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Study of The Risk of CTLA-4 Gene Polymorphism with Changes in Protein Level in Development of Cardiovascular Diseases

A Thesis

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيم

وَلَوْلَا فَضْلُ اللَّهِ عَلَيْكَ وَرَحْمَتُهُ لَهَمَّت طَّائِفَةٌ مِّنْهُمْ أَن يُضِلُّوكَ وَمَا يُضِلُّونَ إِلَّا أَنفُسَهُمْ ۖ وَمَا يَضُرُّونَكَ مِن شَيْءٍ ۚ وَأَنزَلَ اللَّهُ عَلَيْكَ الْكِتَابَ وَالْحِكْمَةَ وَعَلَّمَكَ مَا لَمْ تَكُن تَعْلَمُ ۚ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيمًا

صدق الله العليّ العظيم

النساء (١١٣)

Dedication

To Allah who blessed me with his gifts

To the imam Al-Mahdi

To my supervisors who kept giving me support

To my beloved father and mother and siblings

To everyone who gave me the support to continue

Mohammed

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First, all the thanks and gratitude to Allah, helping me in everything, And for Greatest Messenger Muhammad and his family (may Allah bless him and his family).

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And finally, I wish to express my warmest love to my family and the friend who supported me through all.

SUMMARY

This study is a case–control study of 126 individuals were included in this study, 69 of them were patients with CVD (32 with heart failure and 37 myocardial infraction), with a mean ± standard deviation (SD) of age (52.18 ± 7.9]) and 57 apparently healthy subjects, with a mean ± SD of age (49.5 ± 6.4). The current study was performed at the laboratory of chemistry and biochemistry department, college of medicine, University of Babylon. All samples were collected during the period from the 20th of October 2020 till 20th January2021.The samples were collected from Marjan and Imam Al-Sadiq teaching Hospitals in Babylon province. DNA was extracted from blood then genotyping of SNPs (rs231775) was carried out by Real time -PCR by using special primers and probes. The serum CTLA-4 concentration assessed by using sandwich ELISA technique.

In the patient group, AA, AG, and GG genotypes occurred in position 49 A/G in the *ctla-4* gene with the frequency of 2 (4.3%), 41 (59.4%), and 25 (36.9%), respectively. With respect to the control group, they occurred with the frequency of 58 (8.7%), 39 (87.7%), and 8 (3.6%), respectively. As far as the frequency of A and G alleles in this position was concerned, respectively, 44(34.06%) and 76 (65.94%) for patients and, respectively, 55 (52.63%) and 52(47.37%) for the control group. The calculated values were significantly different between these groups (P = 0.0073). Genotype and allele frequencies of *ctla-4* gene(rs231775) polymorphism were found to be non-consistent with Hardy–Weinberg equilibrium, were examined under the co-dominant, dominant ,over dominant and recessive models with the use of multinomial Abstract logistic regression analysis, the result *ctla-4* A>G rs231775 gene polymorphism in CVD group when compare with control group, show the risk of CVDs was significantly higher among carriers of GG variant under co-dominant GG(OR=20.8 95%CI)=((2.735-158.72), P= 0.003), dominant AG+GG(OR=2.44, 95%CI)=(0.558-10.64, P= 0.2), and

recessive model(OR=15.63, 95%CI)=(3.51-69.598, P= 0.0003).on the other hand, present results showed no difference in serum CTLA-4 concentration between CVD group (1.38 ±1.03 ng/ml) and control group (1.24±0.9 ng/ml).

This study showed that the *ctla-4* +49A>G polymorphism is indeed a risk of CVD and where the presence GG genotype increase the risk of developing CVD also there was no association of the serum CTLA-4 concentration with the risk of CVD

List of Contents

List of Abbreviations

Abbreviation	Details
ACE	Angiotensin-Converting Enzyme
APC	Antigen Presenting Cells
APO B	Apolipoprotein B
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
CBC	Complete Blood Count
CD	Cluster of Differentiation
CHD	Coronary Heart Disease
CI	Confidence Intervals
CSF	Colony Stimulating Factor
CT	Computerized Tomography
CTLA-4	Cytotoxic T lymphocyte Antigen 4
CVD	Cardiovascular Diseases
ECG	Electrocardiogram
ECs	Endothelial Cells
EKG	Electrocardiogram
FOXP3	Fork Head Box P3

GM-CSF	Granulocyte-Macrophage Colony-Stimulating
HDL	High-Density lipoprotein
HF	Heart Failure
HFpEF	Heart Failure With Preserved Ejection Fraction
HFrEF	Heart Failure With Reduced Ejection Fraction
HWE	Hardy–Weinberg Equilibrium
IHD	Ischemic Heart Disease
LDL	low-Density lipoprotein
LV	left-Ventricular
LV	Left Ventricle
MCP-1	Monocyte Chemotactic Protein-
MI	Myocardial Infarction
MRI	Magnetic Resonance Imaging
MRNA	Messenger Ribonucleic Acid
NO	Nitric Oxide
OD	Optical Density
OR	Odds Ratio
PAD	Peripheral Artery Disease

RA	Rheumatoid Arthritis
ROS	Reactive Oxygen Species
SCTLA4	Soluble Cytotoxic T lymphocyte Antigen 4
SD	Standard Deviation
SLE	Systemic lupus Erytheromatosus
SMCs	Smooth Muscle Cells
SNP	Single Nucleotide Polymorphism
TG	Triglycerides
TCR	T Cell Receptor
Th cells	T Helper Cells
TIAs	Transient Ischemic Attacks
TLR	Toll Like Receptor
VSMC	Vascular Smooth Muscle Cells

List of Figures

List of tables

CHAPTER ONE

INTRODUCTION AND LITRITURE REVIEW

1. Introduction

Cardiovascular disease (CVD) is a term used for a number of linked pathologies such as coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease, rheumatic and congenital heart diseases and venous thromboembolism. Globally CVD accounts for 31% of mortality, the majority of this is in the form of CHD and cerebrovascular accidents. [1]

A major factor that contributes to the development of CVD is the immune and inflammatory factors. These factors are major contributor to the formation of atherosclerotic plaque which is the major contributor to the development of many CVDs. Atherosclerosis is an inflammation of chronic nature and caused by dysfunction in immune system with lipid accumulation, in which a plaque formed due to interaction between immune and non- immune cell, lipids and inflammatory mediators. Atherosclerosis is also affected by oxidative damage, which affects antioxidant status and lipoprotein due some conditions such as hyperlipidemia and hyperglycemia. [2,3]

Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) also known as Cluster of differentiation152 (CD152) is a receptor found on the surface of $CD4^+$ and $CD8^+$ T cells. CTLA-4 is an activation-induced glycoprotein of the Immunoglobulin superfamily, expressed with highest levels in lymphoid tissues whose primary function is to down-regulate T cell responses. CTLA-4 is coded by the *ctla-4* gene on the chromosome 2 this gene reported to have several mutations that linked to several diseases one of them is the +49 A>G mutation which cause alteration in amino acid in the CTLA-4 and affect its function which led to the development of atherosclerosis [4].

The CTLA-4 receptor shares its two known endogenous ligands, the B7 molecules B7.1 and B7.2, with the costimulatory receptor CD28(Cluster of Differentiation 28).CD

28 is a co receptor expressed on T cells that provide co-stimulatory signals required for T cell activation and survival. T cell stimulation through CD28 in addition to the T-cell receptor (TCR) can provide a potent signal for the production of various interleukins (IL-6 in particular) [5].

Since CTLA-4 can bind to the ligands expressed on the surface of antigen presenting cells (APCs) which CD28 are binding to CTLA-4 is competing with the CD28, However, the CTLA-4 binding affinity to these ligands (B7 family ligands) is much higher than that of CD28 thus CTLA-4 can suppress the CD28 and interactions of the ligands with CTLA-4 serve to inhibit T cell responses. CTLA-4 interacts with both ligands with higher affinity and avidity than CD28(40) with CTLA-4-CD80 forming the highest avidity interaction and CD28-CD86 the weakest [6,7].

Apart from the membrane CTLA-4 molecule, an alternate transcript of CTLA-4 mRNA that encodes a protein lacking a transmembrane region, which likely represents a native soluble form of CTLA-4 (sCTLA-4).The role of sCTLA-4 in T cell responses and inflammatory diseases have been highlighted by the description of the association of *ctla-4* gene polymorphisms (49 G/G and CT60 G/G) with the risk for common autoimmune diseases, such as Graves' disease, autoimmune hypothyroidism, and type I diabetes . The CTLA-4 CT60G/G genotype was associated with lower SCTLA-4 transcript abundance in CD4+ T cells [7-11].

1.2 Cardiovascular Disease (CVD)

A group of diseases that affects the heart or blood vessels. Cardiovascular disease, also known as heart disease, refers to: coronary artery disease (CAD) which is also known as coronary heart disease (CHD), cerebrovascular disease, peripheral artery disease (PAD), and aortic atherosclerosis. CAD results from decreased myocardial perfusion that causes angina due to ischemia and can result in myocardial infarction (MI). Cerebrovascular disease is associated with strokes and transient ischemic attacks. Peripheral arterial disease (PAD) is arterial disease predominantly involving the limbs that may result in claudication. Aortic atherosclerosis is associated with thoracic and abdominal aneurysms. The most common heart disease is coronary artery disease (narrow or blocked coronary arteries), which can lead to chest pain, heart attacks, or stroke. Other heart diseases include congestive heart failure, heart rhythm problems, congenital heart disease and endocarditis (inflamed inner layer of the heart). There are numerous problems that causes the development of CVD, many of which are related to a process called atherosclerosis. Atherosclerosis is a condition that develops when a substance called plaque builds up in the walls of the arteries. This buildup narrows the arteries, making it harder for blood to flow through. If a blood clot forms, it can stop the blood flow this can cause a heart attack or stroke [1,12].

High levels of triglycerides and total cholesterol and low-density lipoprotein (LDL-c) cholesterol and apolipoprotein B and lower serum levels of high-density lipoprotein, (HDL-c) and disorders such as hyperglycemia contribute to the formation of atherosclerotic plaque which is a major cause of the development of many cardiovascular diseases [13-15].

1.2.1 Types of Cardiovascular Disease

There are many types of heart disease, and each one has its own symptoms and pathogenesis and treatment

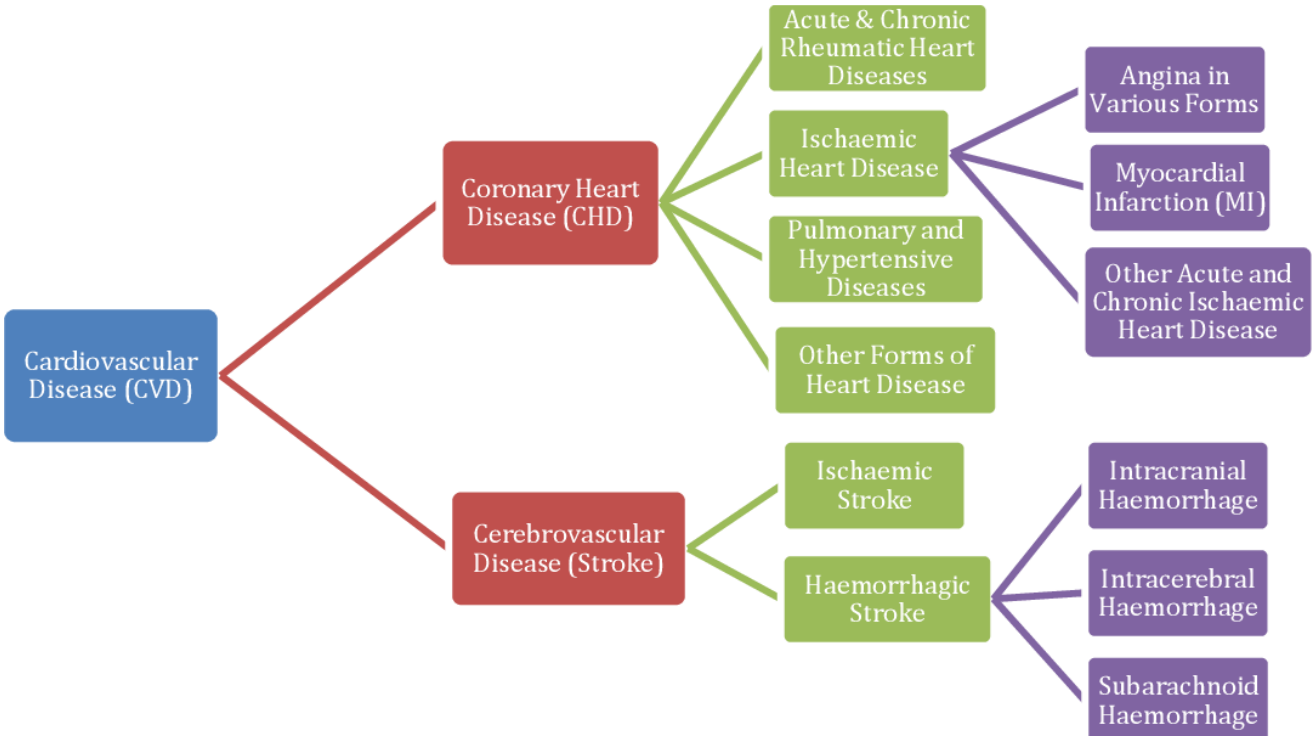


Figure (1-1): Classification and types of cardiovascular disease [16]

- Coronary heart disease – a disease of the blood vessels supplying the heart muscle;
- Cerebrovascular disease – a disease of the blood vessels supplying the brain;
- Peripheral arterial disease – a disease of blood vessels supplying the arms and legs;
- Rheumatic heart disease – damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria;
- Congenital heart disease – birth defects that affect the normal development and functioning of the heart caused by malformations of the heart structure from birth; and

- Deep vein thrombosis and pulmonary embolism – blood clots in the leg veins, which can dislodge and move to the heart and lungs [12,17].

