Company/Country
15	Primers	Bioneer/ Korea
16	TBE (Tries Borate EDTA) buffer solution	Bio basic/ Korea

2.1.2. Equipments

Equipments and apparatus used in this study were listed in table (2-2).

Table (2-2): Instruments and equipments

No.	Apparatus & Equipments	Company/Country
1	Autoclave	LabTek/ Korea
2	Balance	Precis / Switzerland
3	Clean View – UV Cabinet	Cleaver / USA
4	ELISA	Human/Germany
5	Digital camera	Canon/ England
6	Electrophoresis apparatus	Consort / Belgium
7	Electrophoresis power supply and tank	Consort / Belgium
8	High speed Centrifuge	Mikro 200R Hettich / Germany
9	Hot Plate Magnetic Stirrer	LabTech DAIHAN / Korea
10	Micro-Centrifuge	BIONEER / Korea
11	Micropipettes	Eppendorf / Germany
12	Nano drop UV-Spectrophotometer	Thermo Fisher Scientific / USA
13	Shaking Water bath	Biobase / China
14	Thermo cycler PCR instrument	Cleaver / USA
15	UV-VIS Spectrophotometer	UV-VIS /Japan
16	Vortex – mixture	(Karlkole) / Germany

2.1.3. The position of rs7844023 (C/T) SNP and rs2427746 (A/G) SNP

The position of rs7844023 (C/T) SNP on STK11 gene was illustrated in figure (2-1) and of rs2427746 (A/G) in figure (2-2).

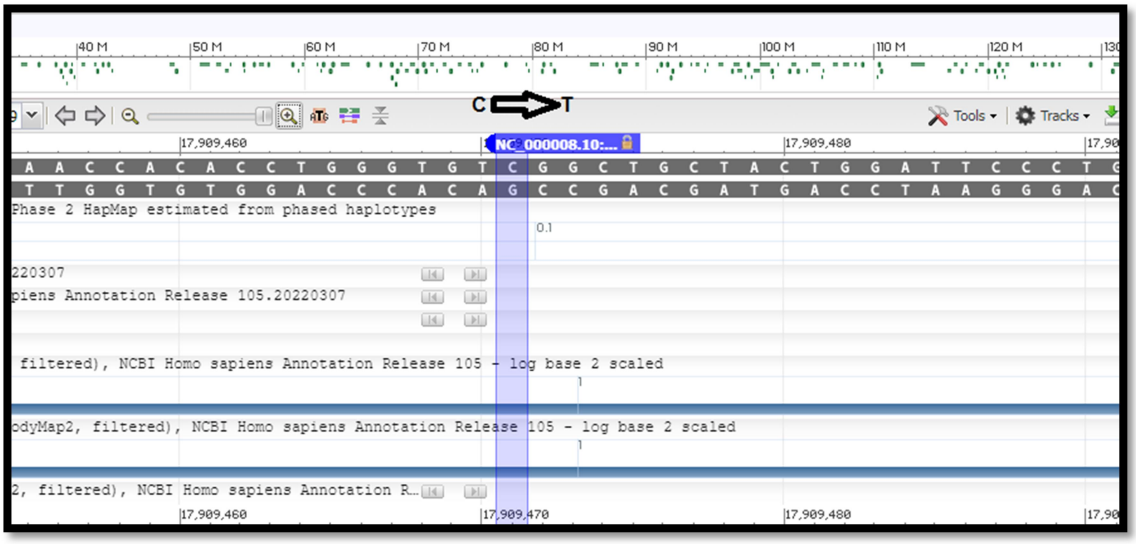


Figure (2-1): Position of SNP rs7844023 (C/T) on gene sequence of acid ceramidase gene; chromosome number 8.

https://www.ncbi.nlm.nih.gov/snp/rs7844023

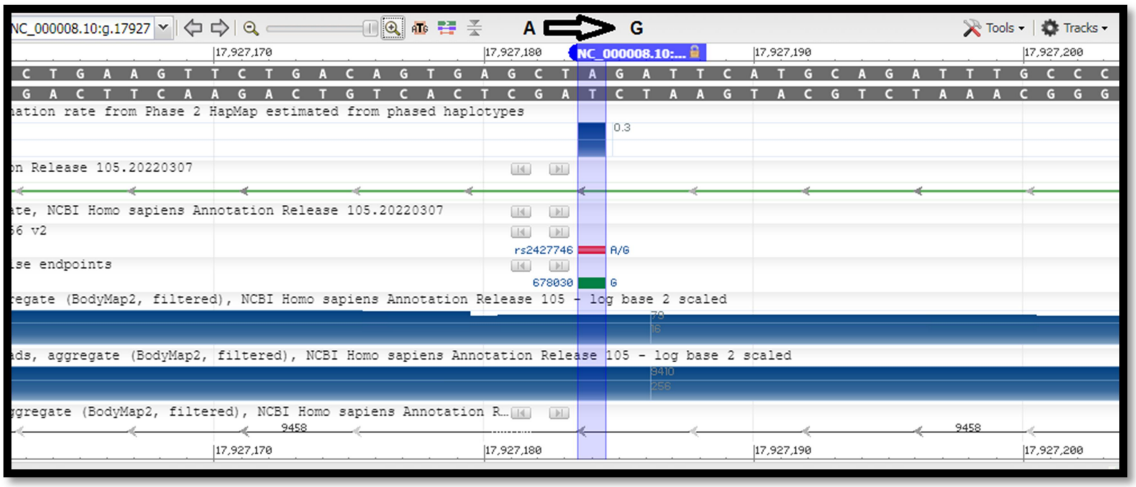


Figure (2-2). Position of SNP rs2427746 (A/G) on gene sequence of acid ceramidase gene; chromosome number 8.

https://www.ncbi.nlm.nih.gov/snp/ rs2427746

2.2. Method

2.2.1.Patients and Controls:

The study was a case-control study. The study was carried out on patients suffering from acute myocardial infarction admitted to Imam Al-Hussein medical city and Karbala center for cardiac diseases and surgery, in Karbala City during the period from March, 2021 till the end of December, 2021. The diagnosis of symptomatic acute myocardial infarction was done by the cardiologist physician depending on the presence of typical symptoms, electrocardiogram (ECG), and biomarkers.

A total number of 120 participants: 60 patients were included in this study, among which 20 were females and 40 were males in age group ranged between 24-80 years with a mean ±SD (56.61 ± 13.32). These were compared with 60 (age and sex matched) apparently healthy controls (20 females, 40 males), with mean age ±SD (54.9 ± 10.03).

At the time when the blood samples were obtained, each patient and apparently healthy control was questioned about demographic parameters.

Questionnaire:

Subjects were asked for information about name, age, past medical history, past surgical history, family history, drugs used, smoking habit, occupation, address, presence of other diseases, and duration of the disease.

2.2.2. Patient's Exclusion Criteria:

Any patient suffered from the following:

- Chronic Inflammatory disease.
- Chronic Infectious disease.
- chronic liver disease.
- chronic renal disease.

- Anemia.
- Thyroid dysfunction.
- Patient on chemotherapy.

2.2.3. Approval of the Ethical Committee:

All subjects involved in this work were informed and the agreement was obtained verbally from each one before collection of the samples. This study was approved by the committee of publication ethics at college of medicine, University of Babylon, Iraq.

2.3.1. Collection and Preparation of the Blood Samples:

After approval by the Ethical Committee of College of medicine, venous blood, (5 ml), was withdrawn from each patient and healthy control who participated in this study.

From withdrawn blood sample of each patient and healthy control, (2ml) was placed in Ethylenediaminetetraacetic acid (EDTA)-tube and used for genetic study, then (3ml) was collected in gel tubes (EDTA-free) and used for serum separation after centrifugation of blood at 1109 Xg for 10 minutes to measure the following parameters: Human high sensitivity C- reactive protein (hs-CRP), Human acid ceramidase (AC). Human nitric oxide synthase (NOs), and Human beta-catenin (BC).

All the laboratory investigations were performed in Imam AL-Hussein Medical City, in Karbala City.

2.3.2. Serum Analysis:

2.3.2.1.Measurement of Serum Human High Sensitivity C-reactive protein (hs-CRP) Concentration:

The Sandwich-Enzyme-Linked Immunosorbent Assay (ELISA) principle was used in this ELISA kit. The antibody specific to Human hs-CRP has been pre-coated on the micro ELISA plate included in this kit.

Standards or samples were mixed with the specific antibody in the micro ELISA plate wells. After that, each micro plate well was treated with a biotinylated detection antibody specific for Human hs-CRP and an Avidin-Horseradish Peroxidase (HRP) conjugate and incubated. The components that weren't needed were rinsed away. Each well was received the substrate solution. Only the wells containing Human hs-CRP, biotinylated detection antibody, and Avidin-HRP conjugate will be colored blue. The addition of stop solution stopped the enzyme-substrate reaction, and the color changed to yellow. At a wavelength of 450 ± 2 nm, the optical density (OD) was determined spectrophotometrically. Human hs-CRP concentration is proportional to the OD value. By comparing the OD of the samples to the standard curve, it may calculate the concentration of Human hs-CRP in the samples.

Preparation of reagents:

1- Before using, all reagents was brought to room temperature (18-25°C).

2. Wash Buffer: To make 750 mL of Wash Buffer, 30 mL of Concentrated Wash Buffer was diluted with 720 mL deionized or distilled water.

If crystals had formed in the concentrate, reheated it in a 40°C water bath and gently mixed it until the crystals had dissolved.

3- The standard was centrifuged at 10,000×g for 1 minute to obtain the standard working solution, 1.0 mL of Reference Standard and Sample Diluent were added, wait 10 minutes, then gently flip it several times. With a pipette, it was thoroughly mixed when it had completely dissolved. This reconstitution yields a 1000 pg/mL working solution. Then, as needed, serial dilutions were made.

The following was the recommended dilution gradient: 1000, 500, 250, 125, 62.5, 31.25, 15.63, 0 pg/mL, 7 tubes were Filled with 500uL each of Reference Standard and Sample Diluent. To make a 500 pg/mL working

solution, 500uL of the 1000 pg/mL working solution was pipetted into the first tube and mixed well. These instructions were followed to pipette 500uL of the solution from the first tube to the second. The illustration below serves as a guide (figure 2-3). The final tube was treated as a blank.

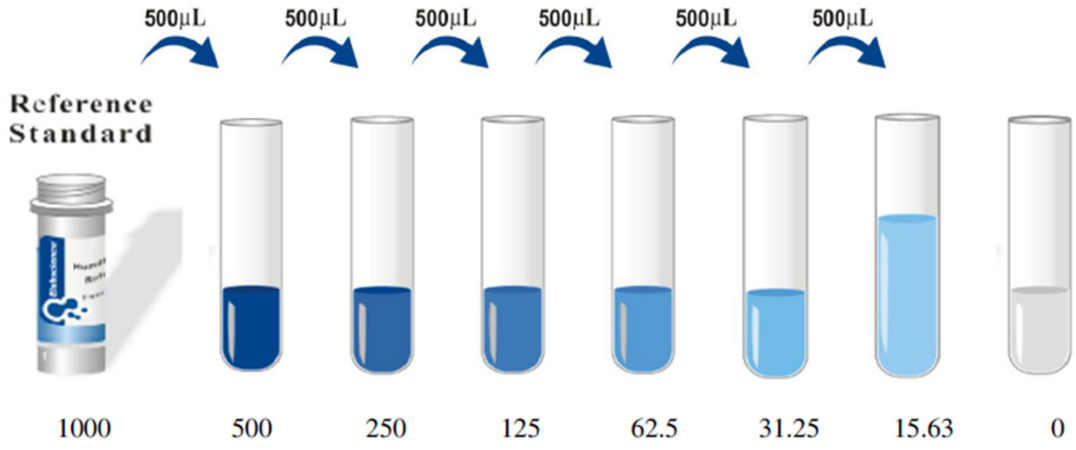


Figure (2-3): Guide for dilution gradient of Human high sensitivity C- reactive protein (hs-CRP) concentration.

4. Each concentration of the solution was added in two wells, side by side (100 uL for each well). The plate was sealed by using the sealer that came with the kit. At 37°C, it was incubated for 90 minutes. To avoid hitting the interior wall and producing foaming, note that solutions should be placed to the bottom of the micro ELISA plate well as much as possible.

5. The liquid from each well was removed without washing it. To each well, immediately 100 µL of Biotinylated Detection Ab working solution was added, the plate was sealed with the Plate Sealer. Everything was mixed together gently. At 37°C, it was incubated for 1 hour.

6. The solution from each well was removed by aspirating or decanting it, then 350 µL of wash buffer was added to each well. After 1 minute of soaking, the solution was aspirated or decanted from each well and pat it dry with clean absorbent paper. This wash procedure should be repeated

three times. This and other wash stages can be done with a microplate washer.

7. Each well was filled with 100 µL of HRP Conjugate working solution. The plate was sealed with the Plate Sealer. At 37°C, it was incubated for 30 minutes.

8. The solution from each well was aspirated or decanted, then the wash process was repeated for a total of five washes.

9. Each well was filled with 90 µL of Substrate Reagent. A fresh coat of plate sealer was applied. At 37°C, it was incubated for around 15 minutes. Light should be kept away from the plate. Note: depending on the actual color change, the reaction time can be cut or extended, but not more than 30 minutes.

Preheated the Microplate Reader for 15 minutes before OD measurement.

10. Each well was filled with 50 µL of Stop Solution. It's important to add the stop solution in the same sequence as the substrate solution.

11. After set microplate reader to 450 nm, the optical density (OD value) of each well was determined at the same time.

Calculation of Results:

The average zero standard optical density was subtracted from the duplicate values for each standard and sample. On log-log graph paper, a four-parameter logistic curve was drew with standard concentration on the x-axis and OD values on the y-axis.

Table (2-3): The Absorbance of Standard Concentration of Human high sensitivity C- reactive protein (hs-CRP)

Concentration (pg/ml)	Absorbance
15.63	0.183
31.25	0.419
62.5	0.506
125	0.827
250	1.498
500	2.381

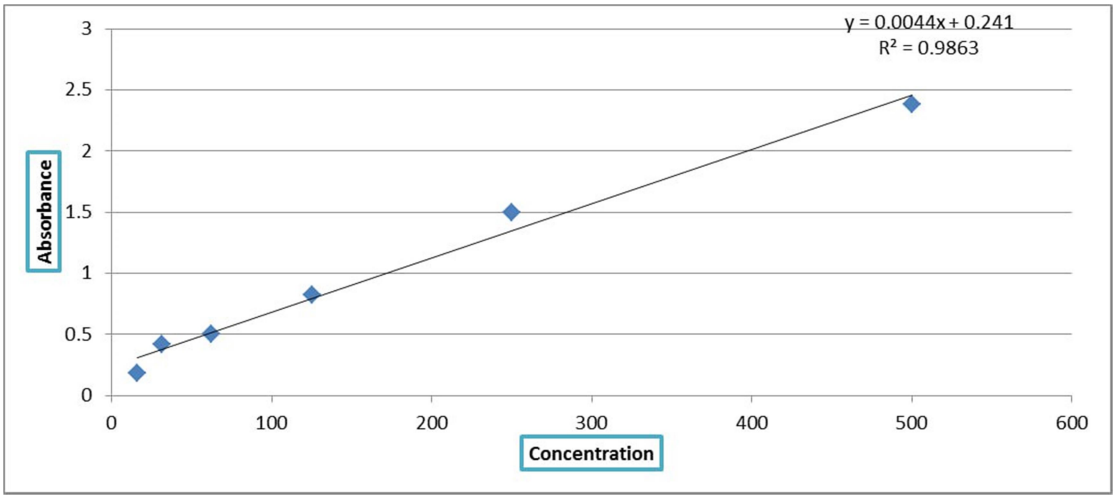


Figure (2-4): The Absorbance of Standard Concentration of Human high sensitivity C- reactive protein (hs-CRP)

2.3.2.2.Measurement of Serum Human Acid Ceramidase (ASAH1) Concentration:

Principle:

An Enzyme-Linked Immunosorbent Assay was included in this kit (ELISA). Human ASAH1 antibody has been pre-coated on the plate. ASAH1 was introduced to the sample and binds to antibodies that had been coated on the wells. The biotinylated Human ASAH1 Antibody was then added to the sample and binds to ASAH1. The Streptavidin-HRP was then added, which binds to the ASAH1 antibody that had been

biotinylated. During the washing phase after incubation, all unbound Streptavidin-HRP was rinsed away. After that, the substrate solution was added, and the color develops in direct proportion to the amount of Human ASAH1. The reaction was stopped by adding an acidic stop solution and measuring the absorbance at 450 nm.