1.2.2 Common Types of Cardiovascular Diseases

1.2.2.1 Stroke

A stroke is a clinically defined syndrome of rapidly developing symptoms or signs of focal or global loss of cerebral function with Symptoms last more than 24 h or more with no apparent cause other than vascular origin (18,19).

Stroke is an abrupt neurological outburst caused by impaired perfusion through the blood vessels to the brain. It is important to understand the neurovascular anatomy to study the clinical manifestation of the stroke. The blood flow to the brain is managed by two internal carotids anteriorly and two vertebral arteries posteriorly (the circle of Willis). Ischemic stroke is caused by deficient blood and oxygen supply to the brain; hemorrhagic stroke is caused by bleeding or leaky blood vessels [20,21].

In thrombosis, the blood flow is affected by narrowing of vessels due to atherosclerosis. The build-up of plaque will eventually constrict the vascular chamber and form clots, causing thrombotic stroke. Other key events contributing to stroke pathology are inflammation, energy failure, loss of homeostasis, acidosis, increased intracellular calcium levels, excitotoxicity, free radical-mediated toxicity, cytokine-mediated cytotoxicity, complement activation, impairment of the blood–brain barrier, activation of glial cells, oxidative stress and infiltration of leukocytes [22,23].

1.2.2.2 Ischemic Heart Disease (IHD)

Ischemia is defined as inadequate blood supply to a local area due to blockage of the blood vessels supplying the area. Ischemic means that an organ (e.g., the heart) is not getting enough blood and oxygen. Ischemic heart disease, also called coronary heart disease (CHD) or coronary artery disease, is the term given to heart problems caused by narrowed heart (coronary) arteries that supply blood to the heart muscle. Although the narrowing can be caused by a blood clot or by constriction of the blood vessel, most often it is caused by buildup of plaque, called atherosclerosis. When the blood flow to the heart muscle is completely blocked, the heart muscle cells die, which is termed a heart attack or myocardial infarction (MI). Most people with early (less than 50 percent narrowing) CHD do not experience symptoms or limitation of blood flow. However, as the atherosclerosis progresses, especially if left untreated, symptoms may occur. They are most likely to occur during exercise or emotional stress, when the demand for the oxygen carried by the blood increases [24,25].

1.2.2.3 Heart Failure (HF)

Heart failure (HF) is a clinical syndrome caused by cardiac dysfunction. Most common cause for HF is reduced left ventricular myocardial function. dysfunction of the pericardial diseases, myocardium, endocardium, heart valves or great vessels alone or in combination is also associated with HF. major pathogenic mechanisms leading to HF are increased hemodynamic overload, ischemia-related dysfunction, ventricular remodeling, excessive neuro-humoral stimulation, abnormal myocyte calcium cycling, excessive or inadequate proliferation of the extracellular matrix, accelerated apoptosis and genetic mutations [26,27].

Heart failure can be classified as predominantly left ventricular, right ventricular or biventricular. Depending on the time of onset, HF is classified as acute or chronic.

Clinically, it is typically classified into two major types based on the functional status of heart: heart failure with preserved ejection fraction (HFpEF) and heart failure with reduced ejection fraction (HFrEF). In patients with HFpEF who are mostly females and older adults, EF is usually more than 50%; the volume of the left-ventricular (LV) cavity is typically normal, but the LV wall is thickened and stiff; hence, the ratio of LV mass/end-diastolic volume is high [28].

1.2.3 Epidemiology

An estimated 17.9 million people died from CVDs in 2019, representing 32% of all global deaths. Of these deaths, 85% were due to heart attack and stroke. More than four out of five CVD deaths are due to heart attacks and strokes, and one third of these deaths occur prematurely in people under 70 years of age. Over three quarters of CVD deaths take place in low- and middle-income countries. Most cardiovascular diseases can be due to behavioral risk factors such as tobacco use, unhealthy diet and obesity, physical inactivity and harmful use of alcohol [29,30].

Globally, death due to CVDs is high because of common CVDs risk factors such as hypertension, cigarette smoking, elevated cholesterol, elevated glucose levels/diabetes, obesity, and physical inactivity comprise the top six leading causes of death globally. Coronary heart disease is the most common type of heart disease, killing 365,914 people in 2017. About 18.2 million adults age 20 and older have CAD (about 6.7%) About 2 in 10 deaths from CAD happen in adults less than 65 years old [17].

According to a study in 2015, Afghanistan had the highest age-standardized death rate from CVD, followed by Iraq and Yemen. In most of the East Mediterranean Region

countries, age-standardized death rates for CVD decreased between 1990 and 2015, with the highest decreases in Bahrain, Qatar, Lebanon, and Jordan [31].

Hypertension is a common medical condition; its prevalence increases with age, and is estimated to affect 65% of those ≥60-years-old.[32].

1.2.4 Risk Factors:

Cardiovascular diseases are large number of disorders thus have many risks factor, risk factors that linked to CVDs are includes:

I. Hypertension

Hypertension significantly increases the risk of myocardial infarction, heart failure, atrial fibrillation, stroke, and renal failure. High blood pressure (BP) is one of the most important risk factors for cardiovascular disease (CVD), which is the leading cause of mortality. Approximately 54% of strokes and 47% of coronary heart diseases, worldwide, are attributable to high BP [33].

II. Hyperlipidemia

Hyperlipidemia is considered to be a very dangerous factor leading to cardiovascular or cerebrovascular diseases, especially atherosclerosis which is a main contributor to the development of many CVDs. Dyslipidemia is increased levels of serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), or a decreased serum high-density lipoprotein cholesterol (HDL-C) concentration. Dyslipidemia is an established risk factor for cardiovascular disease (CVD). Elevated serum lipid levels are the greatest contributor to development of IHD worldwide,

Hypercholesterolemia is a major risk factor for coronary heart disease (CHD) and to lower plasma cholesterol were shown to reduce cardiovascular risk and low-density lipoprotein cholesterol (LDL-C) reduction with statins leads to improved CVD outcomes [14,35,36].

III. Smoking

Tobacco increases the risk of developing the CVD, including progression of atherosclerosis, coronary disease and sudden cardiac death. Smoking is a stronger risk factor for MI in middle-aged women compared to men, with a 6-fold increased risk vs. 3-fold increased risk in men. Smoking even only 2–3 cigarettes per day increases the risk of CVD; smoking cessation decreases the risk within 3 years [37,38]

IV. Obesity

Obesity is an established risk factor for cardiovascular disease. Obesity increases the risk of cardiovascular disease and premature death. Adipose tissue releases a large number of bioactive mediators that influence not only body weight homeostasis but also insulin resistance — the core feature of type 2 diabetes — as well as alterations in lipids, blood pressure, coagulation, fibrinolysis and inflammation, leading to endothelial dysfunction and atherosclerosis [39].

V. Vitamin D Deficiency

Vitamin D deficiency has been linked to several health outcomes, including musculoskeletal (rickets, bone fractures, osteocalcin, osteopenia, osteoporosis and muscle weakness) and non-skeletal complications. Non-skeletal complications include cardiovascular diseases and risk factors such as congestive heart failure, impaired systolic and diastolic function, myocardial infarction, peripheral vascular disease, abdominal aortic aneurysm in older men, nonvalvular AF and hypertension [40]

VI. Lifestyle Factors

- Alcohol: Excessive alcohol consumption is associated with hyperlipidemia, hypertension, vasoconstriction, hypercoagulability, and a lower ventricular fibrillation threshold [41].
- Physical Activity: By enhancing coronary endothelial function, improving ventricular systolic and diastolic function, and decreasing CVD risk factors, exercise is important for the management of angina and prevention of MI [42]
- Nutrition: several nutrients, minerals, food groups and dietary patterns associated with an increased or decreased risk of CVD. Dietary fats associated with an increased risk of CHD include trans–fats and saturated fats, while polyunsaturated fats are known to be protective. Dietary sodium is associated with elevation of blood pressure, while dietary potassium lowers the risk of hypertension and stroke. Regular frequent intake of fruits and vegetables is protective against hypertension, CHD and stroke. Composite diets (such as DASH diets, Mediterranean diet, 'prudent' diet) have been demonstrated to reduce the risk of hypertension and CHD. Sufficient knowledge exists to recommend nutritional interventions, at both population and individual levels, to reduce cardiovascular risk. That knowledge should now be translated into policies which promote healthy diets and discourage unhealthy diets [43]

VII. C-reactive Protein

Recent studies provide evidence that inflammation plays a role in the pathogenesis of cardiovascular disease. Some inflammatory or hemostatic markers actually constitute cardiovascular risk factors. In this respect, the acute phase reactant C-reactive protein (CRP) is of special interest: Baseline levels of CRP in apparently healthy persons or patients with stable angina pectoris constitute an independent risk factor for

cardiovascular events, The link between CRP and cardiovascular disease is thought to be indirect in that circulating CRP only reflects the extent of the acute phase reaction in response to nonspecific stimuli such as confounding risk factors, atherosclerosis, vascular injury, ischemia, and necrosis. However, several arguments are against this explanation that increased plasma levels of CRP are merely an epiphenomenon. First, chronic infections that cause a rise in circulating CRP also yield a higher risk for cardiovascular disease [44,45].

1.2.5 Pathogenesis of Cardiovascular Disease

Although CVD may directly arise from different etiologies such as emboli in a patient with atrial fibrillation resulting in ischemic stroke, rheumatic fever causing valvular heart disease or other conditions mentioned in figure (1-2), risks factors associated to the development of atherosclerosis is most important because it is a common denominator in the pathophysiology of CVD.

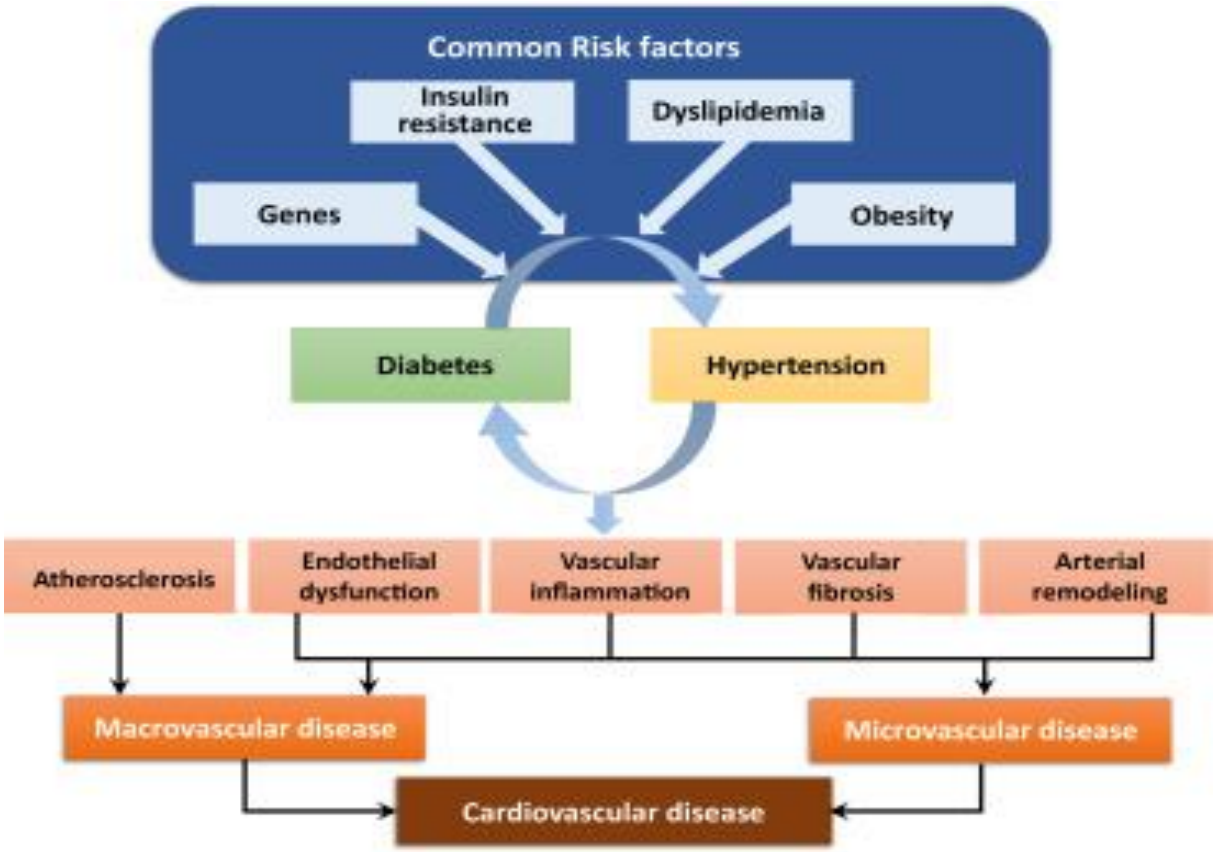


Figure (1-2) Pathogenesis of cardiovascular diseases [46]

Atherosclerosis is the major cause of cardiovascular disease. Hypercholesterolemia, hypertension, cigarette, smoking, obesity, diabetes, genetic predisposition, hyperlipidemia, and hypertension are the common risk factors for atherosclerosis. These risk factors unite behind a convergence of mechanism, involving oxidation and inflammation in the artery wall that, with time, gives rise to characteristic fatty-fibrous lesions. Physical trauma and inflammation produce lesion rupture, which can lead to clinical events such as heart attack and stroke, or resolve with plaque growth.

Atherosclerosis characterized by slow progressing inflammation in conductance and resistance arteries, in which there is an accumulation of cholesterol-containing low-density lipoprotein (LDL) particles beneath the endothelial layer [47,48].

Atherosclerosis starts passive diffusion of LDL into the arterial intima, this occurs in regions of higher blood turbulence or parts where there is endothelial damage After diffusing to the sub-endothelial space, LDL binds to the proteoglycans via apolipoprotein B-100 (ApoB100), subsequently becomes permanently retained. The sequestered LDL undergoes oxidative modification forming old which in turn causes aggregation and increased proteoglycan binding. Oxidation of LDL is mediated by reactive oxygen species (ROS) produced by smooth muscle cells (SMCs), endothelial cells (ECs), neutrophils, and macrophages. These events are potentiated by production and release of monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (m-CSF), which, respectively, attracts circulating monocytes to the plaque and activates them to release more ROS, nitric oxide (NO), and pro-inflammatory cytokines, such as TNF-α and IL-1β. In a positive feedback loop, ROS induces expression of TLRs in ECs, which perpetuates the inflammatory response via the expression of adhesion molecules, which cause circulating monocytes and other leukocytes to enter the tissue via trans-endothelial migration. Monocytes become differentiated into macrophages. which, once present within intima layer of the arteries, engulf old. through the scavenger receptors SR-A and CD36. This uptake leads to the formation of foam cells, which have compromised migratory capacity. Consequently, these cells accumulate in the intima and die, resulting in the formation of a plaque with a necrotic core. Growth factors and cytokines released by ECs and macrophages induce multiple effects including phenotypic changes within vascular SMCs, from the quiescent “contractile” phenotype state to the active “synthetic” state, that can migrate to and proliferate within the intima. The

migratory and proliferative capacities of VSMC's increase the size of the atherosclerotic plaque. Some of the emigrated VSMCs become less differentiated, senescent, or undergo apoptosis, which contributes to plaque instability and rupture. This leads the formation of a traveling thrombus which can occlude smaller arteries, resulting in myocardial infarction (MI) or ischemic stroke [49 – 53]

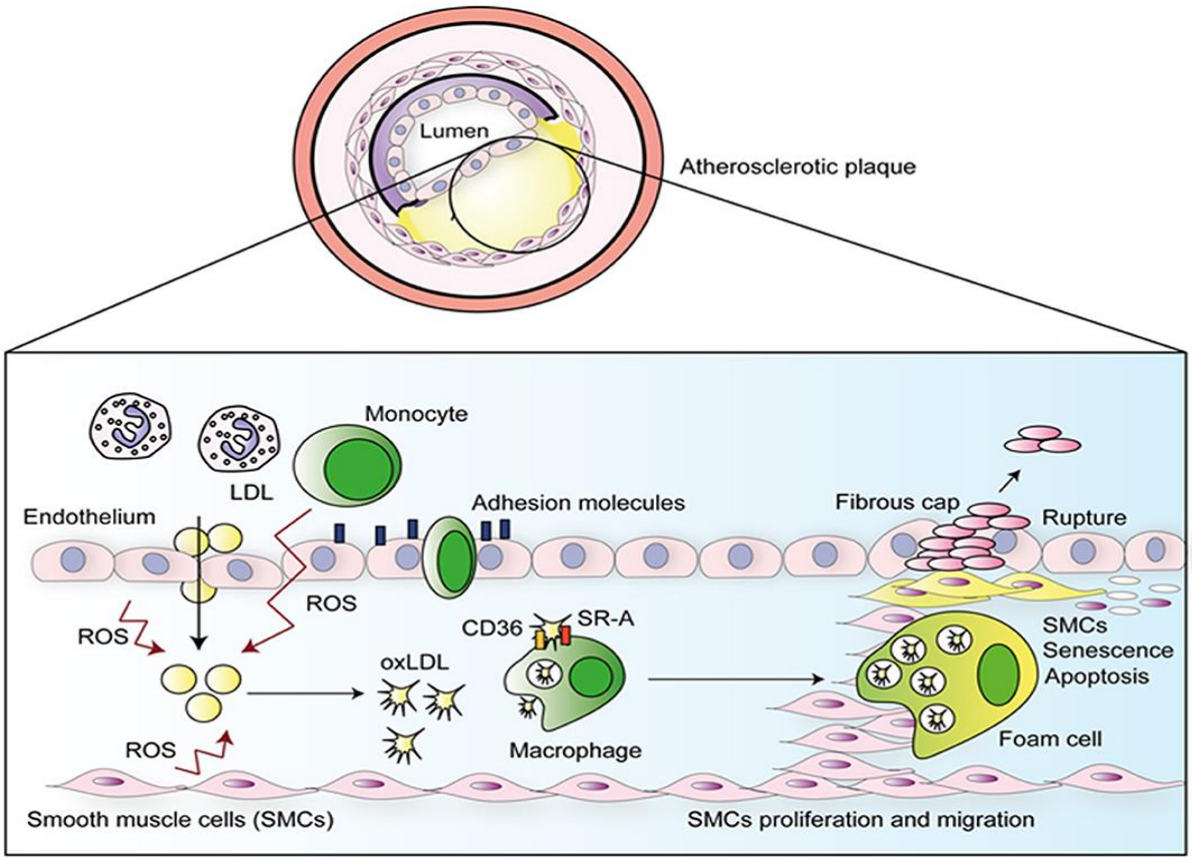


Figure (1-3) Pathogenesis of atherosclerosis [54]

1.2.5.1 Pathogenic Role of T Cells in Atherosclerosis

The Th1 mediated effect inflammatory processes occur over long period that enhance accumulation of macrophage foam cells and smooth muscle cells, which comprise the

bulk of the cellular mass of lesions. This pathogenic role of T cells in atherosclerosis is called chronic Th1-mediated delayed hypersensitivity type (DTH) response. The DTH responses involve interaction of Th1 cells with antigen-presenting macrophages, and the bidirectional activation of each cell type [54,55].

The Antigen Presenting Cells travel to the lymph nodes and present the antigens for recognition by T-cells. This antigen recognition results in the clonal expansion of both CD8+ and CD4+ T-cells. CD4+ T-cells secrete cytokines such as IL-17 and IFN-γ (42,), which facilitate the inflammatory process. Furthermore, the production of IL-4 and IL-13 by the activated CD4+ T-cell leads to a B-cell activation, clonal expansion, and subsequent immunoglobulin production. Antibodies play a prominent role in atherosclerosis, arising due to increased number of immunogenic neo-epitopes which are typically present in the disease [55,56].

The most important effector molecules produced by the activated Th1 cell in this response are membrane-bound CD40 ligand (CD40L), which binds to CD40 on the macrophage, and IFNγ, which binds to the IFNγ receptor on the macrophage. Signals from both CD40 and IFNγ receptor synergistically induce the expression of multiple proinflammatory genes in the macrophage. This type of response promotes further inflammation by elaboration of cytokines like the IL-1 and TNF and causes tissue destruction by upregulation of inducible nitric oxide synthase and phagocyte oxidase activity and the release of ROS [57-59].

The Th1 also has the ability to stimulate the release of matrix-degrading enzymes, including matrix metalloproteinases, from lesional macrophages. These enzymes can reduce the collagen content of fibrous caps and render the plaques more likely to rupture and acutely precipitate intraluminal thrombus formation and ischemic damage of

downstream tissues. Th2 and Th17 cells have also been found in atherosclerotic lesions but at lower frequencies. Th2 cells release IL-4, IL-5, IL-13, and support B-cell activation and antibody production [55,59].

The Th2 cell differentiation leading to upregulation of IL-5 which inhibits Th1 differentiation and therefore IFN-γ production. As a result of this regulatory role Th2 cells were initially assumed to be beneficial in the setting of atherosclerosis. However, recent evidence indicates that these cells may be both helpful and disadvantageous depending on disease stage and/or lesion site. Th17 cells produce IL-17 which is a pro-inflammatory mediator. Th17 development is promoted by TGFβ in the presence of IL-6 and IL-23. Similar to Th2, Th17 cells have been reported to have both positive and negative roles in atherosclerosis. Th17 cells produce factors such as IL-6, IFNγ, and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are proatherogenic. However, opposing this proatherogenic role, Th17 cells can also convert into other cell types such as Tregs; by gaining expression of fork head box P3 (FOXP3) and can subsequently exert suppressive effects on effector Th1 and Th2 cells [59-61].

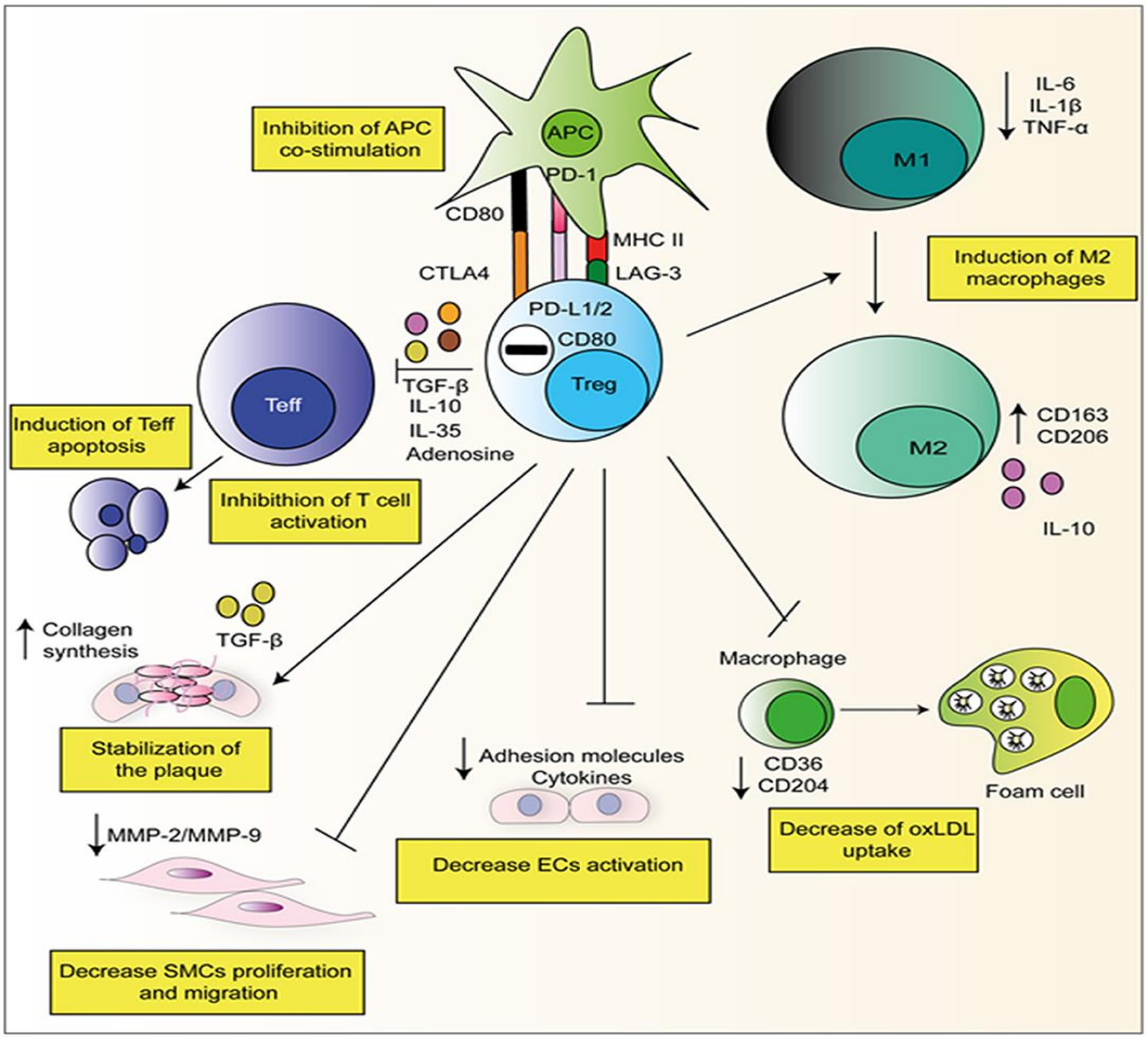


Figure (1-4) Regulatory T cells role in atherosclerosis [54]

The Tregs can directly produce suppressive cytokines and degradative enzymes such as perforin and granzyme that leads to apoptosis. Furthermore, Tregs also have a direct effect on APCs via interaction with via CTLA-4, PD-L1/2, and LAG-3. They can also skew monocyte class switching, encouraging anti-inflammatory M2 macrophages formation which produced collagen and stabilizes the plaque. They can also decrease foam cell formation via the down-regulation of CD36 and CD204 [61,62].

1.2.6 Signs and Symptoms of CVD

• Chest Tightness or Pressure.

• Difficulty Catching Your Breath.

• Dizziness or Fainting.

• Fatigue.

• Fluid Buildup.

• Heart Palpitations (heart pounding or racing).

• Pain or Numbness in Your Legs or Arms.

• Abdominal Pain, Nausea, Vomiting. [63,64]

1..2.7 Diagnosis of Cardiovascular Diseases

1 - Laboratory Tests

a) Lipid Profile

The Lipid Profile Includes:

- Total Cholesterol
- LDL (Low-Density Lipoprotein), the so-called "Bad" cholesterol
- HDL (High-Density Lipoprotein), the so-called "Good" cholesterol
- Triglycerides

b) Lipoprotein (a), or Lap (a)

c) C-reactive Protein (CRP)

d) Markers: Troponin-T, fibrinogen and PAI-1, high levels of homocysteine, elevated asymmetric dimethylarginine and elevated brain natriuretic peptide (also known as B-type) (BNP) [36,45,64]

2 - Blood Tests for Other Body Systems

1. Complete Blood Count (CBC)
2. Sodium and Potassium Levels
3. Blood Urea Nitrogen and Creatinine
4. Fasting Glucose
5. ALT and AST
6. TSH [64-66]

3 - Screening tests

A- Electrocardiogram (ECG or EKG).