Reagent preparation:

• Before using, all reagents were brought to room temperature.

• Standard, to make a 100ng/ml standard stock solution, 120ul of the standard (200ng/ml) was combined with 120ul of standard diluent. Before producing dilutions, the standard was let set for 15 minutes with gentle agitation. Duplicate standard points were prepared by diluting the standard stock solution (100ng/ml) 1:2 with standard diluent to obtain solutions of 50ng/ml, 25ng/ml, 12.5ng/ml, and 6.25ng/ml. The zero standard was standard diluent (0ng/ml). Any leftover solution should be frozen at -20°C and used within one month of freezing. The following were suggested dilutions of standard solutions:

100ng/ml	Standard No.5	120ul Original standard + 120ul Standard diluent
50ng/ml	Standard No.4	120ul Standard No.5 + 120ul Standard diluent
25ng/ml	Standard No.3	120ul Standard No.4 + 120ul Standard diluent
12.5ng/ml	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
6.25ng/ml	Standard No.1	120ul Standard No.2 + 120ul Standard diluent

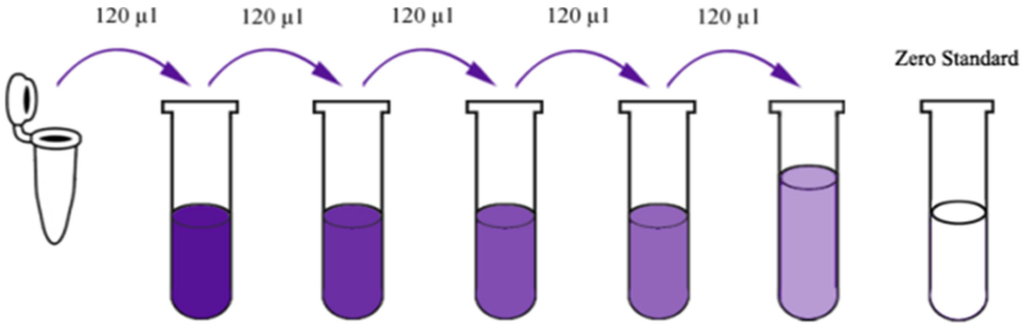


Figure (2-5): Guide for dilution of Human acid ceramidase concentration

Assay Method:

1.Preparation of reagents, standard solutions, and samples. All reagents were pre-heated before used. The test was done at room temperature.

2. The number of test strips needed were calculated. The strips were placed in the frames. Unused strips were stored at 2-8°C.

3. Standard, (50ul), was added to standard well. The standard solution comprises biotinylated antibody.

4. Sample (40ul), 10ul Human ASAH1 antibody, 50ul streptavidin-HRP were added to sample and standard wells (Not blank control well), well blended. the plate was sealed. Incubated 60 minutes at 37°C.

5.With wash buffer, the sealant was removed. Each wash was soaked wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute. Each well was washed 5 times with wash buffer for automated washing. The plate was blotted onto absorbent material.

6.After that, 50ul of substrate solution A was added and then 50ul of substrate solution B was added to each well. Incubated for 10 minutes at 37°C in the dark with a fresh sealer.

7. Stop Solution (50ul) was added to each well, the blue will turn yellow.

8.Within 10 minutes of adding the stop solution, the optical density (OD) of each well was determined using a microplate reader set to 450 nm.

Calculation of Results:

1. This standard curve (figure 2-6) was used to determine the amount in an unknown sample. The standard curve was generated by plotting the average A (450 nm) obtained for each of the six standard concentrations on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis.

The average zero standard optical density was subtracted from the duplicate values for each standard and sample.

Table (2-4): The Absorbance of Standard Concentration of Human acid ceramidase

Concentration (pg/ml)	Absorbance
12.5	0.354
25	0.648
50	0.88
100	0.994
200	1.3
500	2.381

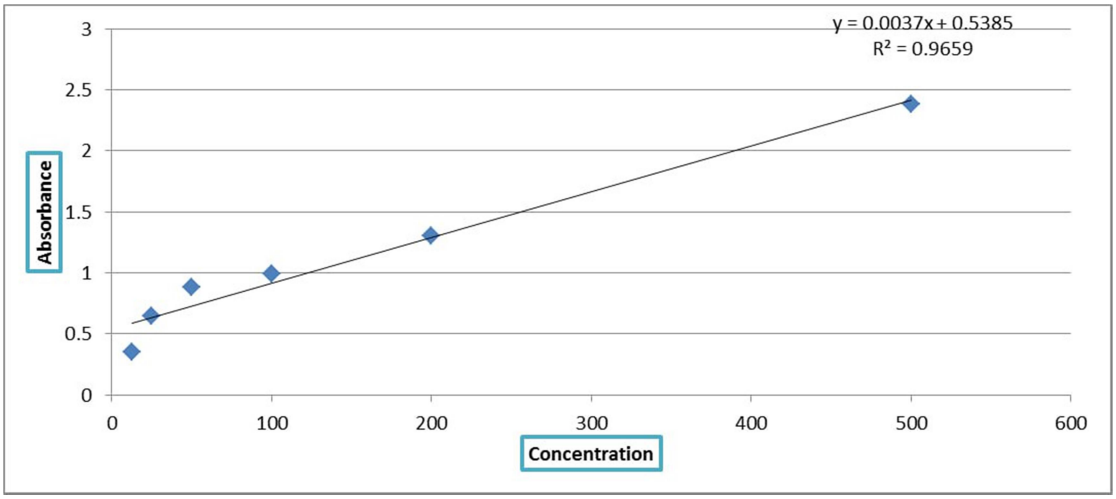


Figure (2-6): The Absorbance of Standard Concentration of Human acid ceramidase

2.3.2.3. Measurement of Serum Human Nitric Oxide Synthase (NOS3/eNOS) Concentration:

Principle:

The Sandwich-ELISA principle was used in this ELISA kit. The antibody specific to Human NOS3/eNOS had been pre-coated on the micro ELISA plate included in this kit. Standards or samples were mixed with the specific antibody in the micro ELISA plate wells. Then a biotinylated detection antibody specific for Human NOS3/eNOS and an Avidin-based detection system were used, each microplate well was incubated after horseradish peroxidase (HRP) conjugate was introduced. Components that were free had been swept away. Each well received the substrate solution. Only those wells containing Human NOS3/eNOS, biotinylated detection antibody and the Avidin-HRP conjugate will be blue. The reaction between the enzyme and the substrate is known as the enzyme-substrate reaction. The color was turned yellow as a result of the addition of stop solution. The optical density (OD) was measured at a wavelength of 450 nm ±2 nm, spectrophotometrically. The OD value was proportional to the amount of human NOS3/eNOS present. The concentration of Human NOS3/eNOS in the samples can be calculated by comparing the OD of the samples to the standard curve samples.

Reagent preparation:

1- Before carrying out the assay procedure, the kit was left at room temperature (18-25°C) for 30 minutes to equilibrate as suggested by the manufacturer; preheated it for 15 min before OD measurement.

2. **Wash Buffer:** Concentrated Wash Buffer (30 mL) was diluted with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer.

3. **Standard working solution:** The standard was centrifuged at 10,000×g for 1 min, 1.0 mL of Reference Standard and Sample Diluent were added, it was let stand for 10 min and inverted it gently several times. After it dissolved fully, it was mixed thoroughly with a pipette. This reconstitution produced a working solution of 4000 pg/mL. Then serial dilutions were made. The recommended dilution gradient was as follows: 4000, 2000, 1000, 500, 250, 125, 62.50, 0 pg/mL.

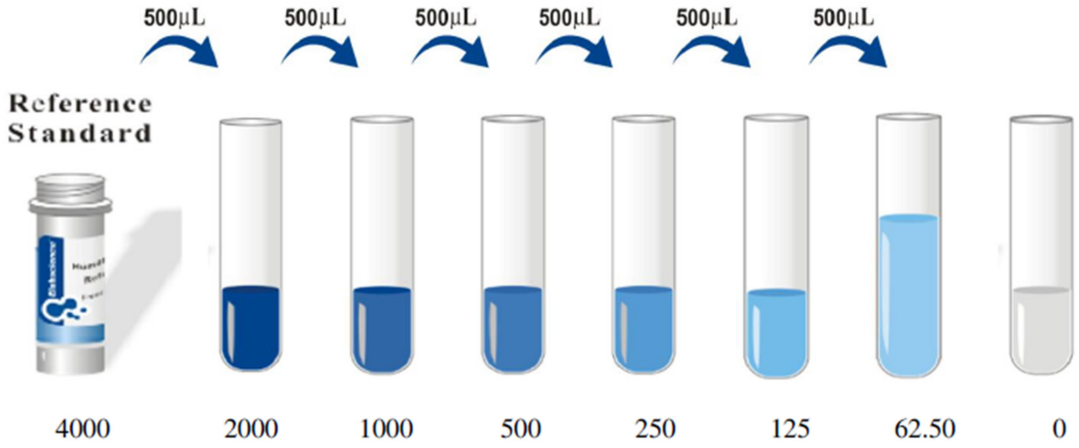


Figure (2-7): Guide for dilution gradient of Serum Human nitric oxide synthase NOS3/eNOS concentration

Assay procedure:

1- One hundred uL of standards and samples were added to each wells then the plate was covered with the sealer provided in the kit. Incubated for 90 min at 37°C.

2- The liquid was removed out of each well, did not washed. Immediately 100 µL of **Biotinylated Detection Ab working solution** was added to each well. Covered with the Plate sealer. Gently it was mixed up. Incubated for 1 hour at 37°C.

3- The solution was aspirated or decanted from each well, 350 uL of wash buffer was added to each well. Soaked for 1~2 min and aspirated or decanted the solution from each well and pat it dry against clean absorbent paper. This wash step was repeated 3 times.

4. **HRP Conjugate working solution** (100 µL) was added to each well. Covered with the Plate sealer. Incubated for 30 min at 37°C.

5. The solution was aspirated or decanted from each well, the wash process was repeated for five times.

6. **Substrate Reagent** (90 µL) was added to each well. Covered with a new plate sealer. Incubated for about 15 min at 37°C.

7. Fifty µl stop solution was added to each well. Adding the stop solution should be done in the same order as the substrate solution.

10. The optical density (OD value) was read at once to 450 nm using a microtiter plate reader.

Calculation the Results:

The average zero standard optical density was subtracted from the duplicate values for each standard and sample.

On log-log graph paper, a four-parameter logistic curve was drew with standard concentration on the x-axis and OD values on the y-axis.

Table (2-5): The Absorbance of Standard Concentration of Human nitric oxide synthase NOS3/eNOS

Concentration (pg/ml)	Absorbance
62.5	0.175
125	0.395
250	0.652
500	0.993
1000	1.558
2000	2.482

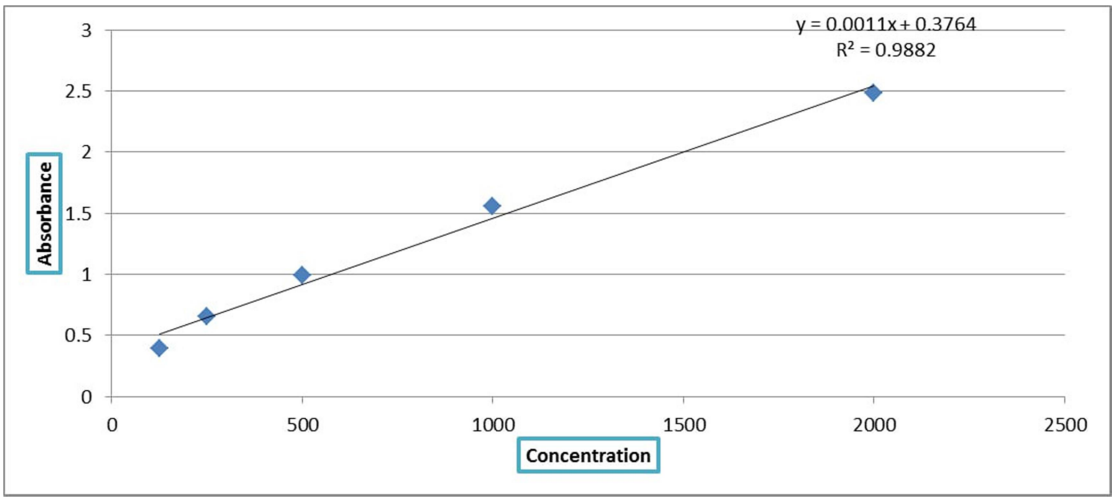


Figure (2-8): The Absorbance of Standard Concentration of Human nitric oxide synthase NOS3/eNOS

2.3.2.4.Measurement of Serum Human Beta-Catenin (CTNNβ1) Concentration:

Principle:

The Kit employs Sandwich-ELISA principle. This kit's micro ELISA plate was pre-coated with an antibody specific for Human CTNNβ1. The specific antibody was applied to the micro ELISA plate wells with standards or samples. Then each microplate well gets a biotinylated detection antibody specific for Human CTNNβ1 and an Avidin-Horseradish Peroxidase (HRP) conjugate and incubated. Free elements washed away. Each well gets a substrate solution. Human CTNNβ1, biotinylated detection antibody, and Avidin-HRP conjugate will appear blue. The stop solution stopped the enzyme-substrate reaction and the color turned yellow. Spectrophotometrically, the optical density was measured at a wave length of 450nm ±2nm. The OD value was proportional to Human CTNNβ1 concentration. Comparing the samples' OD to the standard curve yields the concentration of Human CTNNβ1.

Preparation of the reagent:

1. Before using, all reagents was brought to room temperature (18-25°C). The Microplate Reader was set up according to the instructions in the manual, then it was preheated for 15 minutes before taking the OD measurement.

2. **Wash Buffer:** To make 750 mL of Wash Buffer, 30 mL of Concentrated Wash Buffer was diluted with 720 mL deionized or distilled water.

If crystals had formed in the concentrate, reheated it in a 40°C water bath and gently mixed it until the crystals had dissolved.

3. **The Standard working solution:** The standard was centrifuged for 1 minute at 10,000 ×g, 1.0 mL of Reference Standard and Sample Diluent were added, waited 10 minutes, then gently flipped it several times. With a pipette, thoroughly it was mixed when it had completely dissolved. This procedure yields a workable solution of 10 ng/mL. Then, as needed, serial dilutions were made.

The following was the recommended dilution gradient: 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0 ng/mL.