B- Holter Monitoring.

C- Echocardiogram

D- Stress Test.

E- Cardiac Catheterization.

F- Cardiac Magnetic Resonance Imaging (MRI).[67]

1.2.8 Treatment of Cardiovascular Diseases

- Lifestyle Changes. You can lower your risk of heart disease by eating a low-fat and low-sodium diet, getting at least 30 minutes of moderate exercise on most days of the week, quitting smoking, and limiting alcohol intake.
- Medications.

1. Statins: to lower LDL cholesterol

2. Aspirin: to prevent blood clots

3. Clopidogrel: to prevent blood clots

4. Warfarin: to prevent blood clots

5. Beta-blockers: to treat heart attack and heart failure and sometimes used to lower blood pressure

6. ACE inhibitors: to treat heart failure and lower blood pressure

ACE (angiotensin-converting enzyme) inhibitors prevent the body from producing the artery-constricting hormone angiotensin. Arteries relax with ACE inhibitors and this lowers blood pressure.

- Medical procedures or surgery. [68,69]

1.3 *Cytotoxic T Lymphocyte Antigen 4 (ctla-4)* gene

This gene is a member of the immunoglobulin superfamily and encodes a protein which transmits an inhibitory signal to T cells. The protein contains a V domain, a transmembrane domain, and a cytoplasmic tail. Alternate transcriptional splice variants, encoding different isoforms, have been characterized. The membrane-bound isoform functions as a homodimer interconnected by a disulfide bond, while the soluble isoform functions as a monomer. and consists of four exons and three introns, comprises more than 100 polymorphic sites and is converted into a peptide of 233 amino acids. The CTLA-4 protein consists of a signal peptide and a main chain (the first 35 amino acids) Mutations in this gene have been associated with insulin-dependent diabetes mellitus, Graves' disease, Hashimoto thyroiditis, celiac disease, systemic lupus erythematosus, thyroid-associated orbitopathy, and other autoimmune diseases [70-72].

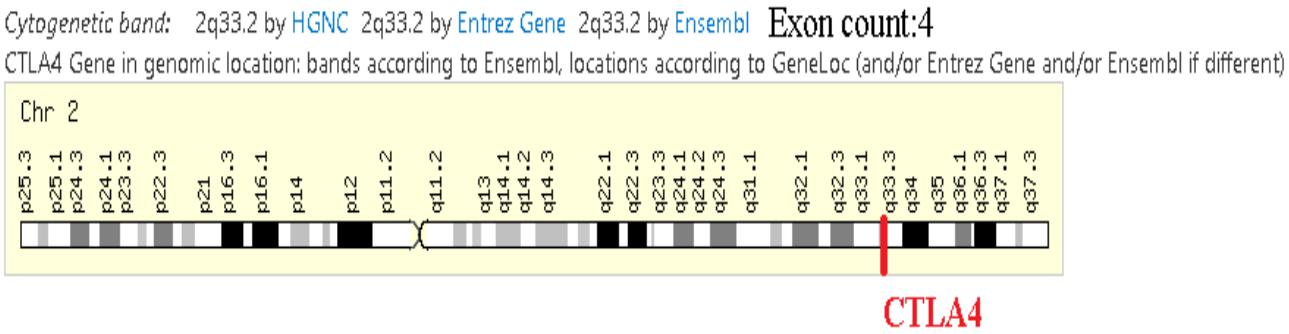


Figure (1-5) The *ctla*-4 gene location on chromosome 2[72].

Change of A-to-G at exon 1 position 49 (+49A/G (rs231775) that occurs in alanine threonine substitution at codon 17 of the fusion protein, contributes to the production of a faulty receptor and impairs the inhibition activity of CTLA-4 on the activation of lymphocyte T cells. Expression of gene products can be impaired by genetic variations [73].

The CTLA-4 is an adhesion receptor expressed on activated T cells. The amino acid sequence of CTLA-4 is related to CD28, and although the function of CTLA-4 remains unknown, it shares several features with CD28, including a common counter-receptor, B7, that is present on Ag-presenting cells.CD28 and CTLA-4 are expressed at the mRNA level on activated T cells but that only CD28 was expressed on resting T cells. Here we show that within the T cell population, CTLA-4 expression is restricted to the subset of T cells that also express cell surface CD28. CTLA-4 mRNA expression can be induced on quiescent T cells via phorbol ester-mediated activation of protein kinase C but not with calcium ionophore treatment alone. Phorbol ester-induced expression of CTLA-4 mRNA could be enhanced with calcium ionophore treatment, and treatment of cells in this manner resulted in a reciprocal decrease in expression of CD28 mRNA. Ligation of CD28 with monoclonal antibody also resulted in the specific and rapid induction of CTLA-4 mRNA [74-76]

1.3.1 Cytotoxic T Lymphocyte Antigen 4

Cytotoxic T Lymphocyte antigen 4 (CTLA-4) also known as CD152 is receptors expressed by both $CD4^+$ and $CD8^+$ T cells. Expressed with highest levels in lymphoid tissues. Exists primarily an intracellular antigen whose surface expression is tightly regulated by restricted trafficking to the cell surface and rapid internalization.

CTLA -4 is an inhibitory receptor acting as a major negative regulator of T-cell responses. The affinity of CTLA4 for its natural B7 family ligands, CD80 and CD86, is considerably stronger than the affinity of their cognate stimulatory coreceptor CD28. [4-7,76].

T-cell activation is a complex process that requires more than one stimulatory signal. TCR binding to MHC provides specificity to T-cell activation, but further costimulatory

signals are required. Binding of B7-1 (CD80) or B7-2 (CD86) molecules on the APC with CD28 molecules on the T cell leads to signaling within the T cell. Sufficient levels of CD28:B7-1/2 binding lead to proliferation of T cells, increased T-cell survival, and differentiation through the production of growth cytokines such as interleukin-2 (IL-2), increased energy metabolism, and upregulation of cell survival genes. [75-77].

CTLA-4 is a CD28 homolog with much higher binding affinity for B77,8; however, unlike CD28, binding of CTLA-4 to B7 does not produce a stimulatory signal. As such, this competitive binding can prevent the costimulatory signal normally provided by CD28:B7 binding (Fig.). The relative amount of CD28:B7 binding versus CTLA-4:B7 binding determines whether a T cell will undergo activation or +anergy.4 Furthermore, some evidence suggests that CTLA-4 binding to B7 may actually produce inhibitory signals that counteract the stimulatory signals from CD28:B7 and TCR:MHC binding.8 Proposed mechanisms for such inhibitory signals include direct inhibition at the TCR immune synapse, inhibition of CD28 or its signaling pathway, or increased mobility of T cells leading to decreased ability to interact with APCs.[76-80].

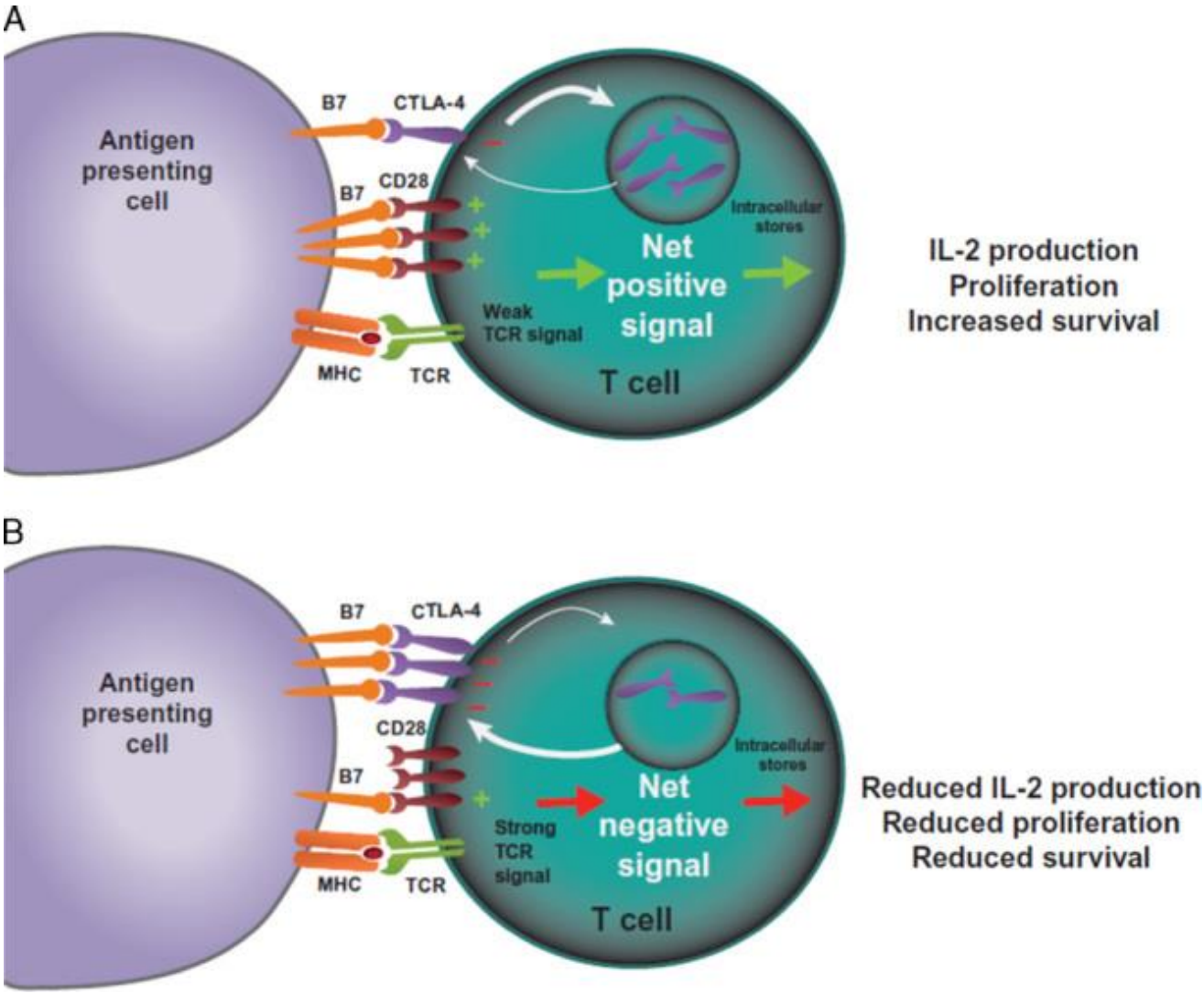


Figure (1-6) CTLA-4-mediated inhibition of T cells. T cells are activated when TCRs bind antigen displayed in the MHC (major histocompatibility complex) on antigen-presenting cells in concert with CD28:B7-mediated stimulation. A, In the case of a weak TCR stimulus, CD28:B7 binding predominates, resulting in a net positive activating signal and IL-2 production, proliferation, and increased survival. B, In the case of a strong TCR stimulus, CTLA-4 expression is upregulated by increased transport to the cell surface from intracellular stores and decreased internalization. CTLA-4 competes with CD28 for binding of B7 molecules. Increased CTLA-4: B7 binding can result in a net negative signal, which limits IL-2 production and proliferation, and limits survival of the T cell. CTLA-4 indicates cytotoxic T-lymphocyte–associated antigen 4; IL-2, interleukin-2; MHC = major histocompatibility complex; TCR = T-cell receptor [81].

1.3.1.1 Cytotoxic T Lymphocyte Antigen -4 Structure:

CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) are the most characterized of the immunoglobulin (Ig) family of coreceptors. Its genes are located on human chromosome 2q33. It forms a homodimer and bind the ligands CD80 (B7-1) and CD86 (B7-2), using a signature MYPPPY binding motif .CD80 binds CTLA-4 and CD28 with different affinities (K_d values of approximately 12 and 200 nM, respectively) The higher affinity binding of CTLA-4 is due to a periodic arrangement in which bivalent homodimers bridge bivalent CD80 molecules. Although T-cell activation can occur with a potent T-cell receptor (TCR) signal alone (i.e., high avidity peptide), CD28 is required in most responses to peptide antigen [82].

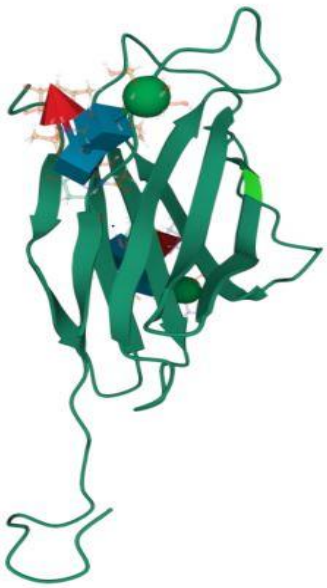


Figure (1-7) Structure of CTLA-4 protein [83]

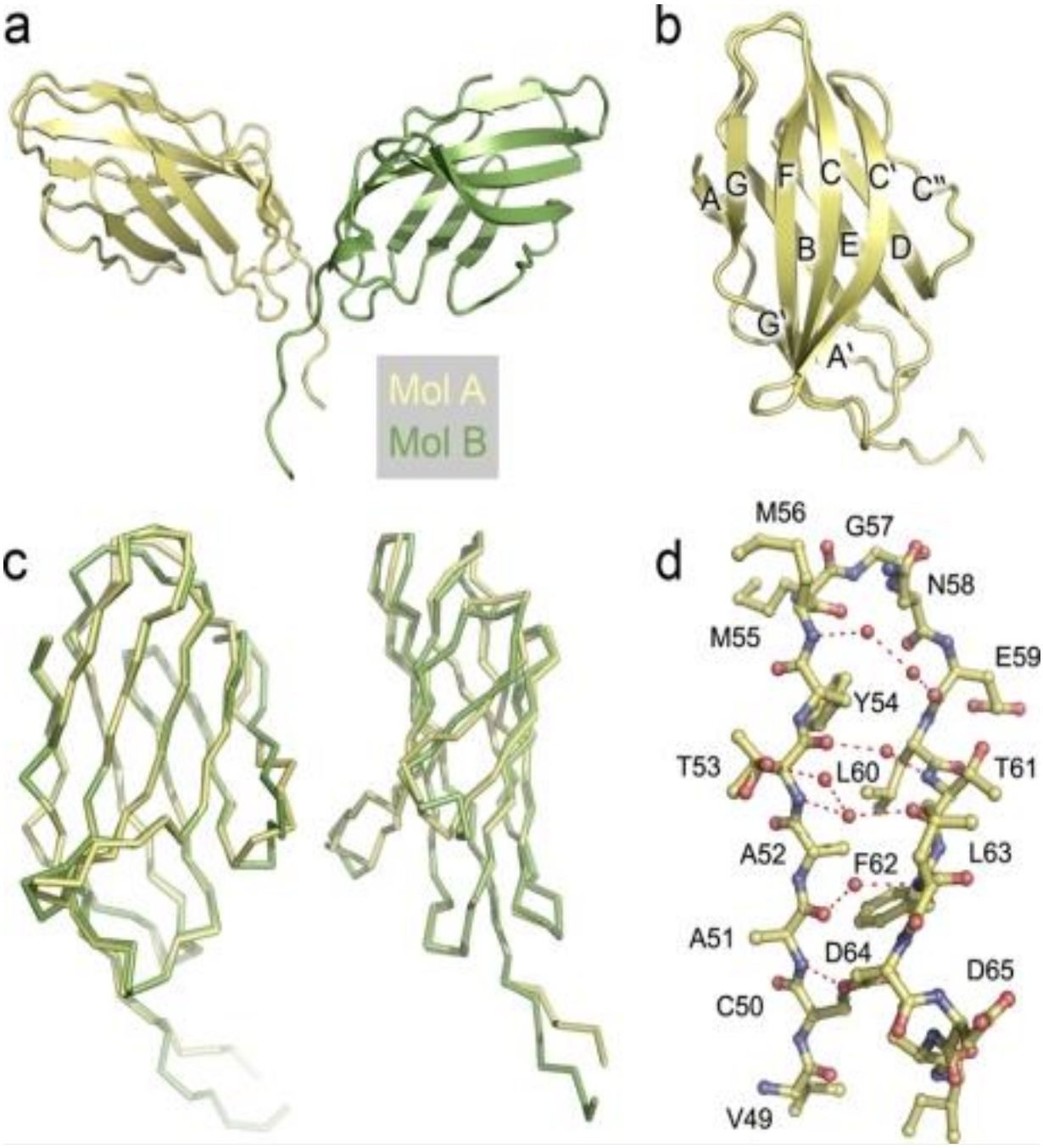


Figure (1-8) The structure of apo-CTLA-4. a, the asymmetric unit of the CTLA-4 crystals containing the apo-CTLA-4 homodimer. Mol A, which had the lowest B factors is colored yellow and mol B, green. b, secondary structure of the IgSF V-set domain of CTLA-4 (mol A). c, two orthogonal views of mol A and mol B of CTLA-4 shown as α-carbon. The position of the domains is similar to b. d, detail of the structural water in the region of the C′ and C″ strands and the C′C″ loop of mol A [83].

1..3.1.2 Function

CTLA-4 can bind to ligands expressed on the surface of antigen presenting cells (APCs). CD28 can bind the same. CD28 interacts with the CD80 dimer with relatively high affinity and the CD86 monomer with lower affinity, mediating T cell co-stimulation in conjunction with T cell receptor (TCR) signals. In contrast, interactions of the ligands with CTLA-4 serve to inhibit T cell responses, although the precise mechanisms are not fully understood. CTLA-4 interacts with both ligands with higher affinity and avidity than CD28(40) with CTLA-4-CD80 forming the highest avidity interaction and CD28-CD86 the weakest. Amongst several possibilities, this raises the concept that CTLA-4 can compete with CD28 for ligand binding and thereby act as an antagonist of CD28-mediated co-stimulation (41). These interactions are thought to take place at the immune synapse between T cells and APCs where CTLA-4 has been showing to recruit CD80 thereby limiting its interactions with CD28[77,80,84].

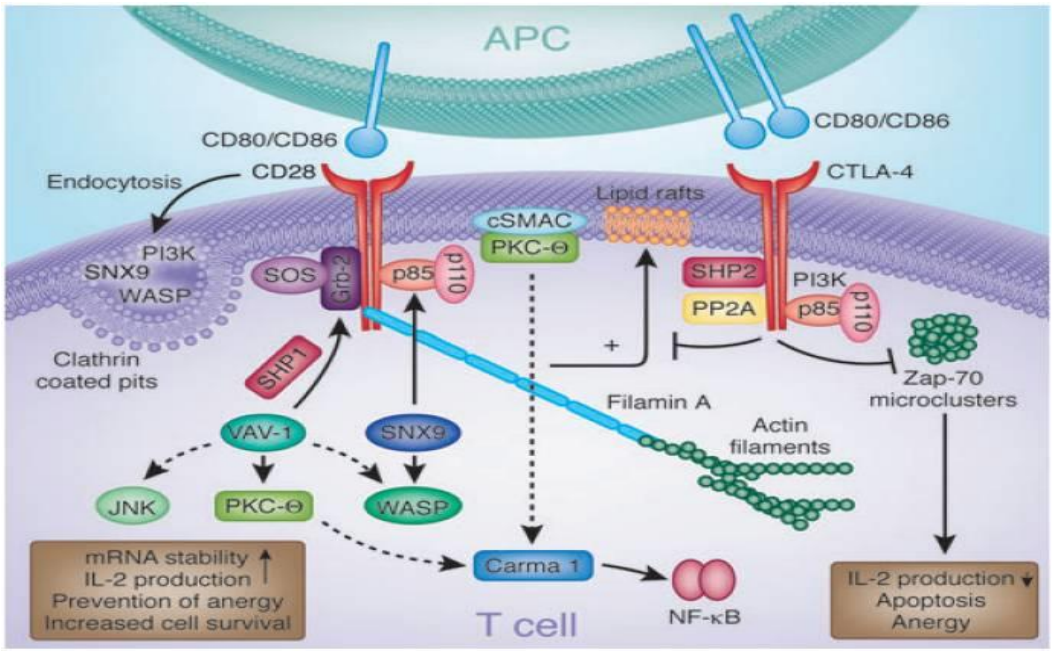


Figure (1-9) CTLA-4 and CD28 role in T cell Activation [84]

1.3.2 Cytotoxic T Lymphocyte Antigen 4 and Cardiovascular Diseases

The inhibitory role of CTLA-4 on T cell activation makes it important for preventing inflammatory and autoimmune diseases. mutation in the CTLA-4 such as CT60A>G and +49A>G reported to have association with several diseases such as Type 1 diabetes athyroid diseases, RA, Graves' disease. CTLA4 inhibits activation of either pro- or anti-inflammatory T cells. It has been known that inflammation is a mediator of CVD. Thus, any changes in the activation of T cells can affect the state of inflammation in the heart, which promotes the development of atherosclerosis which is a major contributor to the development of CVD [6,7].

Multiple studies also found that inflammation and autoimmunity are a big risk factors in the development of cardiovascular diseases. The inflammatory process is a major contributor to the formation of atherosclerotic plaque [3,48-50]

The most commonly studied polymorphisms in the *ctla*-4 gene. The first one is located in the promoter region (the −318C>T polymorphism), and can affect its protein expression. In addition, the exon 1 (+49A>G) polymorphism of CTLA-4 may affect the interaction between the protein and its ligand, B7.1, which is required for its T-cell inhibitory activity. These polymorphisms could potentially influence the risk of CVD. Several studies have investigated the association between these polymorphisms and CVD risk, and found a positive correlation between them [85,86].

Aims of The Study:

The current study aims to analyze the role of:

1- The Polymorphism of *ctla-4* gene +49 A>G.(rs231775).

2 – Serum CTLA-4 Protein Concentration.

As a risk factor in the development of CVD

CHAPTER TWO

MATERIALS AND METHODS

2.1 Subjects and Study Design

A case–control study of 126 individuals were included in this study, and divided into two groups; the first was patient group those who complain from cardiovascular disease (CVD), and the second was apparently healthy control group. The study was conducted to assess the association of SNP rs231775 of *ctla-4* gene and its protein levels with cardiovascular disease, 69 were suffering from cardio vascular disease. The CVD group included 37 patients with heart failure and 32 with MI. The CVD group included (36 male and 33 Female), and 57 control subjects (26 males 31 females).

Current study was performed at the laboratory of chemistry and biochemistry department, college of medicine, University of Babylon. All samples were collected during the period from the 20th of October 2020 till 20th January2021.The samples were collected from Marjan and Imam Al-Sadiq teaching Hospitals in Babylon province.

2.1.1 Inclusion Criteria

a- Ages between 40 – 60 years.

b- Diagnosed with a CVD (according to the specialized cardiologist)

2.1.2 Exclusion Criteria

Subjects who have one or more of the following criteria were excluded from the study:

1-Smokers.

2- Obese

3- History of diabetes, hypertension and liver, kidney and chronic disease.

4- Ages other than 40 – 60 years.

2.2 Questionnaire: Attached in the appendix (1).

2.3 **Ethical Issues**

Ethical issues achieve base on:

A) Agreement of Babylon medical college (University of Babylon, Iraq) and Biochemistry department at the same institution.

B) Scientific committee agreement of Marjan and Imam Al-Sadiq teaching Hospitals.

C) The goals and methods of this research have been clarified to all members in the present research in order to obtain their oral approval.

The project suggesting and sampling process were approved by the committee of publication principles at college of medicine, Babylon University, Iraq. This project also attains the authorization of research ethics in Murjan teaching hospital and Imam AL-Sadiq teaching Hospital at the date 20/10/2020

2.4 Materials

All kits and materials that used in current study as in table 2.1

Table 2.1. All materials that used in the study

Item	Origin
Absolute Ethanol (100%)	China
CTLA-4 ELISA Kit	Ela Science /USA
Disposable Syringes (5 mL)	Medical Jet / Syria
DNA Extraction Kit	Intron / Korea
EDTA Tube (5ml)	AFCO / Jordan
Eppendorf Tube (1.5ml,0.2ml)	China
Nuclease Free Water	Promega / USA
Primers	IDT / USA
Prob qPCR Master Mix	Promega / USA
Proteinase K (10 mg/mL)	Intron / Korea
RNase	Intron / Korea
TaqMan Probe (FAM and HEX)	IDT / USA

2.5 Apparatus

All instruments and devices that used in this work were summarized in table 2.2

Table 2.2 Instruments and devices used in this study

Instrument	**Origin**
Distiller	GFL / Germany
Electrophoresis System	Auto / Japan
ELISA Reader	Biotech / USA
ELISA Washer	Biotech / USA
Hot Plate	Grant / England
Incubator	Fisher Scient. /Germany
Laminar Flow	4labtech / Korea
Micropipettes	Slammed / Germany
Photo documentation	E-Graph/ Japan
Sensitive balance	Sartorius / Germany
UV Trans-Illumination	UV Trans-illumination E- graph / Japan
Vortex (Electronic)	Cleaver / UK
Water Bath	GFL / Germany

2.6 Methods

2.6.1 Collection of Blood Samples

Five milliliters of whole blood were collected from all participant's individuals. The collected blood samples were divided into an EDTA tube for molecular study, and another part was used to obtain serum for CTLA-4 estimation. The serum samples were stored at -20 until use.

2.6.2 BMI Measurement

Body max index (BMI) of both groups' subjects were calculated by using the formula:

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

2.6.3 Determination Blood Glucose Concentration

Principle: Enzymatic reference method with hexokinase, hexokinase was used to assess the levels of Glucose in sera. The phosphorylation of glucose by ATP to form glucose-6-phosphate and ADP is catalyzed by Hexokinase (HK). A second enzyme, glucose-6-phosphate dehydrogenase (G6PDH), is used to catalyze NAD+ glucose-6-phosphate oxidation in order to form NADH+.

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{D-glucose-6-phosphate} + \text{ADP}$$

$$\text{D-glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{D-6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

Reagents:

R1	MES buffer: 5.0 mmol/L, Ph 6.0 Mg2+: 24 mmol/L; ATP: >4.5 mmol/L; NADP: >7.0 mmol/L; preservative.
R2	HEPES Buffer: 200 mmol/L, pH 8.0; Mg2+: 4 mmol/L; HK (yeast): >300 ukat/L; G-6-PDH (E. coli): >300 ukat/L; preservative

Procedure:

1. Wavelength……505nm (500-510)

2. Cuvette……1cm.light path

3. Temperature…….37C/15-25C.

4. Adjust the instrument to (zero) with distilled water.

5. Into clean dry test tubes labeled as Blank (B), Standard(S), and Sample Pipette the reagent. 6. The tubes mixed and incubate at 37°C for 5 min.