Method of dilution:

Seven tubes was taken and each was filled with 500uL of Reference Standard and Sample Diluent, 500uL of the 10 ng/mL working solution should be pipetted into the first tube and mixed to make a 5 ng/mL working solution. These instructions were followed to pipette 500uL of the solution from the first tube to the second. The illustration below served as a guide. The final tube was treated as a blank, solution did not pipetted from the previous tube into it.

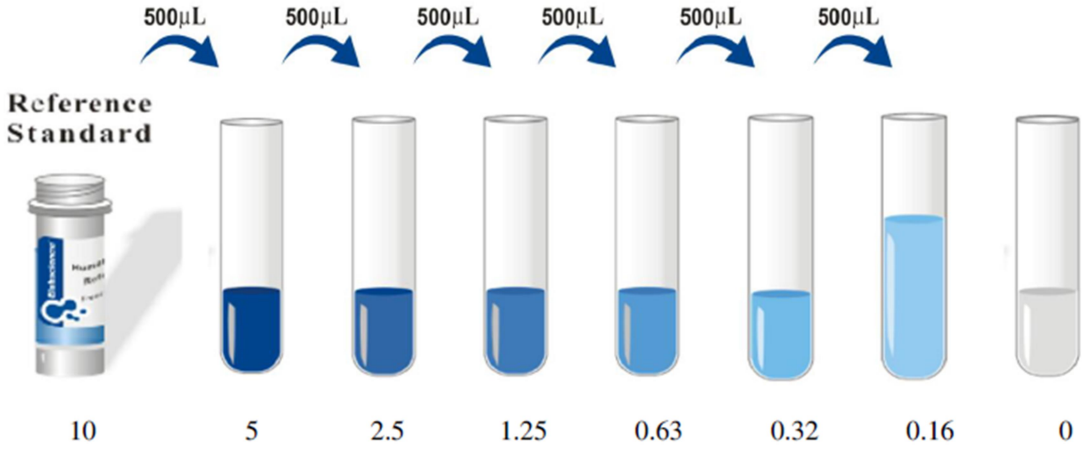


Figure (2-9): Guide for dilution gradient of Serum Human Beta-Catenin (CTNNβ1) Concentration

4. **Biotinylated Detection Ab working solution:** The required amount was calculated before the experiment (100 µL/well).

In preparation, slightly more than calculated should be prepared. The stock tube was centrifuged before used, the 100× Concentrated Biotinylated Detection Ab was diluted to 1×working solution with Biotinylated Detection Ab Diluent.

5. **Concentrated HRP Conjugate working solution:** The required amount was calculated before the experiment (100 µL/well). In preparation, slightly more than calculated should be prepared. The 100× Concentrated HRP Conjugate was diluted to 1× working solution with Concentrated HRP Conjugate Diluent.

Assay Procedure:

1. To the first two columns, the Standard working solution was added: Each concentration of the solution was added in two wells, side by side (100 uL for each well). The remaining wells were filled with the samples (100 uL per well). The sealer that came with the kit was used to seal the plate. At 37°C, incubated for 90 minutes. The solutions should be placed

to the bottom of the micro ELISA plate well as much as possible to avoid hitting the interior wall and producing foaming.

2. The liquid was removed from each well without washing it. To each well, immediately 100 µL of Biotinylated Detection Ab working solution was added. The plate was sealed with the Plate Sealer. Everything was mixed together gently. At 37°C, it was incubated for 1 hour.

3. The solution from each well was removed by aspirating or decanting it, then 350 µL of wash buffer was added to each well. After 1-2 minutes of soaking, the solution from each well was aspirated or decanted and pat it dry with clean absorbent paper. This wash procedure should be repeated three times. This step and other wash stages can be done with a microplate washer.

4. Each well was filled with 100 µL of HRP Conjugate working solution. The plate was sealed with the Plate Sealer. At 37°C, it was incubated for 30 minutes.

5. The solution from each well was aspirated or decanted, then step 3 was repeated for a total of five washes.

6. Each well was filled with 90 µL of Substrate Reagent. A fresh coat of plate sealer was applied. At 37°C, it was incubated for around 15 minutes. Light should be kept away from the plate. Note: depending on the actual color change, the reaction time can be cut or extended, but not more than 30 minutes.

7. Each well was filled with 50 µL of Stop Solution. It's important to add the stop solution in the same sequence as the substrate solution.

8. Microplate reader was set to 450 nm, the optical density (OD value) of each well was determined at the same time.

Calculation of Results:

The average zero standard optical density was subtracted from the duplicate values for each standard and sample.

On log-log graph paper, a four-parameter logistic curve was drew with standard concentration on the x-axis and OD values on the y-axis.

Table (2-6): The Absorbance of Standard Concentration of Human Beta-Catenin (CTNNβ1)

Concentration (pg/ml)	Absorbance
0.16	0.101
0.32	0.158
0.63	0.253
1.25	0.578
2.5	0.898
5	1.562

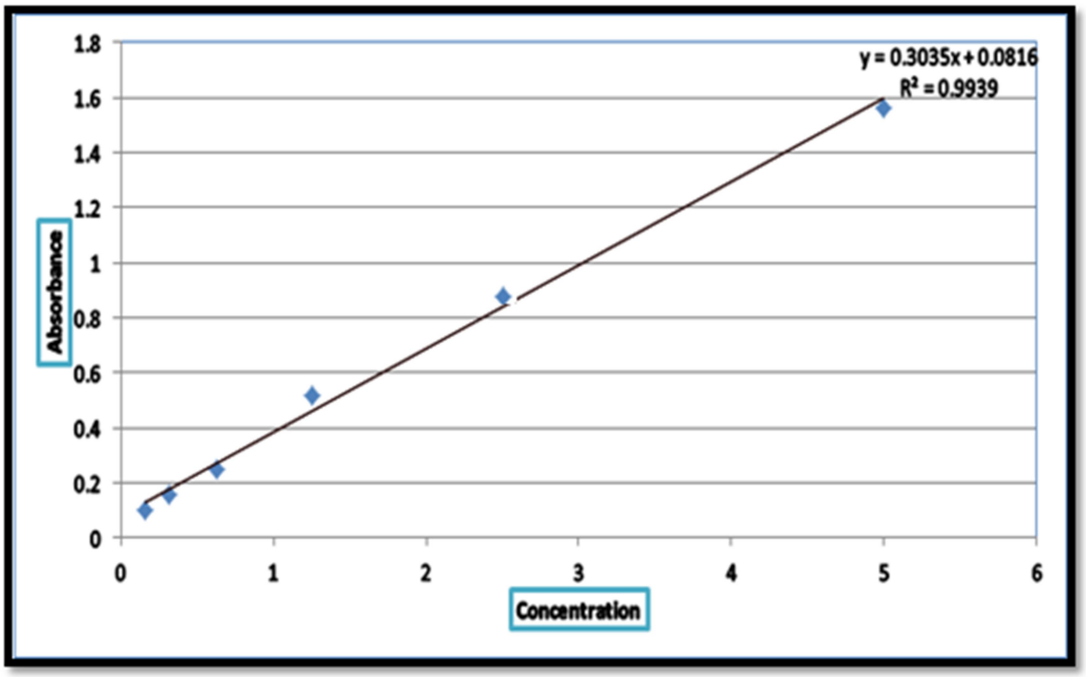


Figure (2-10): The Absorbance of Standard Concentration of Human Beta-Catenin (CTNNβ1)

2.4. Genotyping:

2.4.1. DNA Extraction:

DNA Genome was extracted from blood sample according to protocol G-spin™ Total DNA Extraction Kit.

Principle:

G-spin™ Total DNA Extraction Mini Kit provides a quick, simple technique for preparation of purified and intact DNA from human blood. Samples are processed using a binding column in a micro-centrifuge tube. Up to 200 µl of blood can be processed per purification. The DNA of the isolated genome is of high quality and can be used in common applications such as agarose gel analysis, restriction enzyme process and PCR analysis.

Procedure:

1- A microcentrifuge tube halfway (1.5 ml) was filled with 200 µl of whole blood or bodily fluids, Buffer CL or PBS Buffer was used if the sample volume was less than 200µl.

2. In a sample tube, 20 µl Proteinase K and 5 µl RNase A Solution were combined and mixed carefully.

3- Buffer BL (200 µl) was added into upper sample tube and mixed thoroughly.

4- Two minutes were allowed for the mixture at room temperature.

5. The lysate was incubated for 10 minutes at 56°C.

6- The 1.5 ml tube was centrifuged for a few seconds to remove any drips from the inside of the lid.

7. Vortex 200 µl of 100% ethanol was pulsed into the lysate and it was mixed thoroughly. After mixing, the 1.5 ml tube was centrifuged briefly to remove any remaining drips from the inside of the lid.

8- Carefully the mixture was transferred from step 7 to the Spin Column (in a 2 ml Collection Tube), the cover was closed, and centrifuged at 13,000 rpm for 1 minute. The Spin Column was placed in a new 2 ml Collection Tube and the filtrate was discarded.

9- Buffer WA (Buffer WB) (700 µl) was added to the Spin Column and it was centrifuged for 1 minute at 13,000 rpm without wetting the rim. The flow-through should be discarded and the Collection Tube should be reused.

10- The Spin Column was filled with 700 µl of Buffer WB without soaking the rim, then centrifuged for 1 minute at 13,000 rpm. The flow-through was removed and the Column was inserted in a new 2.0 ml Collection Tube, centrifuged for an additional 1 minute to dry the membrane. The flow-through was removed and Collection Tubes were throw them away.

11- The Spin Column was placed into a new 1.5 ml tube, 100 µl of Buffer CE was poured directly onto the membrane in a fresh 1.5 ml tube, incubated for 1 minute at room temperature before centrifuging for 1 minute at 13,000 rpm.

2.4.2. Determination the Concentration and Purity of the Extracted DNA

Spectrophotometric methods were used to estimate the concentration and purity of extracted DNA. The purity and concentration of DNA were measured by absorbance method using the Nano drop instrument. The absorbance readings were done at 260 nm and at 280 nm. At 260 nm the DNA strongly absorbs light while at 280 nm the protein absorbs light most strongly. DNA purity was measured by the A260/A280 ratio. The A260/A280 ratio 1.8-1.9 is commonly accepted. Initially 1 µL of nuclease free water was smeared on the highly sensitive micro detector of

nano-drop as blank. The micro detector was cleaned up from blank. Then 1 µL of DNA sample was applied on the micro detector of nano-drop. The concentration and A260/A280 ratio of DNA were documented from the instrument.

2.4.3. Primers:

A primer is a short single strand of DNA fragments consisting from (20-27) bases known as oligonucleotides that are a complementary sequence to the target DNA region. It is used to start the process of amplification, without such substance the reaction is not initiated on a single DNA molecule. Thus it should be first annealed to the single strands obtained from denaturation of the double stranded DNA.

Polymerase chain reaction was performed using a specific primer pairs designed for acid ceramidase (AC) gene. Based on National Center for Biotechnology Information (NCBI) database, all gene information and SNPs detail, were collected using Genius software designed.

Preparation of the primers in the following steps: -

Materials: Lypholized primers, Sterile ddH_2O .

1. The tube was spin down before opening the cap.
2. Prepare Master Stock, pmoles/µl, the desired amount of sterile ddH_20 was added according to the manufacturer to obtain a 100 pmoles/µl (Master Stock).
3. The tube was mixed properly to re-suspended the primers equally.
4. Preparing Working Stock, 10 pmoles/µl, ten microliters of the master stock were transferred to a 0.5 ml eppendorf tube that contains 90µl of sterile dH_2O to obtain a 10 pmoles/µl (Working Stock).
5. The master stock was stored at -20 C^o.

The sequence of primers used for PCR amplification of acid ceramidase gene (AC) rs7844023 and rs2427746 SNPs was illustrated in tables (2-7) and (2-8) respectively.

Primer designing:

The study was described a PCR-based identification procedure for (rs7844023, rs2427746) SNPs. It was utilized recently designed species-specific primers targeting acid ceramidase gene, tables (2-7) and (2-8).

Table (2-7): Specific primers of rs7844023 SNPs

Primer	Sequence (5´–3´)	Allele	Size (bp)	Company/ Country
Outer Forward	GAAACTGTCATTTGCAGCTCACA	355		BIONEER/ Korea
Outer reverse	GTGACCAGCGACACATGACTTAA			
Inner forward	TCCAACCACACCTGGGTTTT	T	172	
Inner reverse	CAGGGAATCCAGTAGCAGACG	C	244	

Table (2-8): Specific primers of rs2427746 SNPs

Primer	Sequence (5´–3´)	Allele	Size (bp)	Company/ Country
Outer Forward	CTCTGGATGTCCCTGAGACACTT	379		BIONEER/ Korea
Outer reverse	CCAAGTGGAAAAGTTATGCAGGT			
Inner forward	CCTCTGAAGTTCTGACAGTGAGATG	G	118	
Inner reverse	GGGCAAATCTGCATGAAGCT	A	243	

In order to amplify the target acid ceramidase gene (AC) polymorphism, T.ARMS-PCR was performed in 25 µl volumes in PCR tubes under sterile conditions, all the volume of the reaction mixture was completed to 25 µl with using double-distilled water (ddH_2O) and the master mix

which contained optimum concentrations of reaction requirements (MgCl2 1.5 mM, Taq polymerase 1 U, each dNTPs 200 µM) had been used, table (2-9).

Table (2-9): Components of Master Mix of Specific Site on Ceramidase Gene Polymorphism

No.	Material	Volume(µl)
1	Master Mix	12.5
2	Outer Forward	1
3	Outer Reverse	1
4	Inner Forward	1
5	Inner Reverse	1
6	Template DNA	4
7	DDH_2O	4.5
Total		25 µl

Table (2-10): Specific primers of RFLP for rs2427746 SNPs

Primer	Sequence (5′ –3′)	Size (bp)	Restriction enzyme
Forward	CTCTGGATGTCCCTGAGACACT	514	BFaI
Reverse	CTCTTTTAGCTACATATGGGTAAGCCA		

2.4.4. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) has become one of the most important techniques in modern biological and medical science (Kadri, 2019). It amplifies a specific region of a DNA strand to generate thousands to millions of copies of a particular DNA sequence.

A basic PCR usually requires the following:

1) DNA template containing the target DNA region.
2) Two primers to initiate DNA synthesis.
3) A thermostable DNA polymerase to catalyze DNA synthesis.

4) Deoxynucleoside triphosphates (dNTPs, the building blocks of new DNA strand).

5) Buffer including bivalent cations, usually Mg^{+2}.

There are three steps of a PCR that are cycled about 25-35 times, this steps including the following:

- **Denaturation:** This step includes separation of the double DNA strands into two single strands are accomplished by heating for about 94-95°C.
- **Annealing:** At lower temperature (40-45°C), DNA primers (which are short single strand DNA fragments) attach to the ends of each strands of the DNA and initiates the reaction.
- **Extension:** This step occurs at 72°C, where each primer binding to the DNA template will be extended complementary to the template DNA. This process is carried out in the presence of the Taq DNA polymerase, because of its ability to operate efficiently at high temperatures.

2.4.5. Optimization of PCR Conditions

Different volumes of primer (0.5 µl,1 µl, 1.5 µl,) with different volumes of template DNA (1 µl,2 µl,3 µl,4 µl,5 µl, 6 µl) and different experiments of the reaction conditions were tried in order to optimize the conditions of the reaction. PCR tube centrifuged for 30 seconds at 2000 relative centrifugal force (rcf) in a micro-centrifuge in order to mix solutions well at room temperature, then the tubes were placed in the thermocycler to start the reaction.