7. The absorbance of the standard and test of sample against blank was measured.

8. At the end incubation the color is stable between 15-30min and the read should be done with this period.

Calculation:

Glucose (mmol/L.) = (A) Sample/(A) STD x (Standard Conc.) normal range of fasting blood glucose concentration is 3.5–5.5 mmol/L

2.6.4. Measurement of Serum Urea Level.

Principle: Kinetic test with urease and glutamate dehydrogenase was depended Urea is hydrolyzed to form ammonium and carbonate through urease., in the second reaction 2-oxoglutarate reacts with ammonium in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH are oxidized into NAD for each mole of urea hydrolyzed.

$$\text{Urea} + 2H_2O \xrightarrow{UREASE} 2\ NH_4+ + CO_3^{2-}$$

$$NH_4^{+1} + \text{2-oxoglutarate} + \text{NADH} \xrightarrow{GLDH} \text{L-glutamate} + \text{NAD+} + H_2O$$

The rate of decrease in the NADH concentrations is directly proportional to the urea concentration in the specimen. It is determined by measuring the absorbance at 340 nm.

Reagents

R1	TRIS buffer: 220 mmol/L; 2-oxoglutarate: 73 mmol/L; NADH: 2.5 mmol/L; ADP: 6.5 mmol/L; urease (jack bean): ≥ 300 µkat/L; GLDH (bovine): ≥ 80 µkat/L; stabilizers; pH 8.6

Procedure:

1. Bring reagents and samples to room temperature.

2. Pipette into a cuvette:

Tubes	blank	stander	Sample
Working reagent	1.0ml	1.0ml	1.0ml
stander		10 µL	
sample			10 µL

3. The additive was mixed and incubated for 5 minutes at 37 ºC 4. Finally, the mixture was subjected to addition Pipette: 45 R3 1.0ml 1.0 ml 1.0 ml The contents of mixture mixed thoroughly and incubated the tubes for (5 minutes) at (37ºC) or for (10 minutes) at room temperature (16-25ºC).

absorbance (A) of the samples and the standard at 600 nm against the reagent blank was read. The color is stable for at least 2 hours protected from light. **Calculations**

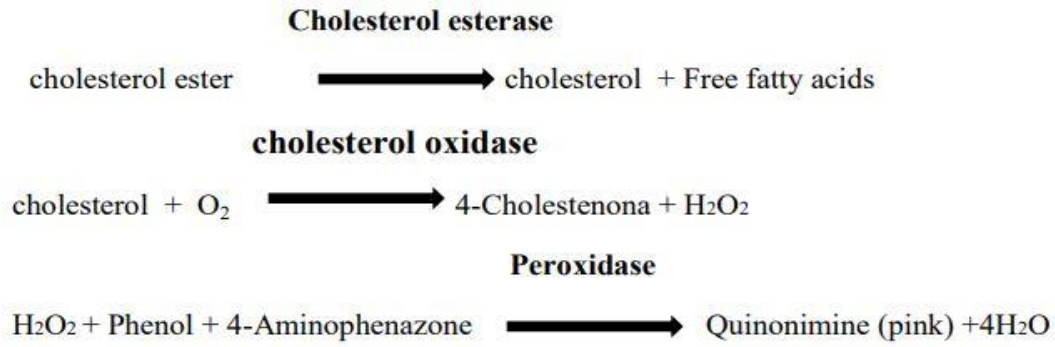
Conc. urea = ΔA Sample / ΔA Standard x standard concentration mg/dl

2.6.5 Measurement of Serum Lipid Profiles

2.6.5.1 Estimation of Fasting Total Cholesterol Level

The concentration of cholesterol was enzymatically calculated based on

:



Reagents

Content of Reagents	Composition of Reagents vial	
Reagent of Vial 1 (Buffer)	Sodium chlorate	2.3 mmol/L
	Triton x 100	1.5 mmol/L
	Phosphate buffer	100mmol/L
	Chloro-4-phenol	5mmol/L
	Preservative	
Reagent of Vial 2 (Enzymes)	Peroxidase	> 1200 IU/L
	4- Amino-antipirina	0.25µmol/L
	Polyethyleneglycol	6000 167 µmol/L
	cholesterol oxidase	>100 IU/L
	Cholesterol esterase	>170 IU/L
Reagent of Vial 3 (Standard)	Cholesterol	5.17 mmol/L

Reagent preparation:

According to the manufacturer company; both reagents 1 and 2 are mixed for two minutes .

Calculation

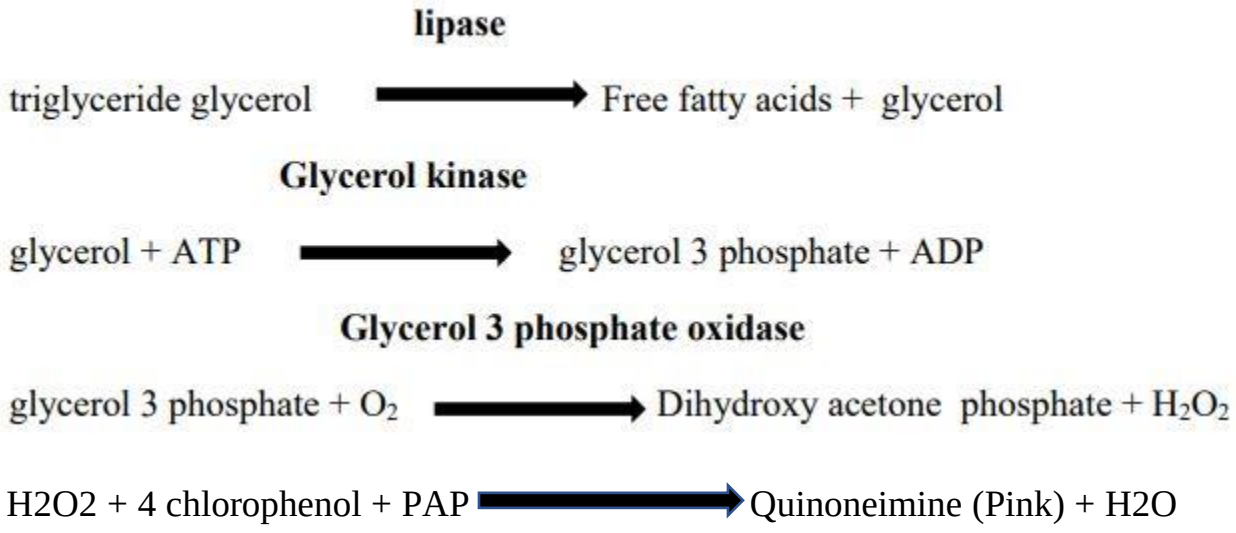
The result was calculated as follows:

Total cholesterol = Absorbance of the sample / Absorbance of the standard x concentration of standard

Standard concentration = 5.17 mmol/L

2.6.5.2 Determination of Fasting Triglycerides (TG)

Concentration Principle:



H2O2 + 4 chlorophenol + PAP → Quinoneimine (Pink) + H2O

The absorbance of quinonimine, which is the colored compound, was directional to the sample amount.

Calculation:

The result was calculated as follows:

Triglyceride = Absorbance of the sample / Absorbance of the standard x concentration of standard

Standard concentration = 2.28mmol/L

2.6.5.3 Assay of High -Density Lipoprotein Level

At the start Very Low-Density Lipoprotein Cholesterol (VLDLc), Low Density Lipoprotein Cholesterol (LDLc), and Chylomicrons have been precipitated from phosphotungstic acid and Magnesium Chloride specimens. After centrifugation, HDLc was acquired in supernatant and then treatment as total cholesterol.

Procedure:

In a clean plain tube, a volume of 0.5 ml of serum was added, then the precipitant 50µl was added. The tubes were mixed in a vigorous manner and let stand for ten minutes at room temperature. Then centrifuge for 15 minutes at 3,000- x g. The following procedure was applied for the measurement of HDL-cholesterol in the supernatant:

Reagents	Blank	Standard	Assay
Working reagent	1ml	1ml	1ml
Standard	-	25µl	-
Supernatant	-	-	25µl
Distilled water	25µl	-	-

The tubes were mixed, and leave stands for five minutes at 37°c or ten minutes at room temperature. Absorbance was recorded at 500nm against blank. The color was stable for 1 hour.

Calculation:

HDL-cholesterol = Absorbance of the sample / Absorbance of the standard x concentration of standard

Standard concentration =2.58mmol/L.

2.6.5.4 Very Low-Density Lipoprotein Cholesterol Assay

Method Very Low-Density Lipoprotein Cholesterol was calculated according to the following equation.

VLDL (mg / dl) = Triglyceride / 5

2.6.5.5 Determination of LDL-Cholesterol

It determined by Friedewalds equation which is an indirect method

LDL- cholesterol = Total cholesterol – (HDL- cholesterol + VLDL- cholesterol)

2.6.6Measurement of Human CTLA-4

Measurement of Cytotoxic T Lymphocyte Antigen -4 was done using a sandwich ELISA technique

A- Test Principle

The micro-ELISA plate has been pre-coated with an antibody specific to human CTLA4. Standards or samples were added to the micro-ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for human CTLA4 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain human CTLA4, biotinylated detection antibody and Avidin-HRP conjugate will appeared with blue color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) was measured at a wavelength of 450 nm ± 2 nm.

The OD value is proportional to the concentration of human CTLA4.Levels of human CTLA4 in the samples can be by comparing the OD of the samples to the standard curve.

B- Preparation of Reactants:

1. All reagents were brought to room temperature (18~25℃) before use. Then the microplate reader manual for set-up and preheated it for 15 min before OD measurement.

2. Wash Buffer: A volume of 30 mL of concentrated wash buffer was diluted with 720 mL of deionized or distilled water to prepare 750 mL of wash buffer.

3. Standard working solution: Standard solution was subjected to centrifugation at 10.000×g for 1 min, after that 1.0 mL of reference standard & sample diluent was added and let it to stand for at least 10 minutes at room temperature, and inverted gently several times, then mixed thoroughly by pipetting. This reconstitution produces a working solution of 10 ng/milk. Standard curve was built relying a serial dilution as follows: 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0 ng/milk. A total 7 Eppendorf tubes, added 500uL of reference standard & sample diluent to each tube. A volume of 500uL pipetted of the 10 ng/mL working solution to the first tube and mix up to produce a 5 ng/mL working solution. A volume of 500uL of the solution from the former tube into the latter one was pipetted according to these steps, while the last tube left as a blank. The serial dilution method demonstrated in figure 2.1.

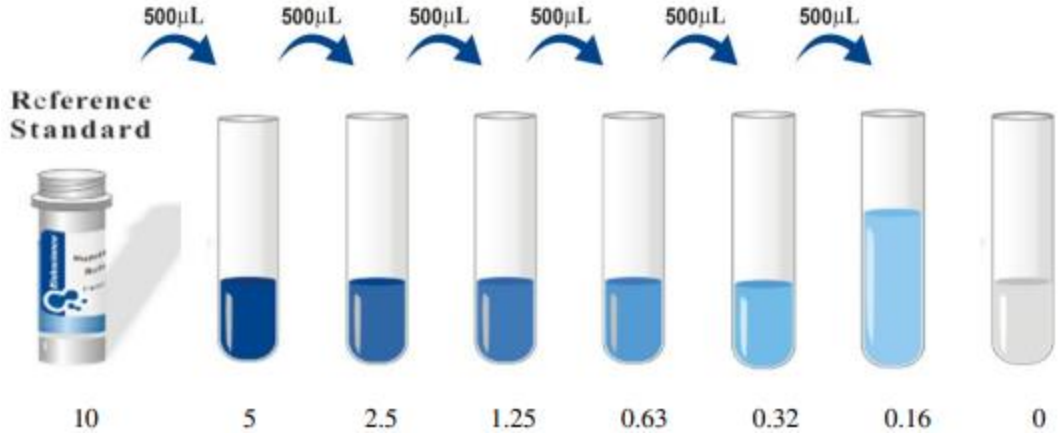


Figure (2-1): Serial dilution of the standard curve of CTLA-4

4. Biotinylated detection antibody working solution: The required amount before the experiment (100 µL/well) was calculated. In preparation, slightly more than calculated should be prepared. The stock tube centrifuged before uses, and diluted the 100× concentrated biotinylated detection antibodies to 1×working solution with biotinylated detection antibodies diluent.

5. Concentrated HRP conjugate working solution: The required amount of HRP was calculated before the experiment (100 µL/well). The 100x concentrated of HRP conjugate was diluted to 1× working solution with concentrated HRP conjugate diluent.

C- Assay Procedure

1. Standard working solution 100 µL was added for each well. The samples were added to the other wells 100 µL. The plate was covered with the sealer provided with kit. The plate was incubated for 90 min at 37℃. All solutions were added to the bottom of the micro-ELISA plate wells, with avoiding touching the inside wall and causing foaming as much as possible.

2. Liquid was removed out from each well, without washing. Then, immediately 100 µL of biotinylated detection antibodies working solution was added to each well. The Plate was covered with sealer and gently mixed, and incubated for 1 hour at 37°C.

3. The solution decanted from each well, and a volume of 350 µL of wash buffer added to each. The solution soaked left for 1~2 min and the solution decanted from each well and put it dries against clean absorbent paper. Washing step repeated 3 times.

4. A volume of 100 µL all HRP conjugate working solution was added to each well. The Plate was covered with sealer and incubated for 30 min at 37°C.

5. Aspirate or decant the solution from each well, and washing process was repeated for five times as conducted in step 3.

6. Substrate reagent 90 ul was added to each well. A new plate sealer was used to cover the plate, and incubated for about 15 min at 37°C with caring plate from light.

7. A volume of 50 ul all of stop solution was added to each well. Stop solution was added at the same order as the substrate solution.