Programs of the PCR protocol reaction for acid ceramidase gene (AC) polymorphism for each rs7844023 and rs2427746 were illustrated in tables (2-11) and (2-12).

Table (2-11): PCR-T.ARMS program for detection of acid ceramidase gene (AC) rs7844023 SNPs

No.	Stage	Cycle	Step	Temp.	Time
1	Initial Denaturation	1	1	92 °C	2min.
2	Denaturation	45	1	92 °C	30 sec.
3	Annealing	45	2	45 °C	30 sec.
4	Extension	45	3	72 °C	20 sec.
5	Final Extension	1	1	72 °C	5 min.
6	Hold Phase			10 °C	

Table (2-12): PCR- T.ARMS program for detection of acid ceramidase rs2427746 SNPs

No.	Stage	Cycle	Step	Temp.	Time
1	Initial Denaturation	1	1	92 °C	2min.
2	Denaturation	45	1	92 °C	30 sec.
3	Annealing	45	2	40 °C	30 sec.
4	Extension	45	3	72 °C	45sec.
5	Final Extension	1	1	72 °C	5 min.
6	Hold Phase			10 °C	

One PCR reaction were performed, one with outer forward primer rs7844023 (OF) and outer reverse primer rs7844023 (OR) and inner forward rs7844023 (IF) and inner reverse rs7844023 (IR) fore detection of acid ceramidase rs7844023 SNPs. The same thing with rs2427746 SNP, one PCR reaction were performed, one with outer forward primer rs2427746 (OF) and outer reverse primer rs2427746 (OR) and inner forward rs2427746 (IF) and inner reverse rs2427746 (IR) fore detection of acid ceramidase rs2427746 SNPs, and run side by side on an agarose gel.

Table (2-13): Bands obtained from the amplification product of rs2427746 and rs7844023 SNPs

Genotype			Size of bands (bp)	
	rs7844023	rs2427746	rs7844023	rs2427746
Wild type	CC	AA	244	243
Heterozygous	CT	AG	244, 172	243, 118
Homozygous Mutation	TT	GG	172	118

2.4.6. Restriction Fragment Length Polymorphism (RFLP)

2.4.6.1. Principle of Restriction Fragment Length Polymorphism:

The principle behind a restriction fragment length polymorphism (RFLP) analysis is that restriction endonucleases recognize short stretches of DNA (generally four or six base pairs) that contain specific nucleotide sequences. These sequences, which differ for each restriction endonuclease, are palindromes, that is, they exhibit twofold rotational symmetry. This means that, within a short region of the double helix, the nucleotide sequence on the "top" strand, read 5′→3′, is identical to that of the "bottom" strand, also read in the 5′→3′ direction. Therefore, if you turn the page upside down—that is, rotate it 180 degrees around its axis of symmetry—the structure remains the same. A restriction enzyme is named according to the organism from which it was isolated and each restriction enzyme has specific requirements to achieve optimal activity. A DNA sequence that is recognized by a restriction enzyme is called a restriction site or enzyme recognition site. These sites are recognized by restriction endonucleases that cleave DNA into fragments of different sizes (Denhart *et al*. 2001).

2.4.6.2.BFaI Restriction Enzyme

The lyophilized vial contained the following components:

1- BFaI restriction enzyme; 1000 unit (10u/µl after reconstitution).
2- Buffer C. 10X composed of 100mM Tris HCL pH(7.9), 500mM NaCl,100mM MgCl2 and 10m M DTT at 37^0 C. This buffer always yield 100% activity for the enzyme it accompanies.
3- **Bovine serum albumin (BSA) in restriction enzyme reaction.100x**

All Promega restriction enzyme activity assays were performed with the addition of acetylated BSA to a final concentration of 0.1 mg/ml. Bovine serum albumin (BSA) had been shown to increase the activity and efficiency of many restriction enzyme.

2.4.6.3.Characteristics of Restriction Enzyme

Characteristics of restriction enzyme were displayed in table (2-14).

Table (2-14): Characteristics of Restriction Enzyme

Restriction Enzyme	BFaI
Recognition sequence	5..C▼TAG..3 3..GAT▲C..5
Source	*Bacteroides fragilis*
Optimal assay temperature	37 ^{0}C
Incubation time	1-4 hours

2.4.6.4.Optimization of Digestion Conditions

Optimization of digestion conditions was done by using the following:

1) Different volumes of enzyme (0.5 µl,0.7 µl and 1 µl).

2) Different volumes of PCR product (7 µl, 8 µl and 10 µl).

3) Different incubation time (2 hrs, and 3hrs).

Digestion conditions that gave best result were summarized in table (2-15). The digestion reaction volume was 20 µl in 0.5 ml PCR tube, done on ice, then it was centrifuged in a microcentrifuge for few seconds at 2000 relative centrifugal force (rcf), mixed by pipetting and incubated for the intended period.

Table (2-15): Digestion Reaction Volumes

Reagents	Volumes for 1x
PCR product	8 µl
Buffer C 10X	2 µl
Nuclease free water	8.8 µl
BSA 1X	0.2µl
Restriction enzyme	1 µl (10u)
Incubation time 3 hours	

2.4.7. Agarose Gel Electrophoresis:

Electrophoresis through agarose is a standard method used to separate, identify and purify DNA fragments and PCR product. The technique is simple and rapid to perform and capable of resolving fragments of DNA that cannot be separated adequately by other procedures.

The agarose gel electrophoresis was done, and ethidium bromide staining was added (2-3 µl) , which can bind with DNA by forming close van der Walls contacts with the base pairs and that's why it binds to the hydrophobic interior of the DNA molecule. Molecules that bind in this manner are called intercalating agents because they intercalate into the compact array of stacked bases, 2% agarose was prepared using this same protocol.

The separation in this way uses an electric current to migrate the biomolecules through a porous gel matrix at a rate that is proportional to the particle charge, size and shape (Lee *et al*., 2012).

Procedure:

One hundred milliliters of a 2% agarose solution were prepared by the following steps (Green & Sambrook, 2019):

A. Preparation of TBE buffer solution:

1X TBE buffer (tris borate EDTA) was prepared by diluting 10 TBE buffer with deionized water (one volume of 10X TBE buffer with 9 volume of deionized water 1:10 dilution).

B. Preparation of the agarose gel:

1. Two grams of agarose were weighted and placed inside a conical flask and 100 mL of 1x buffer (TBE) was added with gentle mixing.
2. The solution was placed in the microwave for one minute until the agarose was completely dissolved and the solution became clear and then allowed to cool to about 55 °C before pouring.
3. Ethidium Bromide dye solution (2µl) was added to the solution.
4. The gel tray ends were closed with tape.
5. The comb was placed in the gel tray about 1 inch from one end of the tray.
6. The gel solution was poured into the chamber and allowed to solidify for 30 minutes at room temperature.
7. The comb was removed from the gel and the chamber was placed in a horizontal electrical system and was covered (only until the wells were flooded) with the same buffer TBE used to prepare the gel.
8. From the samples, (6 µl) were loaded on each well with extreme care to avoid damage to wells and contamination of adjacent wells.

9. Cathode pole was connected to the well side of the unit and the anode to the other side.
10. Electrophoresis was performed at 75 V, for 60-100 min or until the dye markers were migrated at an appropriate distance, depending on the size of the DNA to be visualized.

2.4.8. Photo Documentation:

The agarose gel was placed over an ultraviolet transilluminator device and subjected to ultraviolet light and the images were captured using a digital camera and conceived by a computer connected to the transilluminator.

2.4.9. DNA Sequencing:

Samples of DNA from patients and normal people were sent to South Korea for a Sanger sequencing techniques, The samples included a total of 20 microns of PCR product and 17 picomoles of both primers (Outer Primers) belonging to both rs7844023 and rs2427746 SNPs.

2.5. Limitation of Study:

1- Pandemic Corona virus.

2- Cost of kits.

3- Time limit.

2.6. Statistical Analysis:

Microsoft Excel program (Version 2016) with Statistical package for the social sciences (SPSS) (Version 25) were used in current study. Chi-square test was used for comparison of demographic parameters. All values were expressed as mean± standard error (M± SE). Student's t-test was used to collectively indicate the presence of any significant difference between the two groups for a normally distributed quantitative variable. Pearson's correlation analysis was used to show the strength and

direction of association between two quantitative variables, the Shapiro–Wilk test are most widely used methods to test the normality of the data.

The alleles and genotypes differences between each case compared with controls were determinated by odd ratio and 95% confidence interval. PAleontological STatistics Past 3.0 (Texas, United States) was used to generate the receiver operating characteristic (ROC) curve and for calculating the area under the curve.

Chapter Three

Results and Discussion

Chapter Three

Results and Discussion

3.1. Demographic Characteristics of Patients

3.1.1. Gender

Patients with acute myocardial infarction were classified according to the gender into two groups, as shown in figure (3-1). There were 40 males and 20 females, and this represents 66.7% and 33.3% of patients respectively.

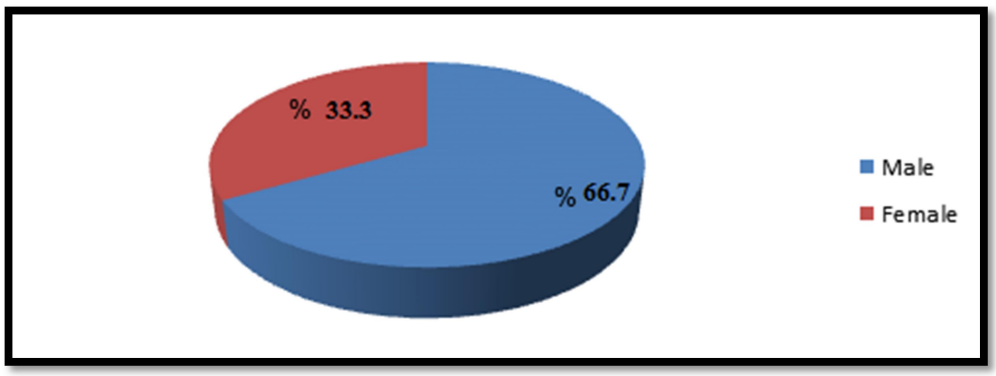


Figure (3-1): Gender distribution of patients group

The results of the current study were showed an increasing percentage in the number of males compared to females.

Regarding to the study subjects gender, the results indicate, that the higher percentage of the study sample were male, figure (3-1). This result comes along with (Saleem *et. al*., 2011) all of them mentioned that the male is the dominant gender for patients with myocardial infarction.

There was an increase in the mean of males compared to females and this is consistent with some studies that consider males have slightly higher rates of cardiovascular disorders than females (Millett *et al*., 2018). In the general

population, women tend to have more infarctions involving the anterior circulation (Claire , 2013).

The incidence of MI in men is higher than women in all age groups but this difference narrows with advanced age. Women suffer less from heart disease due to vasculoprotective action of oestrogen which helps in preventing atherosclerosis (Vaccarino *et al*., 2018).

Studies have shown that changes in serum sex hormone levels may contribute to CVD etiology (Suthahar *et al*., 2020).

3.1.2. Age

Patients with acute myocardial infarction were classified according to the age into two groups, as shown in figure (3-2).

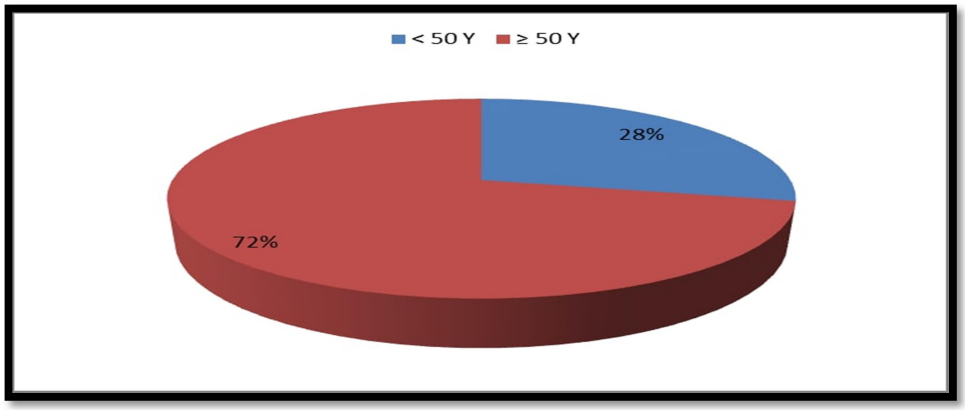


Figure (3-2): Age distribution of patients group

Patients were involved in this study had an age of 56.61 ± 13.32 years. The minimum and maximum ages were 24 and 80 years respectively, in which about 28% of the patients were in the age <50 years of old and 72 % were in the age ≥ 50 years of old. The mean ± SD age of patients more than and equal 50 years groups 63.32 ± 6.4, and 40.41 ± 2.9 for less than 50 years. T-test was found to be (0.001), and there was a significant difference between acute myocardial infarction with age groups.

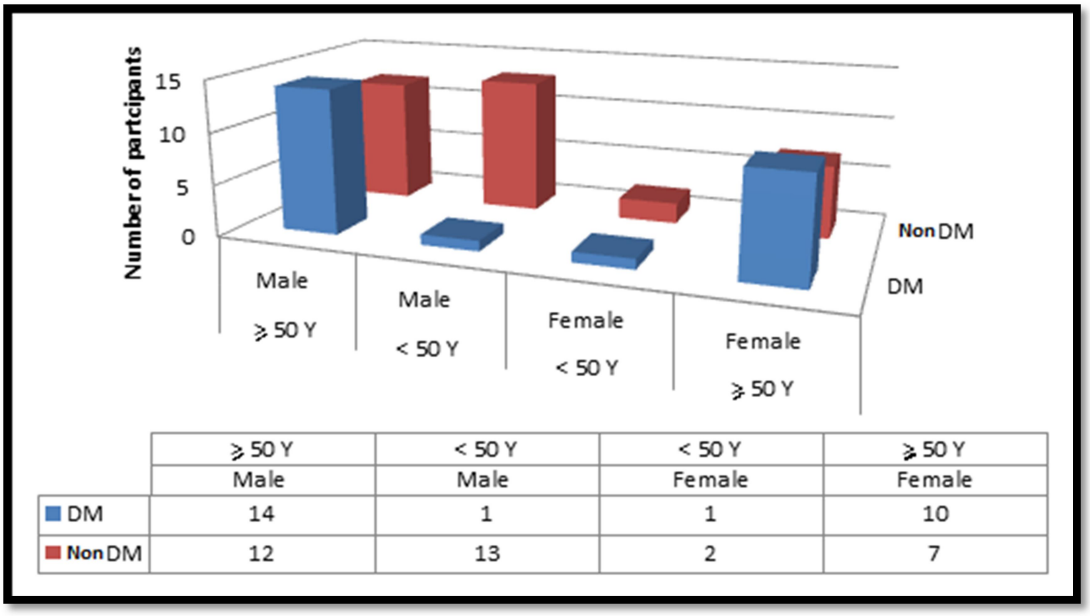
Although MI may occur at any age, but it occurs mainly in the age between 55-85 years of old (Groenewegen, *et al*., 2020). The frequency of MI rises sharply beginning at the age of 40, partly because traditional risk factors started to accumulate around the age of 44 years (Putaala, *et al*., 2009). The prevalence of acute myocardial infarction was higher in men as compared to women. This difference is present in all age groups. The age of onset also appears to be earlier in men. The age distribution showed expected patterns of the rising prevalence and incidence with increasing age (Khan, *et al*., 2020).