8. The optical density measured foreach well at once with a micro-plate reader steed to 450 nm.

E- Calculation of Results

The readings for standard and samples were taken, then subtract the average zero standard optical density. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis. If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by

the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, should re-test it with an appropriate dilution. The actual concentration was calculated concentration multiplied by the dilution factor.

F- Typical Data

As the OD values of the standard curve may vary according to the conditions of the actual assay performance that lead to establish a standard curve for each run.

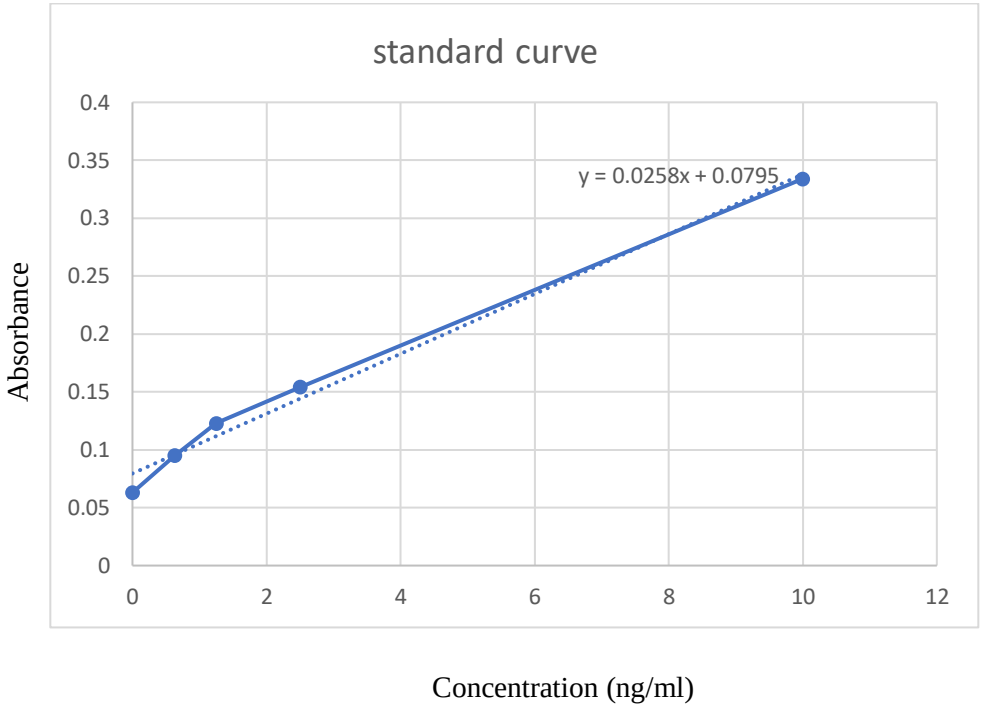


Figure (2-2) Standard curve of CTLA-4

2.7 Genotype Analysis

DNA was extracted from whole blood using intron DNA extraction kit. Genotyping was carried out by the Real-Time Polymerase Chain Reaction. Primers, TaqMan Probes (FAM and HEX), and a probe master mix kit was used in the amplification reaction with standard conditions.

2.7.1 DNA Extraction

The DNA was extracted using extraction kit from Intron company

2.7.1.1 DNA Extraction Procedure

1- A volume 200µl of whole blood was added to Eppendorf tube (1.5 ml).

2- A volume 20µl of proteinase K and 5µl of RNase A solution were added and mixed.

3- A volume of 200µl of BL Buffer added to the mix and thoroughly mixed.

4- The mixture left for 2 minutes at room temperature then incubated for 10 min at 56°c.

6- The lysate centrifuged for 30 seconds.

7- A volume of 200µl of pure ethanol were added and then mixed using vortex.

8- The mixture then transferred to the spin column and centrifuging for 1 minute at 8000 xg. The filtrate was discarded and the spin column transferred to new 2 ml collection tube.

9- A volume of 700µl of "Buffer WA" added to the spin column and then centrifuged for 1 minute at 8.000 xg.

10- A volume of 700µl "buffer WB" was added to the spin column and centrifuged for 1 minute at 19.000 xg the then centrifuged again to dry the membrane from the ethanol for another 1 min.

11- The spin column was transferred to a 1.5 ml tube, and then 100µl of "Buffer CE" were added and incubated at room temperature for 1 minute, and then centrifuged to elute DNA for 1 minute at 8.000 xg.

12- The extracted DNA was stored at -20 C.

2.7.2 Real Time PCR

Real-time polymerase chain reaction (RT-PCR) method is widely used for the quantification of genes from cells, and tissues. This method allows for a direct detection of PCR product during the extension phase of the reaction, which combine the amplification step and the detection in a single step [87].

2.7.2.1 TaqMan Probe

TaqMan probes are labeled with two fluorescent dyes that emit light at different wavelengths. The probe sequence is intended to hybridize specifically in the DNA target region of interest between the two PCR primers. Typically, the probe is designed to have a slightly higher annealing temperature compared to the PCR primers so that the probe will be hybridized when extension (polymerization) of the primers begins. A minor groove binder is sometimes used near the 3′-end of TaqMan probes to enable the use of

shorter sequences that have higher annealing temperatures than would be expected for sequences of equivalent length.

The “reporter” (R) dye is attached at the 5′-end of the probe nucleotide sequence while the “quencher” (Q) dye is synthesized and labeled at the 3′-end. A popular combination of dyes is FAM or VIC for the reporter dye and TAMRA for the quencher dye. When the probe is intact and the reporter dye is in close proximity to the quencher dye, little to no fluorescence will result because of suppression of the reporter fluorescence due to an energy transfer between the two dyes.

During polymerization, strand synthesis will begin to displace any TaqMan probes that have hybridized to the target sequence. The Taq DNA polymerase used has a 5′-exonuclease activity and therefore will begin to chew away at any sequences in its path (i.e., those probes that have annealed to the target sequence). When the reporter dye molecule is released from the probe and is no longer in close proximity to the quencher dye, it can begin to fluoresce. Increase in the fluorescent signal results when the target sequence is complementary to the TaqMan probe. It is important to note that mismatches between the DNA template sequence and the TaqMan probe can cause failure to detect the DNA template appropriately [88-90].

2.7.2.3 Stock Primers Preparation

1- Vial of primers and probes were centrifuged first to ensure not lost the lyophilized primer.

2- Nuclease free water was added to get a 100 Picomolar, the amount of nuclease free water was added is recorded at table (2-3) and left to stand for 2 minutes.

3- Vials were vortexed to re-suspension.

4- Stock solution was stored at -20°C.

Table 2-3 Preparation of stock solutions of primers and probes

Reagents	**Amount of nuclease free water for each**
Allele **A** Probe	173 microliters
Allele **G** Probe	165 microliters
Forward Primer	232 microliters
Reverse Primer	304 microliters

2.7.2.4 Reagent's Preparation

1-The stock solutions were thawed and vortexed before use.

2-The stock solution was diluted 1:10 using nuclease water to obtain the working solutions with 10 Picomolar.

3- Working solutions were stored at -20°C after use.

2.7.2.5 Working Procedure:

The assay was carried out according to table 2-4

Table 2-4 Content of monoplex real time PCR reaction

Item	Volume (ul)
2X qPCR master mix (I-Taq)	10
Forward Primer (10 pmol/ul)	1
Reverse Primer (10 pmol/ul)	1
HEX Probe	1
FAM Probe	1
DNA Template	2
Free Nuclease Water	4
Total Volume	20

2.7.2.6 Steps of PCR Cycle

a. Uncoiling of the double-stranded DNA through denaturation to a two single strands of DNA. This step occurs at 94-95 °C.

b. Annealing: annealing of primers to the DNA.

(primers= forward and reverse) needs to stand at 56 C the end of the DNA template.

c. Extension: occurs at 72° C, the polymerase creates new strand by adding nucleotides.

Table 2-5 The sequence of the primers and TaqMan probes used in this study

	Sequence (5'-3')	**Tm (°C)**	Product Size (bp)
Forward Primer	5'- CCT GAA CAC CGC TCC CAT-3'	57.6	153
Reverse Primer	5'- GCT CCA AAA GTC TCA CTC ACC T-3'	56.9	
Allele **A** (FAM Probe)	5'- AGC TGA ACC TGG CTA CCA GGA CCT-3'	63.8	
Allele **G** (HEX Probe)	5'- CTG AAC CTG GCT GCC AGG ACC T-3'	63.7	

2.8 Protein Modeling Protocol

Homology modeling and sometime known as comparative modeling, it is based on the biological fact that when two sequences share high similarity/identity, their respective structures are also similar. It is greatest accurate computational process to making dependable structural models. Frequently homology model used in several biological applications. It predicts the 3D structure of a requested protein through the sequence alignment of template proteins. Normally, the method of homology modeling includes 4 steps: 1) selecting and identification of intended target,2) optimizing sequence alignment,3) model building for inquired sequence and finally 4) model refinement [91].

When there is only sequence data of amino acids are available homology modeling has become only useful tool for the prediction of protein structure. mostly determination of protein function preferred by depending on structural information is more beneficial

than sequence alone. Many Protein modeling servers were performed to build the predicted 3D protein structure as in flow chart below.

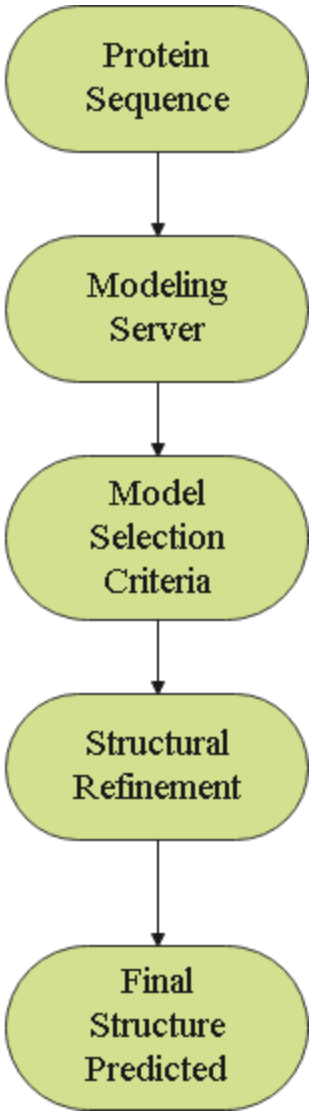


Figure (2-3): Flow chart of predicted protein structure depending protein modeling servers.

2.8.1Computational Assessments of CTLA-4 Variants

The sequence and structure analyses of non-synonymous single-nucleotide polymorphisms (nsSNPs) (rs231775 A>G) in *ctla-4* gene was performed depending on the art of computational approaches. Several predicting modeling of protein were used like I-TASSER, and Ramachandran plot, however the structural, stability, disease related and functional effects were analyzed by many proteins' analytical servers like, polyphen, i-mutant, SNPs &GO, SNAP2, PROVEAN, servers respectively, as demonstrated in flowchart bellow.

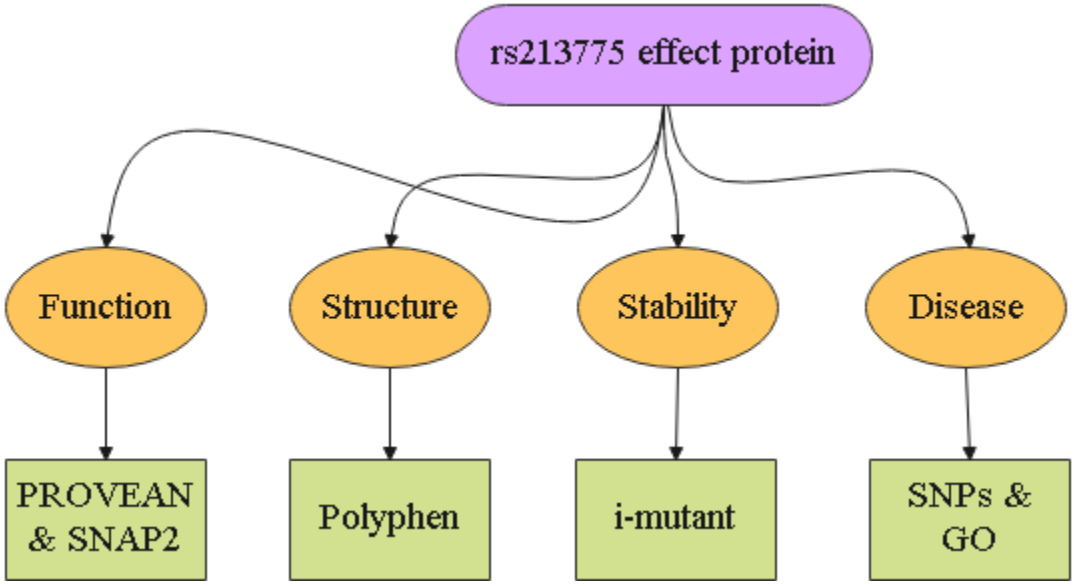


Figure (2-4): Flow chart of computational analysis of rs231775 A>G of *ctla-4* gene polymorphism protocol.

2.8.2 SNAP2 Server

It's based on the prediction of functional effect of mutation. SNAP2 is a trained classifier that is distinguishes between effect and neutral variants/non-synonymous

SNPs by taking a variety of sequence and variant features into account. Reliability score index was calculated from the final prediction score and shows that stronger predictions are more reliable. The score ranges from 0 (very low reliability) to 9 (very high reliability) [92]

2.8.3 PROVEAN Server

It is a tool developed for prediction of human proteins whether and sequence variants effects. It shows SIFT predictions when precomputed scores are available. It was suggested default score equals to -2.5, and reduce the effect to neutral when reach to zero, while far towards more negative result give deleterious effect [93]

2.8.4 Polyphen Server

It is a new and advanced development automatic tool used for annotating coding of most human genetic variation represented as nsSNPs (prediction of possible effect). Polyphen is a high-quality multiple sequence alignment [94]

2.8.5 i-Mutant Server

It is a web server for automatic prediction of protein stability changes upon point mutation mutations. I-Mutant predicts the value of the stability change upon single point mutation [95].