Acute heart diseases such as angina pectoris and myocardial infarctions affected a large percentage of the world's population, the majority of patients (80 %) were males older than 50 years old, while only 30 % of patients were female. The difference between males and females in regards to the incidence rate of various diseases is attributed to many factors, including physiological, psychological, and social factors (Iestra *et al*., 2005).

3.1.3. Study Groups with Diabetic Mellitus

In the present study, acute myocardial infarction patients were classified into sub- groups depending on diabetes mellitus as shown in figure (3-3). It was found that: less than 50 years old male: with diabetes (N=1; 7.14%) and without diabetes (N=13; 92.86%), and males over and equal 50 years: with diabetes (N=14; 53.8%) and without diabetes (N=12; 46.2%).

On the other hand, less than 50 years old female: with diabetes (N=1; 33.3%) and without diabetes (N=2; 66.6%), and females over and equal 50 years: with diabetes (N=10; 58.8%) and without diabetes (N=7; 41.2%).



	⩾ 50 Y Male	< 50 Y Male	< 50 Y Female	⩾ 50 Y Female
DM	14	1	1	10
Non DM	12	13	2	7

Figure (3-3): Acute myocardial infarction patients with Diabetes Mellitus

The result indicated that the most cases of diabetes for heart patients were at the age above and equal 50 years for both sexes, it was recorded (35%) and (50%) for male and female had diabetes mellitus in more than and equal 50 years related with gender, respectively, it was more than in patients without diabetes mellitus. The result found that the most cases of non-diabetes for heart patients for both sexes were recorded (32.5%) and (10%) for male and female less than 50 years related with gender, respectively.

Diabetes patients are more likely to develop vascular diseases, including MI (Glovaci *et al*., 2019). Many factors contribute to the development of MI in diabetic patients. Insulin resistance, hyperinsulinaemia, hyperglycemia, hypertension, obesity, and dyslipidemia are some of factors that promote vascular injury; they not only increase the risk of MI, but they also contribute to the severity of these diseases (Schmidt , 2019). Based on the results of this study, the majority of people with heart disease who also had diabetes are over the age of 50, with the prevalence of diabetes being 35% for male and 50% for female patients over the

age of 50, respectively, compared to patients without diabetes mellitus. However, the study indicated that the highest rates of non-diabetes for cardiac patients were observed for men and women under the age of 50 (32.5%) and 10%, respectively.

Some of previously studies were shown that there is interconnection between hyperglycemia, and the prevalence of coronary heart disease (CHD) (Ford *et al.* (2002) ; Montazerifar *et al.*, 2016).

The results indicated, figure (3-3), that (43.3%) of acute myocardial infarction patients had Diabetes mellitus. This result is supported with the (Holman *et. al.*, 2017) the results indicated that the higher percentages diabetic patients were suffering from angina and myocardial infarction.

3.1.4. Smoking Status

According to the history of smoking among acute myocardial infarction patients, the smokers were 20 (33%), and non-smokers (never) were 40 (67%), as shown in figure (3-4). The percentage of males those were smokers 96% against 4% females.

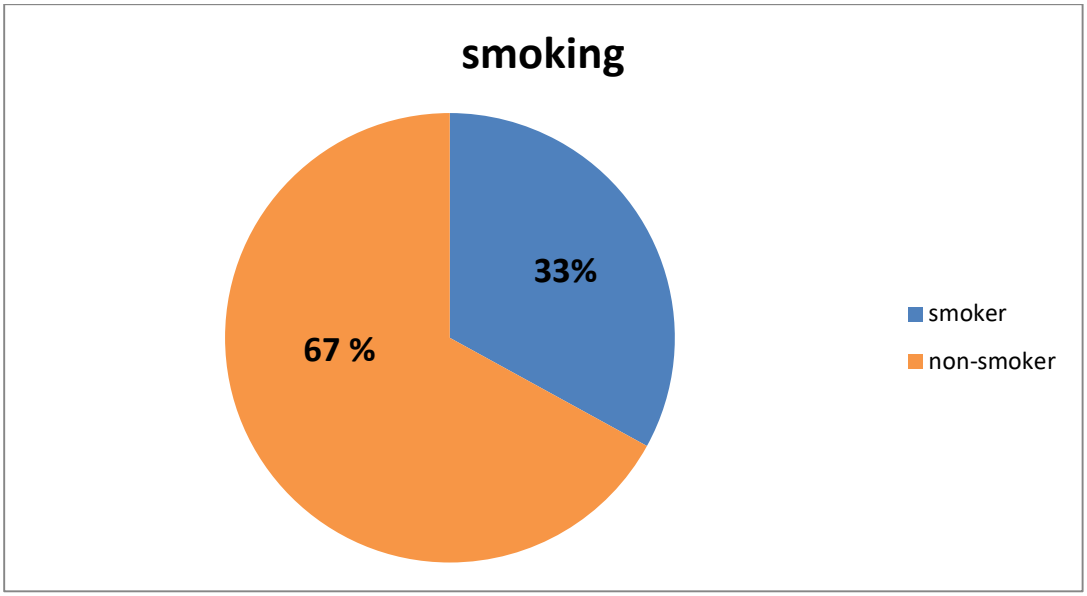


Figure (3-4): Distribution of patients depending on smoking status

The analysis was conducted 2 items of the questionnaire that assess the adherence with smoking and drinking alcohol, on scale of yes and no. This finding was supported by (Jordan , *et al* ., 2011; Koole , *et al* ., 2012) who stated that as a general, there is a link between alcohol and tobacco using and the level of the fat in the blood (triglycerides) with relation to the coronary heart disease.

Cigarette smoking is a significant modifiable risk factor for coronary artery disease, stroke, peripheral vascular disease, and congestive heart failure (Kondo *et al*., 2019). Cigarette smoke induces cerebrovascular and cardiovascular disorders by a variety of mechanisms that are synergistic (Flouris *et al*., 2010). Thrombosis, endothelial dysfunction, atherosclerosis, and hemodynamic consequences are just a few of them (Halperin *et al*., 2010). Smoking promotes thrombosis by increasing platelet adhesion to the endothelium and platelet aggregation, and endothelial dysfunction as a result of oxidative stress mediated by lipid peroxidation and the generation of free radicals (Bazzano, *et al*., 2003).

Health team members such as workers and physicians, occupy position of a large influence in helping the patient to adept anew positive lifestyle activities to the incidence of coronary heart disease, health professional recommended that changing to healthy behaviors, such as healthy diet, medical regimens, stop smoking, have an important role to decrease the incidence of the disease (Rippe, *et., al*. 2007).

3.1.5. Hypertension

The results of the study showed that there were 47 patients (78.3%) suffered from high blood pressure and had heart disease, As for the remaining 13 patients (21.7%), they did not suffer from high blood pressure as shown in the figure (3-5).

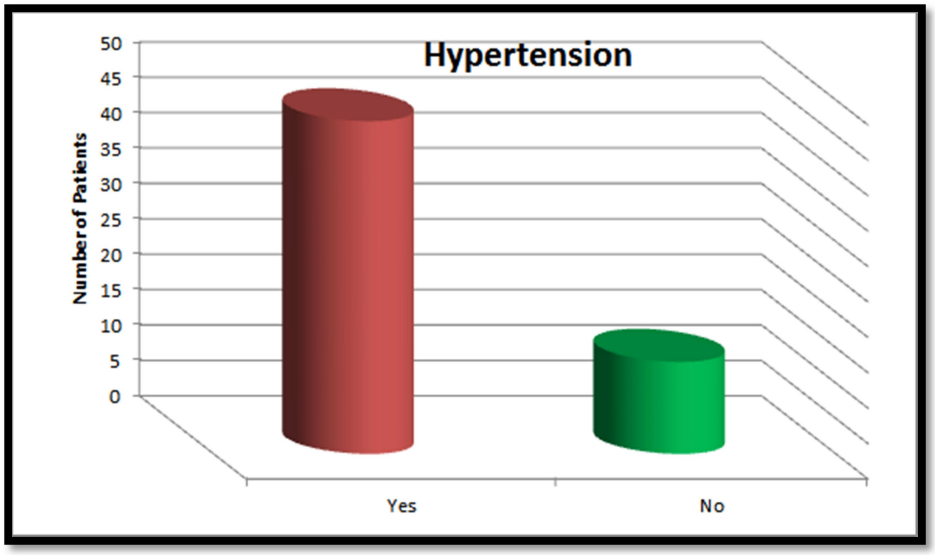


Figure (3-5): Acute myocardial infarction patients with Hypertension

Ischemic heart disease is a condition that can be brought on by hypertension. This condition occurs when the muscle of the heart does not receive sufficient blood supply (Dorobantu *et al*., 2016).

Ischemic heart illness may be accompanied by the following symptoms when dealing with patients who have high blood pressure, it should always be on the lookout for target organ damage (such as left ventricular hypertrophy, microalbuminuria, and retinal angiosclerosis/retinopathy) as well as for the problems that can arise as a result of this damage (ventricular dysfunction and manifestation of ischemic disease) (Mensah *et al*., 2017).

3.1.6. Residence and Acute Myocardial Infarction

The results of the study showed that there were 46 patients (76.7%) who lived in the urban had heart disease, as for the remaining 14 patients (23.3%), they were lived in the rural areas as shown in the figure (3-6).

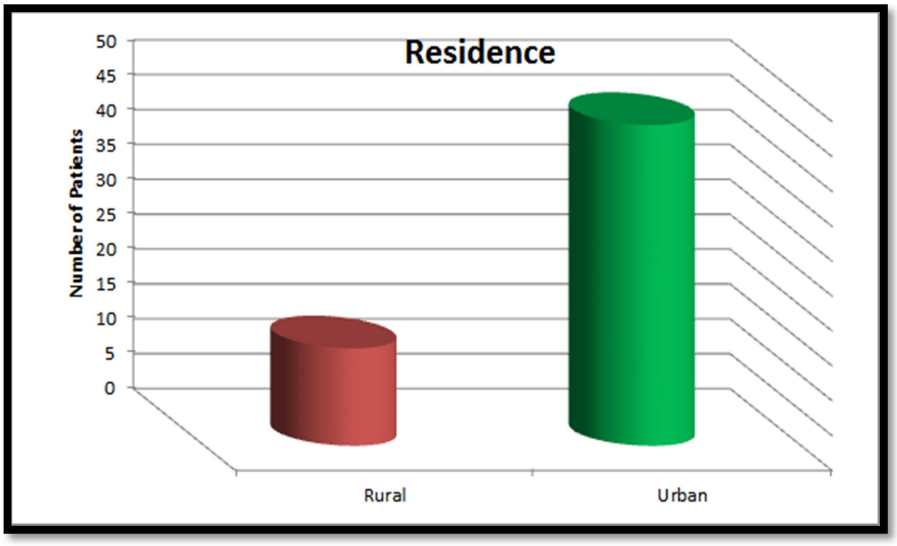


Figure (3-6): Residence and acute myocardial infarction patients

The results of this study indicated that the majority of those included in the sample were residents of urban residential areas, figure (3-6). This findings matches with that of (Ma, *et al*., 2020), the findings of which indicate that the majority of the study subjects live in large cities rather than in rural areas. A statistically significant relationships have been reported between early residency status and later hypertension, diabetes, and coronary heart disease in some studies (Rehkopf *et al*., 2015), an education and economic inequality were the most often linked features (Hamad *et al*., 2016).

Many prior epidemiologic studies have linked air pollution to an increased risk of ischemic heart disease, especially long-term exposure (Huang, *et al*., 2018), A case-crossover research found that a 10 g/m3 increase in fine particle pollution (PM2.5) increased the incidence of ischemic heart disease by 4.5 percent (95 %CI 1.1–8.0). A time-series analysis found that greater exposure to SO2, and NO2 for 3 days increased the probability of ischemic heart disease death (Li *et al*., 2018).

According to the findings of this study, the majority of the sample resides in urban residential areas. This conclusion is consistent with (Okoro and Ngong, 2012),

who's findings indicate that the most of participants in the study are city dwellers, not people living in the countryside.

Furthermore, (Sarrafzadegan *et al*., 2012) reported that the majority of study participants reside in urban residential areas, while the remaining participants reside in rural areas. Also, these outcomes may be related to myocardial infarction associated with sophisticated modern society.

The incidence of ischemic heart disease may be higher among urban residents due to their complex lifestyles than among rural residents. Also, rural residents engaged in more daily physical activity than their urban counterparts, which reduces their chance of developing ischemic heart disease. That's why people living in the country have a lower risk of developing coronary artery disease (Nowicki, *et al*.,2018).

The difference in the type of food also played an important role in increasing the incidence of heart disease, also, the patients with a diverse diet and a middle-class economic position had the highest rates of coronary heart disease (CHD). Patients who were given an extra meal had a higher coronary heart disease risk than those who did not receive an extra meal (Tahir *et al*., 2013).

3.1.7. Employer and Acute Myocardial Infarction

The results of the study showed that there were 38 patients (63.3%) who were non employers had heart disease, as for the remaining 22 employer patients (36.7%) had ischemic heart disease as shown in the figure (3-7).

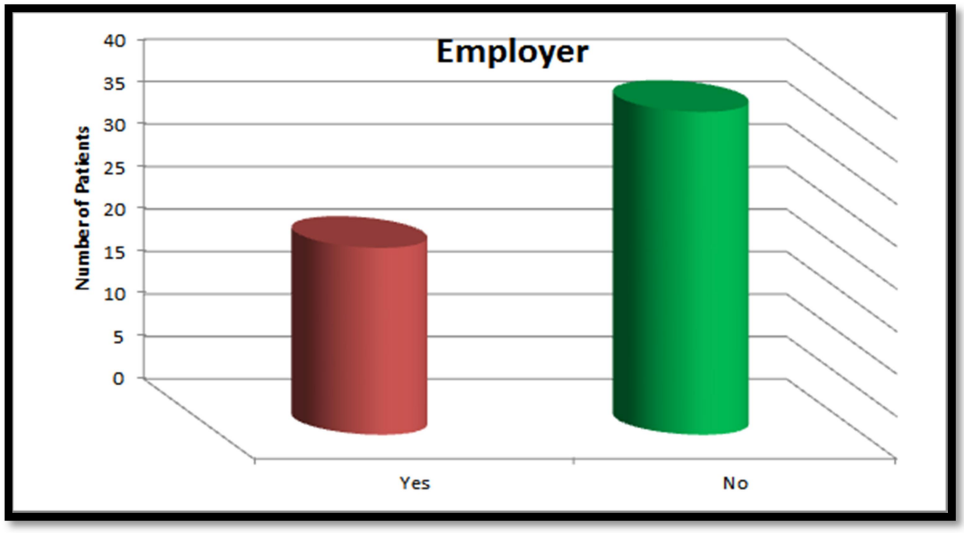


Figure (3-7): Employer status and acute myocardial infarction patients

Concerning the occupation, the figure (3-7) showed that the greater numbers of patients in this study were retired and Housewifery and they were accounted (63.3%), it mean they did not work. This result was agreed with (Akram, *et. al*., 2018) who showed that: there were (39%) housewives have ischemic heart disease. According to one study in Kirkuk, it was found from the total of 160 patients, only 10 patients have CHD with regularly engaged in physical activity, the rate of coronary heart disease was highest among non-employer, followed by housewives and then officials.

Women reported much greater symptoms of emotional discomfort than men, despite their being no difference in their employment level, higher daily stress and marital discontent play very important role in ischemic heart disease with housewives and employer women. Working women were also less likely to report behavior, and marital disputes than housewives (Haynes *et al*., 1980). Some of study revealed that women who worked outside the home did not have significantly higher risks of coronary heart disease than women who stayed at home (Del Brutto, *et al*., 2014).

3.2. Biochemical Parameters:

3.2.1. Measurement of Acid Ceramidase

Serum Acid ceramidase concentration was significantly decreased ($P < 0.05$) in all patients with acute myocardial infarction compared to control group, as shown in table (3-1).

Table (3-1): Serum levels of Acid ceramidase in patients with acute myocardial infarction compared to control group

	Groups	Mean	Std.E	T -test	P value
Acid ceramidase pg/ml	Cases	14.49	1.35	-2.48	0.014*
	Control	21.63	2.47		

* P< 0.05

Cardiac disease, diabetes, heart failure, and other cardio metabolic disorders are fueling a global pandemic of calorie consumption and inactivity. They raise circulating lipid levels, which can accumulate in non-adipose tissues like blood vessel walls and the heart. Deficiencies in lipid metabolism lead to cellular dysfunction and death. Ceramides, for example, are thought to be responsible for much of the tissue damage in these cardiometabolic disorders. Human serum ceramide levels are reliable indicators of cardiovascular disease. One of the most important reasons for the low concentration of enzymes in patients is due to inhibiting or depleting enzymes involved in the ceramide production, which is protect from diabetes, atherosclerosis, hypertension, and heart failure in human (Choi *et al*., 2021). Ceramides have been shown to impair mitochondrial activity, inhibit energy use, cause vasodilation, and induce apoptosis (Havulinna *et al*., 2016). It is also play a different role in the metabolic processes of the cell

depending on the environment, an apoptosis and stress-related cellular responses have both been linked to ceramide's engagement in the processes (Ren *et al*., 2021).

Accumulating evidence indicates that ceramides play an important role in the development of valvular heart disease and atherosclerosis, So that ceramides was biologically active sphingolipids produced by complex enzyme networks. Reducing cellular and tissue levels of ceramides by inhibiting the enzyme that produces ceramides impairs the progression of atherosclerosis and valvular heart disease in animal models (Zietzer *et al*., 2022).

Other observational studies by Meikle *et al*., (2011) have clearly shown that specific ceramide strains are associated with an increased risk of cardiovascular events. Acid ceramidase, widely expressed in somatic cells, regulates cellular ceramide levels (Park and Schuchman, 2006). In the body, ceramide is broken down to sphingosine and Free fatty acids (FFAs). However, abnormally high expression of the enzyme has been documented in various human malignancies (Chavez *et al*., 2005; Park and Schuchman, 2006). Endurance training lowers ceramide levels in skeletal muscle of obese people, improving insulin sensitivity (Adams *et al*., 2004; Bruce *et al*., 2006). The effects of ceramides on insulin sensitivity and exercise are complex and unknown (Błachnio-Zabielska *et al*., 2008; Bergman *et al*., 2016). In addition to acid ceramidase, ceramide control can occur via neutral or alkaline ceramidases, or by sphingomyelin conversion (Duarte *et al*., 2020).

3.2.2. Measurement of High Sensitivity C-reactive protein (Hs-CRP)

Serum Hs-CRP concentration was highly significantly higher ($P < 0.05$) in all patients with acute myocardial infarction compared to control group, as shown in table (3-2)**.**

Table (3-2): Serum levels of Hs-CRP in patients with acute myocardial infarction compared to control group

	Groups	Mean	Std.E	T- value	P value
S.Hs-CRP pg/ml	Cases	36.56	7.18	2.8	0.006
	Control	16.21	1.09		

Coronary artery disease (CAD) is one of the leading causes of morbidity and mortality in the world, accounting for approximately one-third of all cases. Activation and aggregation of platelets, thrombus formation, and subsequent infarction are all characteristics of this condition. (Derer *et al*., 2009). According to various studies, at least 250 factors are associated with the development of coronary artery disease. (Ekmekci *et al*., 2002) demonstrated molecules associated with impaired coagulation or fibrinolysis, cardiovascular remodeling, and inflammation are among those that have been discovered recently and are associated with cardiovascular disease, these molecules include high sensitivity C-reactive protein (hs-CRP) and other similar molecules. C-reactive protein (CRP), the classical acute phase protein, is associated with moderately elevated baseline concentrations and the long-term risk of coronary heart disease in general populations, and with major acute phase response of CRP following myocardial infarction and the risk of death and cardiac complications (Castro *et al*., 2018). There is debate about whether or not these correlations are pathogenic or have clinical importance. Large-scale epidemiological research, innovative tests, and potential specialized medicines are all described in some publications, and the evidence is reviewed. Moreover, in several of the articles, a distinction was made between the potential pathogenicity of high acute phase circulating CRP concentrations in people with considerable tissue damage and the modest but

persistent increases in baseline values in usually healthy persons (Niknezhad *et al*., 2020).

Recent studies have shown that inflammation is an important step in the development of atherosclerosis and is involved in the formation of unstable plaques, the measurement of blood C-reactive protein levels using a high-sensitivity assay (hs-CRP) can show a subclinical inflammatory state (Wilson *et al*., 2006).

Clinical studies have shown that elevated levels of hs-CRP in healthy individuals predict vascular events such as myocardial infarction (MI) and stroke, as well as the development of diabetes. In patients with acute coronary syndrome, elevated hs-CRP levels are associated with adverse outcomes and subsequent vascular events (Strasser *et al*., 2012).

Vidula *et al*., (2008) showed that in 377 patients with peripheral arterial disease (PAD) and C-reactive protein (CRP) were associated with higher all-cause mortality and short-term cardiovascular mortality.

3.2.3. Measurement of Beta-Catenin (CTNNβ1)

Serum CTNNβ1 concentration was highly significantly low ($P < 0.05$) in all patients with acute myocardial infarction compared to control group, as shown in table (3-3).

Table (3-3):Serum levels of CTNNβ1 in patients with acute myocardial infarction compared to control group

	Groups	Mean	Std.E	T value	P value
CTNNβ1 pg/ml	Cases	1.27	0.18	-3.5	0.0006
	Control	3.14	0.19		

The result found decrease in the concentration of CTNNβ1 in patients rather than control. It was recorded (1.27 ±0.18) pg/ml and (3.14±0.19) pg/ml for patients and control, respectively.

One of the most causes about reducing of CTNNβ1 in patients was a protein that is found in adherens junctions (AJs). As the name implies, adherens junctions (AJs) are critical for the establishment and maintenance of epithelial layers, which include those lining organ surfaces and those lining the surface of blood vessels. Adherens junctions are responsible for mediating adhesion between cells, communicating a signal with neighboring cells, and anchoring the actin cytoskeleton to the extracellular matrix, Thus, it was noticed a clear decrease in patients (Colombo *et al*., 2017).

β-catenin is a key component of the canonical Wnt signaling pathway, which promotes cell proliferation and differentiation by activating the transcription of key target genes. When Wnt is missing, cytoplasmic β-catenin is continually destroyed by a degradation complex that includes the scaffolding tumor suppressor protein axin, the adenomatous polyposis coli protein (APC), and glycogen synthase kinase 3 (GSK3), resulting in a low quantity of CTNNβ1 in nuclei (Guan *et al*., 2018).

Some studies found that activating the CTNNβ1 signaling pathway reduced the amount of neuregulin-1 "NRG1" produced by endothelial cells (ECs) both in vitro and in vivo. Whether the stimulation of β-catenin signaling suppresses the expression of the Neuregulin-1 (Nrg1) gene via T-cell factors (Tcf)-dependent classical Transcription factors or by contact with another signaling cascade such as Foxo protein (Nakagawa *et al*., 2016).

In previous studies, CTNNβ1 in experimental animals were utilized to study the functions of endothelial Wnt/-catenin signaling in angiogenesis for both

embryogenesis and ischemia (Grigoryan *et al*., 2008). The activity of beta-catenin within the nucleus of the cell. E-cadherin is responsible for the immobilization of newly generated CTNNβ1 signaling pathway at adherens junctions. At these junctions, E-cadherin is also able to interact with β-catenin, which in turn indirectly affects the actin cytoskeleton. The action of cellular proteins or the dephosphorylation of E-cadherin can cause β-catenin to be discharged from adherens junctions (Valenta *et al*., 2012).

3.2.4. Measurement of Nitric Oxide Synthase (NOS3)

Serum NOS3 concentration was significantly higher (P< 0.05) in all patients with acute myocardial infarction compared to control group, as shown in table (3-4).

Table (3-4): Serum levels of NOS3 in patients with acute myocardial infarction compared to control group

	Groups	Mean	Std.E	T value	P value
S.NOS3 pg/ml	Cases	3296.6	98.9	2.049	0.04*
	Control	2216.24	56.24		

*** P< 0.05**

One of the reasons related to the increase in the concentration of NOS3 in patients (3296.6 ± 98.9) pg/ml is due to the nature of the work of the necrosis factor in the reticuloendothelial system. The release of mediators such as nitric oxide (NO) by the vascular endothelium plays a critical role in the progression of the atherosclerotic process in the body. Endothelial homeostasis is influenced by the presence of nitric oxide, which suppresses platelet aggregation, leukocyte adhesion, smooth muscle cell migration, and proliferation (Rai *et al*., 2014) .

Nitric oxide, is a molecule that is generated by practically all of the types of cells that constitute the heart. It is responsible for regulating cardiac function via both dependent and independent vascular activities, the former category includes things like the modulation of coronary artery tone, thrombogenicity, proliferative and inflammatory qualities, as well as cellular cross-talk that supports morphogenesis (Farah *et al*., 2018).

Gheibi *et al*., (2020) have assessed the prospect of treating endothelial dysfunction by boosting the production of nitric oxide from the endothelial, either by stimulating nitric oxide synthesis or protecting nitric oxide to oxidative deactivation and transformation to harmful compounds including peroxynitrite. Traditional methods of elevating NO levels, such as using nitroglycerin or other organic nitrates, have limited therapeutic usefulness, mainly due to unfavorable pharmacokinetics and the acquisition of tolerance, which are hallmarks of various cardiovascular illnesses (Lundberg *et al*., 2015).

3.2.5. Correlation Between Biochemical Parameters in Patients with Acute Myocardial Infarction

The correlations between biochemical parameters were listed in table (3-5).

Table (3-5): Correlation between biochemical parameters in patients with acute myocardial infarction

Parameters	Acid Ceramidase	NOS3	CTNNβ1	Hs-CRP
Acid Ceramidase	**1**			
NOS3	0.401	1		
CTNNβ1	-0.083	0. 2543	1	
Hs-CRP	0.248	0.35173	-0.04302	1

There was no correlation between biochemical parameters among acid ceramidase, NOS3, CTNNβ1 and HS-CRP in patients with acute myocardial infarction.

(İlikay *et al*., 2019) illustrated that there is a correlation effect in the biochemical marker Caveolin-1 (CAV-1) and eNOS. Caveolin-1 plays a crucial role in endothelial-nitric oxide synthase (eNOS) enzymatic activity. Caveolin-1 and eNOS interactions have a significant impact on endothelial dysfunction, cholesterol levels, atherosclerosis and the coronary heart disease.

(Luo *et al*., 2010) was noted that correlation between high-sensitivity C-reactive protein (hs-CRP) serum levels and severity of coronary atherosclerosis, in a sample of Iranian patients referring to Taleghani Hospital in 2011. Moreover, Luo J G and colleagues investigated the relationship between serum hsCRP and TNFα levels and severity of coronary atherosclerosis in 100 patients. They found a positive and effective correlation between the mentioned indices and the Gensini score (Piranfar, 2014).

3.3. Distribution of Genotype Frequency

A population's genetic risk of disease can be attributed to differences in the DNA of individuals. Single nucleotide polymorphisms (SNPs) are the most common cause of genetic diversity across individuals, although there are a variety of other factors at play. We have an average of 1,000 genetic mutations per human genome. To put this into perspective, an average human gene with 250,000 base pairs has the potential to have 250 SNPs. There are only a small number of SNPs found in exons, which affect the protein's amino acid sequence. The majority of single nucleotide polymorphisms (SNPs) have no influence on gene function, however a small number of SNPs can change gene transcription, splicing, translation, or mRNA stability. Additional genetic variability in the human genome can be attributed to either short or long DNA sequences that have been altered in some way. As a result, disease-causing genetic polymorphisms can modify the amino acid sequence of proteins, as well as the amount of protein that is made as a result of the gene (Liu *et al*., 2016).

3.3.1. Distribution of Genotype Groups "rs7844023" Among Patients and Control

The result found there were 6 patients with Acute Myocardial Infarction had TT genotype in their chromosome, 14 patients had TC and 40 patients had wild CC genotype frequency, on the other hand the result found there were 2 control had TT genotype in their chromosome, 30 had TC and 28 had wild CC genotype frequency, the results did not found any significant association odd ratio 3.06 with 95% confidence interval 1.37-6.79, between wild gene CC and heterozygote in patients and control (TC), also there were no significant differences (P> 0.05) between wild gene CC and mutation factor TT, the odd ratio 0.47 with 95%

confidence interval 0.089-2.53. The results also did not found association significant between allelic frequency, it was found that odd ratio 1.42 with 95% confidence interval 0.621-3.28 in patients and control, table (3-6).

Table (3-6): Distribution of Genotype Groups "rs7844023" Among Patients with Acute Myocardial Infarction and Control

Study of genotypes "rs7844023"	Patients with MI N(%)	Control	Odd ratio	95% CI	P value
CC	40(66.7%)	28(46.7%)	Reference	_	_
TC	14(23.3%)	30(50%)	3.06	1.37-6.79	0.059
TT	6(10%)	2(3.3%)	0.47	0.089-2.53	0.38
Major C	47 (78.3%)	43 (71.6%)	Reference	_	_
Minor T	13 (21.7%)	17 (28.4%)	1.42	0.621-3.28	0.41

The reason for the absence of significant differences (P< 0.05) is due to the sample size used in the study, in addition to the fact that two thirds of patients carry the dominant gene CC genotype frequency.

(Lewis *et al*., 2018) found that the Genetic Variation in Acid Ceramidase T allele frequency was statistically lower in Japanese patients with heart diseases, and this might be explained by the low frequency of homozygotes among Japanese patient.

3.3.1.1. ROC Curve Analysis Between Acid Ceramidase Concentration and Patients with rs7844023 Polymorphism

The Receiver operating characteristic (ROC) curves have two dimensional graphs that are visual depictions of the performance and performance trade-off of a classification model, ROC curves began as tools within the theory of

communication to provide visual determination of optimal operating points in signal discriminators. The ROC curve is a graph of Acid ceramidase versus rs7844023 allelic frequency, which are both independent of disease prevalence. Basically, a traditional ROC curve defines the possible compromises between allele frequency and biochemical markers - thus among the relative frequencies of positive is true, positive is false, negative is true, and false negative decisions – due to the variability of decision thresholds**.**

Table (3-7): Area under the ROC curve (AUC) between acid ceramidase and rs7844023

Area Under the Curve				
Acid ceramidase / rs7844023				
Area	Specificity	Sensitivity	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.646	63.9%	72.6%	.610	.682

In table (3-7): the area (0.646) is moderate gold test; wasn't near the perfect value of 1.0 and more sizeable than worst case value at 0.0. Which means that an individual selected at random from the positive group had a test value larger than that for a randomly selected individual from the Mutant allele (T), figure (3-8).

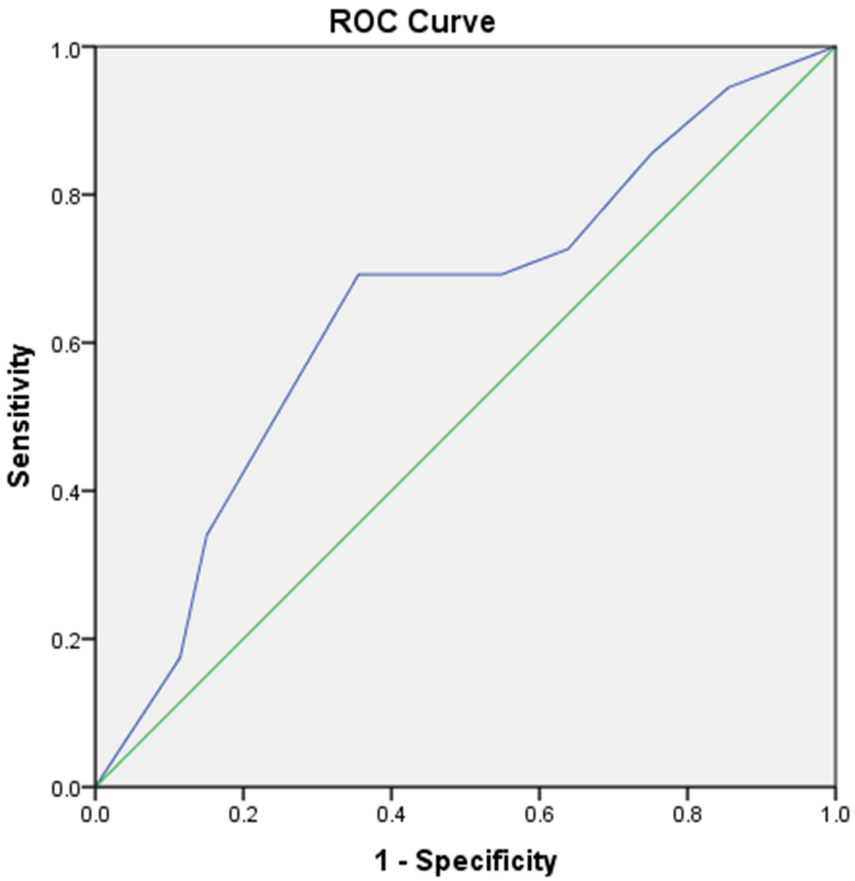


Figure (3-8): The ROC curve graphically shows the relationship between sensitivity and specificity for all possible threshold values (Acid ceramidase and rs7844023 polymorphism)

To demonstrate the use of ROC curves, a study of the ability of serum acid ceramidase and apolipoprotein measures to discriminate among degrees of coronary artery disease in patients undergoing coronary heart disease. ROC curve analysis reveals that none of these indexes is highly accurate, but demonstrates a modest increase (0.646) in the accuracy of acid ceramidase over biochemical indexes, the result was agreement with (Zweig *et al*., 1992) who was record roc curve analysis among lipid and apolipoprotein concentrations in identifying patients with coronary artery disease.

Each application of a diagnostic test is associated with specific consequences of the possible outcomes. When we use ROC analysis for risk stratification systems, AUCs are useful measures for comparison of the overall diagnostic

performance of two tests. However, given the equal weighting attributed to all parts under the curves, it is possible that the comparison of the AUC will be non-significant for two tests that differ in an area of practical relevance. Comparison of crossing ROC curves may also result in misleading inferences from AUC estimates (Vanagas, 2004).

3.3.2. Distribution of Genotype groups " rs2427746" Among Patients and Control

The result was found 23 patients with MI had wild AA genotype in their chromosome, 17 patients had AG and 20 patients had GG genotype frequency, on the other hand, the result found 8 control had GG genotype in their chromosome, 14 control had AG and 38 had wild AA genotype frequency, the result didn't found any significant association odd ratio 0.49 with 95% confidence interval 0.207-1.19, between mutation factor GG and heterozygote AG, otherwise it was a significant difference ($P< 0.05$) between mutation factor AA and wild AA patients, the odd ratio 0.242 with 95% confidence interval 0.091-0.638, as well as it was found an association significant between allelic frequency, where it noticed that odd ratio 0.356 with 95% confidence interval 0.164-0.772, table (3-8).

Table (3-8): Distribution of Genotype Groups " rs2427746" Among Patients with acute myocardial infarction and Control

Study of genotypes " rs2427746"	Patients with MI	Control	Odd ratio	95% CI	P value
AA	23(38.3%)	38(63.4%)	Reference	_	_
AG	17(28.3%)	14(23.3%)	0.49	0.207-1.19	0.119
GG	20(33.4%)	8(13.3%)	0.242	0.091-0.638	0.0045
A	31 (51.6%)	45 (75%)	Reference	_	_
G	29 (48.4%)	15 (25%)	0.356	0.164-0.772	0.008

3.3.2.1. ROC Curve Analysis between Acid Ceramidase Concentration and Patient with Rs2427746 Polymorphism

The ROC curves have two dimensional graphs that are visual depictions of the performance and performance trade-off of a classification model**,** ROC curves began as tools within the theory of communication to provide visual determination of optimal operating points in signal discriminators. The ROC curve is a graph of Acid ceramidase versus rs2427746 allelic frequency, which are both independent of disease prevalence. Basically, a traditional ROC curve defines the possible compromises between allele frequency and biochemical markers - thus among the relative frequencies of positive is true, positive is false, negative is true, and false negative decisions – due to the variability of decision thresholds**.**

Table (3-9): Area under the ROC curve (AUC) between acid ceramidase and rs2427746

Area Under the Curve				
Acid ceramidase / rs2427746				
Area	Specificity	Sensitivity	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.473	55.2%	64.5%	.325	.621

In table (3-9): the area (0.646) is weak test; it is move away the perfect value of 1.0 and approaching than worst case value at 0.0. Which means that an individual selected at random from the positive group has a test value larger than that for a randomly selected individual from the Mutant allele (G) group figure (3-9).

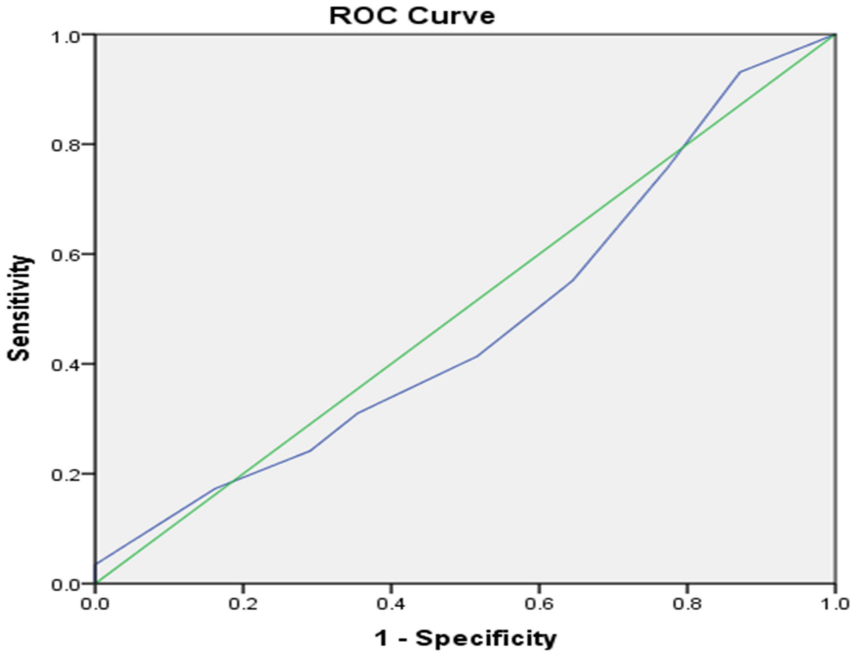


Figure (3-9): The ROC curve graphically shows the relationship between sensitivity and specificity for all possible threshold values (Acid ceramidase and rs2427746 polymorphism)

The study was agree with (Barnabei, *et al*., 2007) who were found an threshold levels to diagnose coronary artery disease on electrocardiographic stress testing by use of ROC curves in diagnostic medicine and electrocardiographic markers of ischemia. In clinical practice, it is exceedingly rare that a chosen cut point will achieve perfect discrimination between normal cases and those with disease, and one has to select the best compromise between sensitivity and specificity by comparing the diagnostic performance of different tests or diagnostic criteria available. Receiver operating characteristic (or receiver operator characteristic, (ROC) curves allow systematic and intuitively appealing descriptions of the diagnostic performance of a test and a comparison of the performance of different tests or diagnostic criteria (Kumar& Indrayan, 2011).

3.3.3. Optimization of T.ARMS -PCR genotyping method

3.3.3.1. Results of Amplification Reactions Among the Genotypes of rs7844023

PCR-T.ARMS was used to assay genotypes of rs7844023 SNPs for rapid screening of polymorphism. Amplification products were obtained to have a size of 355 bp for rs7844023 as outer forward and reverse primer, 172 bp for T allele and 244 bp for C allele. PCR product was electrophoresed and directly visualized with agarose gel which was colored with ethidium bromide under UV light, figure (3-10).

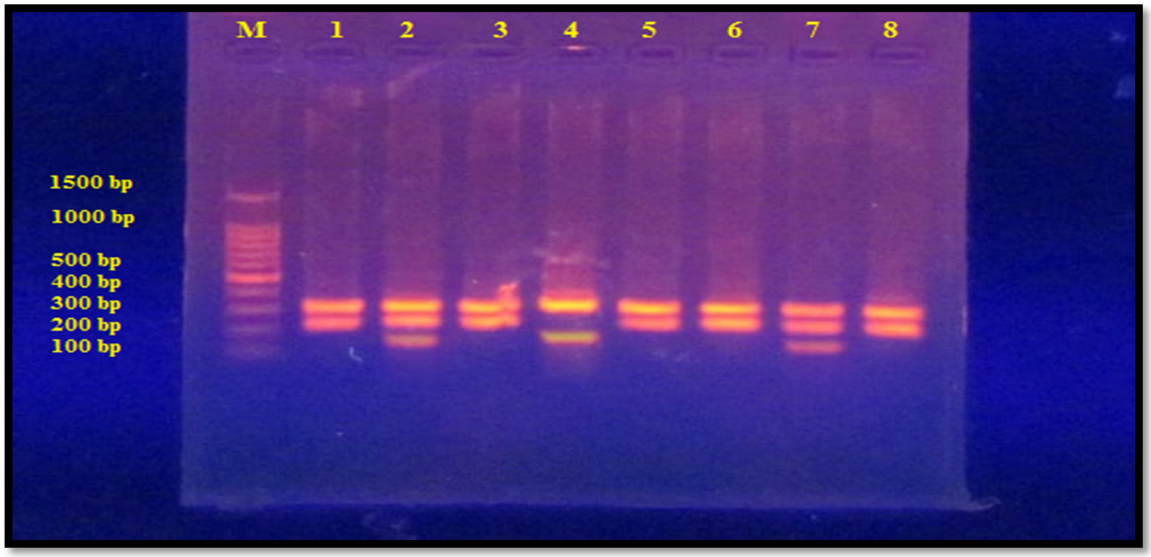


Figure (3-10):The PCR-T.ARMS was used to assay genotypes of rs7844023 SNPs of (C> T) (rs7844023) genetic polymorphism showed: Lane M: Represented DNA ladder 100 - 1000 bp, Lane 1, 3, 5, 6 and 8 : Represented CC genotype (Wild) were showed in (244 bp), Lane 4: Represented TT genotype (mutation) were showed in (172 bp) and Lane 2 and 7: Represented heterozygous TC genotype.

3.3.3.2. Distribution of Allele Frequencies of Acid Ceramidase Gene Polymorphism (C>T) (rs7844023) in Patients

The patients that enrolled in present study were classified into three genotypes, for (rs7844023) (C> T) genetic polymorphism, first: homozygous for the C allele (CC) wild type, second: heterozygous (TC) and the third was homozygous for the T allele (TT) mutant type.

Among 60 patients, there were 14 heterozygous (TC) genotypes (23.33%), 6 (TT) genotypes (10%) and 40 (CC) genotypes (66.67%) for the SNP rs7844023 in the acid ceramidase gene, so minor allele frequency was 21.66% for allele T.

3.3.3.3. Results of Amplification Reactions Among the Genotypes of rs2427746

PCR-T.ARMS was used to assay genotypes of rs2427746 (A>G) SNPs for rapid screening of polymorphism, the amplification products were obtained to have a size of 379 bp for rs2427746 as outer forward and reverse primer, 243 bp for A allele and 118 bp for G allele. PCR product was electrophoresed and directly visualized with agarose gel which colored with ethidium bromide under UV light figure (3-11).

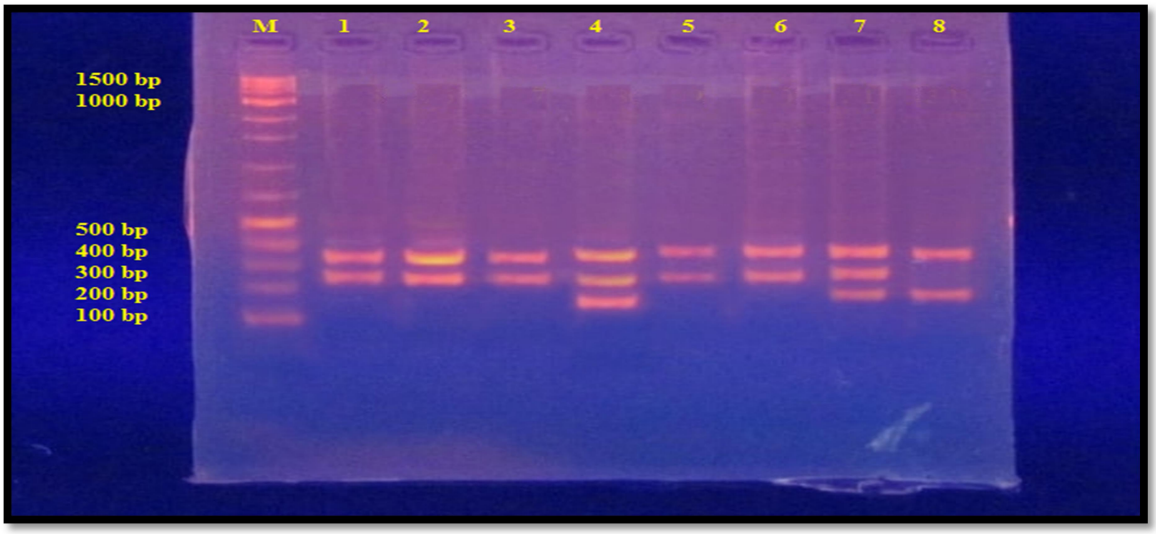


Figure (3-11):The PCR-T.ARMS was used to assay genotypes of rs2427746 SNPs of (A> G) (rs2427746) genetic polymorphism showed: Lane 1: Represented DNA ladder 100 - 1500 bp, Lane 1, 2, 3, 5 and 6 : Represented AA genotype (Wild) were showed in (243 bp), Lane 8: Represented GG genotype (mutation) were showed in (118 bp) and Lane 4 and 7: Represented heterozygous AG genotype.

3.3.3.4. Distribution of Allele Frequencies of Acid Ceramidase Gene Polymorphism (A> G) (rs2427746) in Patients

The patients that enrolled in present study were classified into three genotypes, for (A> G) (rs2427746) genetic polymorphism, first: homozygous for the A allele

(AA) wild type, second: heterozygous (AG) and the third was homozygous for the G allele (GG) mutant type.

Among 60 patients, there were 17 heterozygous (AG) genotypes (28.34%), 23 (AA) genotypes (38.33%) and 20 (GG) genotypes (33.33%) for the SNP rs2427746 in the acid ceramidase gene, so minor allele frequency was 47.5% for allele G.

3.3.4. RFLP Analysis for Genotypes of rs2427746 Confirmation

The amplification product of (rs2427746) polymorphism was digested by BFaI restriction enzymes. The products of digestion were analyzed by agarose gel electrophoresis. Results exhibited one band of : 514 base pair (bp), three bands of : 514bp, 223bp, 291bp and two bands of : 223bp and 291bp and for individuals with the wild type (AA), heterozygous (AG) and homozygous (GG) genotypes, respectively, figure (3-12).

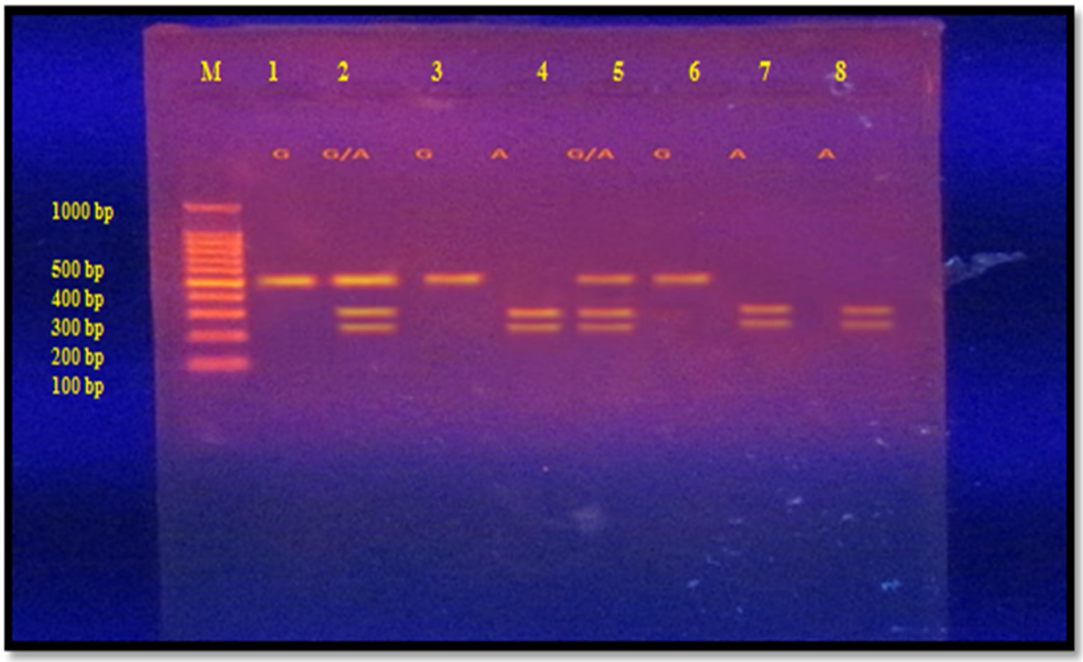


Figure (3-12): Results of rs2427746 Gene Polymorphism Product on Agarose Gel Electrophoresis. Line M : DNA Marker , Line 1,3 and 6: AA genotype (514) bp , Line 2 and 5:AG genotype (514,223,291) bp, Line 4,7 and 8: GG genotype (223 and 291) bp.

3.3.5. DNA Sequencing

3.3.5.1. (C>T) (rs7844023) gene polymorphism

The molecular detection was carried out by using the technology of DNA sequencing, where the sequence of nitrogen bases was determined in acid ceramidase gene polymorphism of (rs7844023) (C> T) region, that amplified 355 bp with both (Outer forward and reverse Primers) by PCR technique. The results were obtained after they were done by Macrogen company/ Korea (Macrogen Inc. Geumchen, Seoul, South Korea) and it was read by using the SnapGene Viewer 3.3.1 software. The results were compared with data obtained from the National Center for Biotechnology Information (NCBI), as shown in figure (3-13).

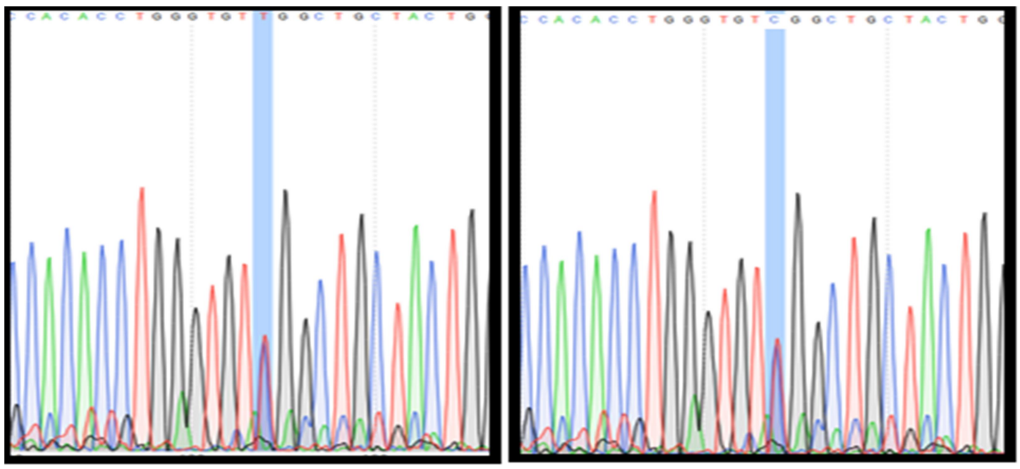


Figure (3-13): Chromatogram Sequence Results Analysis showed the Reading of the Nucleotides (C> T) (rs7844023) Polymorphism. Left figure represented T allele while right figure represented C allele

The figure (3-13) shown multiple sequence alignment analysis to reference rs7844023 gene (NCBI) with samples of current study. The blue color shown site of " rs7844023" polymorphism, The colors were shown the variable sites ,white color shown conserved sites. The results of sequencing of " rs7844023" gene in this research was identical or agreement with (Venter, *et al*., 2001).

3.3.5.2. (A> G) (rs2427746) Gene Polymorphism

The molecular detection was carried out by using the technology of DNA sequencing, where the sequence of nitrogen bases was determined in acid ceramidase gene polymorphism of (A> G) (rs2427746) region, that amplified 379 bp with both (Outer forward and reverse Primers) by PCR technique. The results were obtained after they were done by Macrogen company/ Korea (Macrogen Inc. Geumchen, Seoul, South Korea) and it was read by using the SnapGene Viewer 3.3.1 software. The results were compared with data obtained from the National Center for Biotechnology Information (NCBI), as shown in figure (3-14).

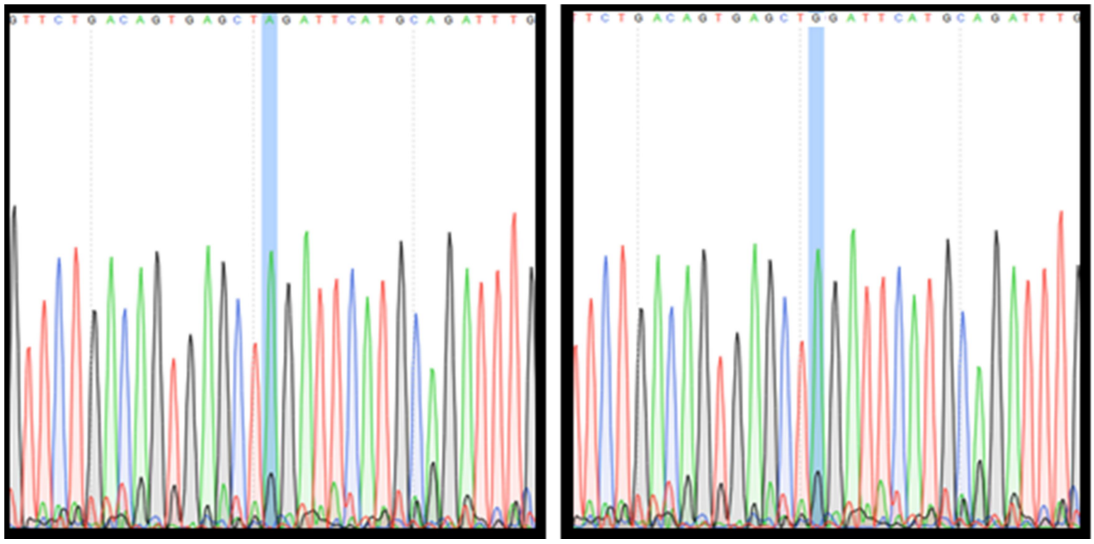


Figure (3-14): Chromatogram Sequence Results Analysis showed the Reading of the Nucleotides (A> G) (rs2427746) Polymorphism. Left figure : represented A allele while right figure represented G allele.

The figure (3-14) shown multiple sequence alignment analysis to reference rs2427746 gene (NCBI) with samples of current study. The blue color shown site of " rs2427746" polymorphism, The colors were shown the variable sites and white color shown conserved sites. The results of sequencing of "rs2427746" gene in this research was identical or agreement with (Waterston, *et al*., 2003).

3.4. Conclusions:

From the current study, we conclude:

1- Genetic variation (rs2427746) is associated with myocardial infarction, otherwise there was no significant association with (rs7844023).

2- It is necessary to measure acid ceramidase for the prediction of myocardial infarction, beside the changes in serum beta catenin levels.

3- ROC curve lying on the diagonal line consider as moderate test (0.646) value for acid ceramidase in patients with myocardial infarction.

4- There was no effect of SNP (rs7844023) (C> T) of Acid Ceramidase gene in development of myocardial infarction.

   Mutant genotype of Acid Ceramidase gene in (rs2427746) (A> G) was high risk factor for development of myocardial infarction.

3.5. Recommendations for Future Work:

1- Future studies are needed to establish the biochemical changes in acid ceramidase enzyme activity in patients with myocardial infarction.

2- Large scale studies with increasing number of participants (patients and control groups) to get more accurate results and to increase power of statistics.

3- Establish a genetic database of Iraqi genome variants for patients with myocardial infarction.

4- Study of others genetic polymorphisms associated with risk of myocardial infraction.

Chapter Four

References

Chapter Four

References

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الملخص

احتشاء عضلة القلب (MI) هو أحد الأسباب الأكثر شيوعًا لدخول المستشفى وهو شائع في جميع السكان في جميع أنحاء العالم.

الهدف من هذه الدراسة هو تقييم التغيرات في بعض المتغيرات البيوكيميائية كمؤشرات على احتشاء عضلة القلب والمرتبطة بتعدد الأشكال الوراثي.

تم تضمين إجمالي عدد 120 مشاركًا 60 مريضًا و 60 يبدو أنهم يتمتعون بصحة جيدة في هذه الدراسة. وأجريت الدراسة على مرضى يعانون من احتشاء قلبي حاد تم إدخالهم إلى مدينة الإمام الحسين الطبية ومركز كربلاء لأمراض وجراحة القلب في مدينة كربلاء.

تم الحصول على عينات الدم والمصل من جميع المشاركين لقياس المعلمات التالية:

Human high sensitivity C- reactive protein (hs-CRP), Human acid ceramidase (AC), Human nitric oxide synthase (NOs) and Beta-Catenin (BC) وكذلك تحديد تعدد الأشكال الوراثي ل acid ceramidase.

أشارت النتائج إلى أن معظم حالات مرضى السكري المصابين بأمراض القلب كانت في سن 50 سنة واكبر لكلا الجنسين وتم تسجيل (35٪) و (50٪) للذكور والإناث مصابون بداء السكري في سن أكبر من 50 سنة مرتبطة بالجنس ،على التوالى.

وكشفت نتائج هذه الدراسة أن المرضى فوق الخمسين سنة هم 72٪ وأقل من خمسين سنة 28٪.

وبحسب نتائج الدراسة فإن 78.3٪ من المرضى يعانون من ارتفاع ضغط الدم واحتشاء عضلة القلب.

كان هناك 46 مريضا يعيشون في المناطق الحضرية وتم تشخيص إصابتهم بأمراض القلب ، بينما يعيش 14 مريضا في المناطق الريفية.

انخفض تركيز مصل acid ceramidase معنويا (P <0.05) في جميع المرضى الذين يعانون من احتشاء عضلة القلب مقارنة بمجموعة التحكم ، وزاد تركيز مصل Hs-CRP بشكل كبير

(P <0.05) في جميع المرضى الذين يعانون من احتشاء عضلة القلب مقارنة بمجموعة التحكم. بالإضافة إلى ذلك ، انخفض تركيز مصل .Beta-Catenin (BC) بشكل كبير (P <0.05) في جميع المرضى الذين يعانون من احتشاء عضلة القلب مقارنة بمجموعة التحكم. من ناحية أخرى ، عند مقارنة مرضى احتشاء عضلة القلب ، كان تركيز Nitric oxide synthase (NOS3) أعلى بشكل ملحوظ في المرضى (P <0.05) من تركيز المجموعة الضابطة.

أظهر توزيع مجموعات النمط الجيني لتعدد أشكال acid ceramidase "rs7844023" في المرضى الذين يعانون من احتشاء عضلة القلب والضوابط أن 3.3٪ من مجموعة الضوابط لديها النمط الجيني TT في كروموسوماتهم ، و 50٪ كان لديهم TC ، و 46.7٪ لديهم ترددات وراثية CC برية ، مقارنة بـ 10٪ مرضى احتشاء عضلة القلب لديهم أنماط وراثية TT ، و 23.3 ٪ من المرضى الذين يعانون من الأنماط الجينية TC ، و 66.7 ٪ من المرضى الذين لديهم ترددات وراثية CC البرية.

من ناحية أخرى، وجد توزيع مجموعات التركيب الوراثي لتعدد الأشكال الجيني acid ceramidase "rs2427746" بين مرضى احتشاء عضلة القلب والضوابط أن 33.4٪ من مرضى احتشاء عضلة القلب لديهم النمط الجيني GG ، و 28.3٪ من المرضى لديهم AG ، و 38.3٪ من المرضى لديهم تواتر وراثي AA بري ، بينما 13.3 ٪ من الضوابط كان لديها النمط الجيني GG، 23.3 ٪ كان لديها AG ، و 63.4 ٪ لديها تردد النمط الجيني AA البري.

الاستنتاجات: الاختلاف الجيني ل acid ceramidase (rs2427746) مرتبط باحتشاء عضلة القلب بينما لم يكن هناك ارتباط كبير بـ (rs7844023) ، من ناحية أخرى ، يمكن استخدام ceramidase acid و beta catenin كمعلمات تشخيصية لاحتشاء عضلة القلب.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل/ كلية الطب
فرع الكيمياء و الكيمياء الحياتية

تقييم مستوى السيراميداز الحمضي في الدم وتعدد الأشكال الجيني في السكان العراقيين المصابين باحتشاء عضلة القلب الحاد

أطروحة
مقدمة إلى كلية الطب / جامعة بابل كجزء من متطلبات نيل درجة الدكتوراه فلسفة في علوم الكيمياء الحياتية السريرية

من قبل
رجاء نعمة أمين علي
ماجستير صيدلة / علوم مختبرية سريرية
جامعة بغداد / كلية الصيدلة 2014

إشراف

الأستاذ الدكتور
مؤيد عمران الغزالي

الأستاذ الدكتور
عدي جاسم الصالحي

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