2.8.6 SNPs & GO Server

The genetic basis of human variability is mainly due to SNPs. The most investigated SNPs are missense mutations [96].

2.9 Statistical Analysis

SPSS version 26 was used for data analysis. Continuous data were presented as mean ± standard deviation and categorical data were presented as frequencies. Chi-square test (for categorical data) or T test (for continuous data) was performed to evaluate the differences between patients and controls and also to assess deviation from Hardy-Weinberg equilibrium. The association of polymorphisms with CVD was examined by calculating the odds ratios (ORs) and 95% confidence intervals (CIs). The wild type genotype was used as reference in the logistic regression model. A P value < 0.05 was considered significant.

2.9.1 Hardy–Weinberg Equilibrium

Hardy–Weinberg Balance (HWE) is a mathematical formula that links genotypes to allele frequencies. Allele frequencies that persist the same between parents and their kids suggested that there were no genetic mutations, and HWE was statistically subjected to the following equation.

$$p2+2pq+q2=1$$

p: refers to the major allele frequency

q: refers to the minor allele frequency.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Demographic Characteristics of the Study Subject

The age of the patients and control groups inducted in this study range between 40 to 70 years. Table (3-1) shows mean differences of study variables including (age and body mass index) according to study group (CVD patients and control).

Table 3-1: Demographic data of study groups.

Variable	Study groups	No.	Mean ± SD	P-value
Age (years)	Patients	69	52.18 ± 7.9	0.09
	Control	57	49.5 ± 6.4	
BMI (kg/m2)	Patients	69	25.85 ± 2.01	0.81
	Control	57	25.97 ± 1.6	

SD = Standard deviation, BMI =Body mass index. p value < 0.5 considered statically insignificant

3.1.1 Age

There was no significant (P value = 0.09) difference in age (as mean) between control and CVD patients, mean ±SD for patients (52.18 ± 7.9) and for controls (49.5 ± 6.4). This age matching helps to eliminate differences in parameters results that may originate due to the significant variation in age. Highest number was seen in the age group from 45 to 55 years old and the lowest number was in the age group from 60 to 70 years.

3.1.2 Body Mass Index (BMI)

Analysis of body mass index (BMI) as shown in table (3.1). The analysis shows no significant difference in body mass index between patients with CVD group and control group P<0.81, mean ± SD for patients (25.85 ± 2.01), and controls (25.97 ± 1.6)

3.1.3 Gender

Gender allotment between control and patient groups as demonstrated in (figure 3.1). Patient group comprised of 48% females and 52% males, while control group comprised of 54% females and 46% males,

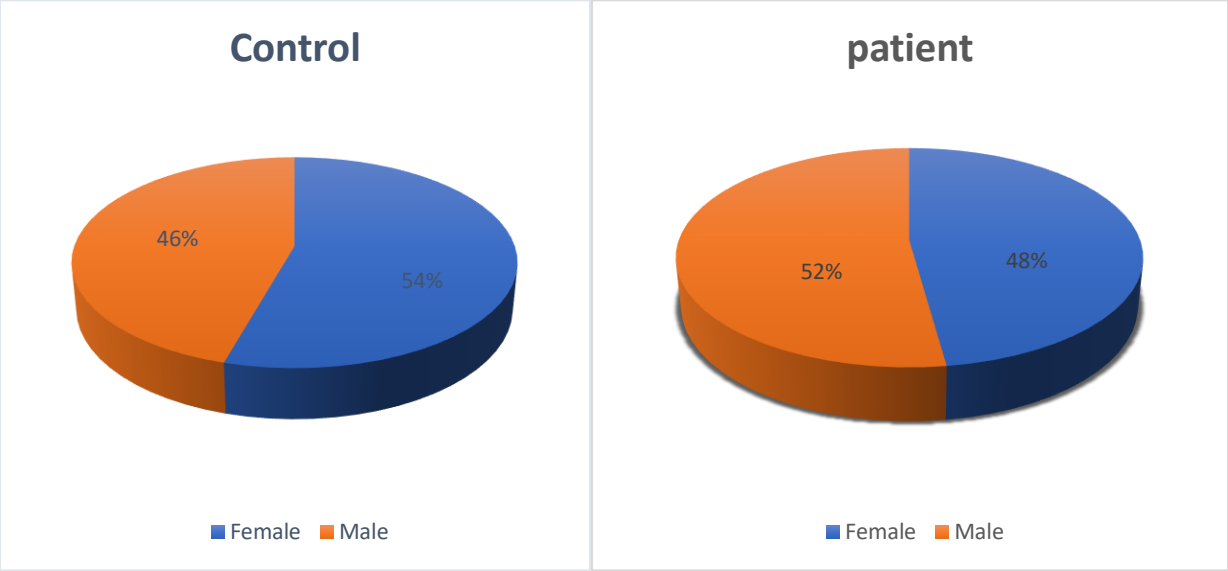


Figure (3-1) Gender distribution of patients group with CVD and control group.

3.2 Biochemical Analysis For Patients Group

3.2.1 Blood Urea and Fasting Serum Glucose

The results show normal levels of blood urea and serum glucose as shown in the table below. The results show no abnormal values since subjects with high blood glucose and urea were excluded from the study

Test	Mean ± SD
Blood urea	**32 ± 6 mg/dl**
Fasting Serum Glucose	**93 ± 20 mg/dl**

3.2.2 Measurement of Lipid Profile

The results showed normal levels of cholesterol (TC) and LDL-c, triglyceride and VLDL-c and serum HDL-c level. . Subjects with hyperlipidemia were excluded from the study hence the results are normal

Test	Mean ± SD
TG	137 ± 23 mg/dl
Total cholesterol	173 ± 28 mg/dl
HDL-c	63 ± 12mg/dl
LDL-c	157 ± 18 mg/dl
VLDL-c	29.3 ± 7.8mg/dl

3.3 Molecular Analysis of *ctla-4* gene

In current study the gene polymorphism of *ctla-4* gene was studied in Iraqi patients with CVD and healthy control groups. The genotyping was detected by allele discrimination real-time PCR using specific probes (Taqman probes) in order to detect the specific allele present. Two Taqman probes were used, each one of them specific to one of the alleles.

3.3.1 Measurement of Concentration and Purity of Deoxyribonucleic Acid

Genomic DNA was extracted from whole blood according to instruction of corporation (Intron; Korea). The concentration and purity of the extracted DNA were measured by the calculation of ultraviolet light absorption. A ratio of absorbance at 260 nm and 280 nm was used to measure for the estimation of deoxyribonucleic acid (DNA) concentration and purity and expressed as (Mean ± SD) =42.09± 3.67 and 1.864± 0.05 respectively, the results as in table (3-2)

Table 3.2: Concentration and purity of DNA

Test	Mean ± SD
DNA concentration(µg/ml)	42.09± 3.67
Absorbance 260/280	1.864± 0.05

3.2.2 Genotyping of *ctla-4* Gene

Genotyping of *ctla-4* Gene rs231775 was done by real- time PCR using the HEX probe for the G allele and FAM probe for A allele. The results are shown in figure (3-2)

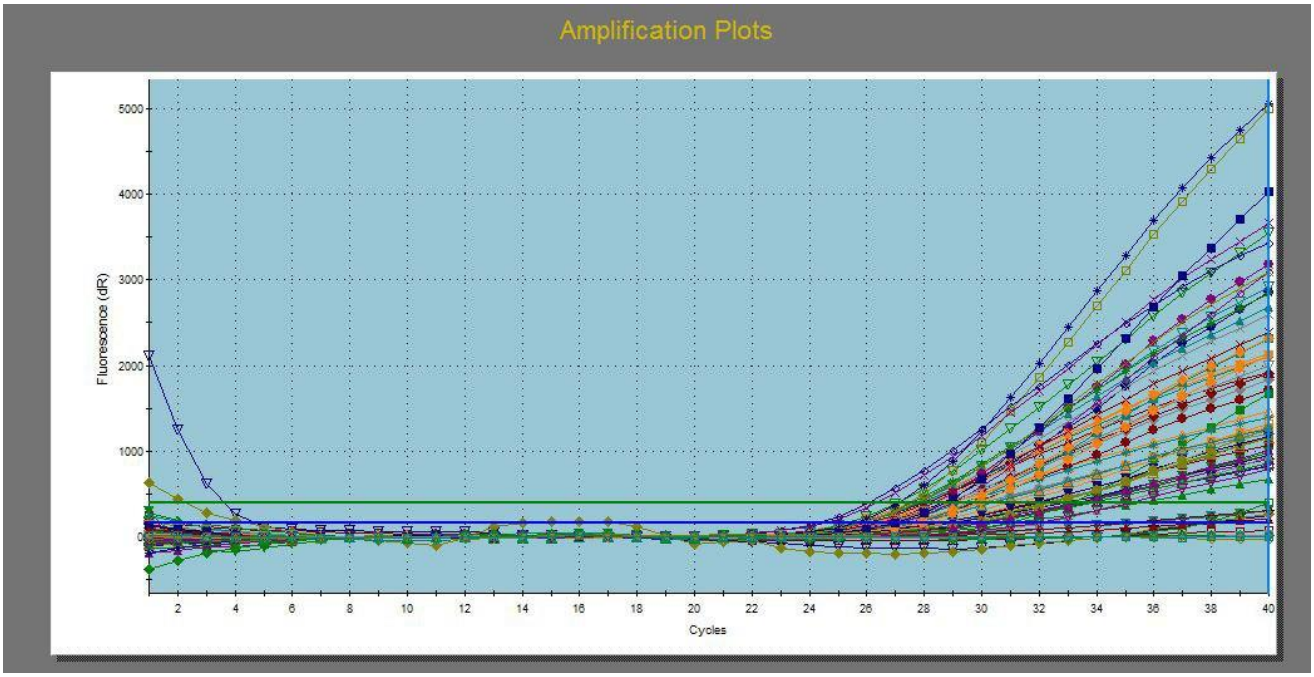


Figure (3-2) Amplification curve of real time-PCR of rs231775 polymorphism of the *ctla-4* gene, the zero line is the threshold line, the (X-axis) is refers to the number of thermal cycles, and the (Y-axis) is the fluorescent intensity, while the plot diagrams is the amplification products that display the FAM probe which have marker reaction to A allele, at the same time display circles marker for HEX probe reaction to G allele.

3.3.3 Genotype Distribution of rs231775

Genotype of rs231775 (A>G) variant of *ctla-4* gene polymorphism were shown in table (3-3). The most genotype found was heterozygote (AG), 72.30 % of the study subjects have this genotype and it is more frequent in control group and patients' groups (91.2% and 60.09% respectively) than the other genotypes; while (GG) genotype found in 21.4%% of the study subjects is more frequent in patients' group (34.8%) than the control group (2.8%). The genotype (AA) appeared the lowest consisting 6.3% of the

study subjects and it appeared more in control (8.7%) group more than patients' group (4.3%).

Table (3-3) Genotypes of *ctla-4* gene polymorphism rs231775 of standard group and patients' groups

Genotype	**Control** **N = 57**	**Patient** **N = 69**	**Total**
AA (Wild Type)	5 8.6%	3 4.3%	8 6.3%
AG (Heterozygous)	50 91.2%	41 60.1%	91 72.3%
GG (Homozygous Mutant)	2 2.8%	25 34%	27 21.4%
Total	57	69	126 100%

The higher frequency of (GG) in patient group also reported by (*Ruppert et al*, *Zhou et al*) which demonstrated that the (GG) genotype is overrepresented in the patient group compared to the control group, the mentioned study also shows the over representativeness of the (AA) and (AG) genotypes in the control group compared to the patient group this also showed in the current study. However, the frequency of the of (GG) allele in the of the first study and current study were both high in the patient group (43.3% and 34.8 respectively) there was difference in the frequency in control group

where the first study showed (GG) frequency of (35.8%) while current study showed (2.8%) in the control group, however the other study showed different results where the genotype was found (14.7%) in patient group and (7.4%) in control group. The differences in the frequency between studies occur due to the difference in the in the geographic area of the studies, number of subjects and the method used for genotyping.

3.3.4 Frequency Assessment of the Alleles According to Hardy–Weinberg Equilibrium Equation.

The result from Hardy-Weinberg equilibrium (WHE) exact test revealed that both control and patients group the genotype frequency not follow the Hardy-Weinberg equilibrium, indicating that the investigated allele frequencies are constant between generations as in tables (3-4 and 3-5)

Table (3-4) *ctla-4* gene in control group according to Hardy-Weinberg equilibrium (HWE)

Genotype of Control	Observed	Expected	Difference	X^2	P value
AA Reference	5	15.79	10.79	32.86	0.000
AG Heterozygote	50	28.42	21.58		
GG Recessive	2	12.79	10.79		
An Allele%	52.63				
G Allele%	47.37				
An Allele frequency	60				
G Allele Frequency	54				

The genotype frequencies of gene *ctla-4* SNP rs231775 wasn't consistent with Hardy–Weinberg equilibrium in control group.

Table (3-5) ctla-4 gene in patient group according to Hardy-Weinberg equilibrium (HWE)

Genotype of Patients	**Observed**	**Expected**	**Difference**	X^2	**P value**
AA Reference	3	8.00	5	7.193	0.0073
AG Heterozygote	41	30.99	10.01		
GG Recessive	25	30.00	5		
An Allele%	34.06				
G Allele%	65.94				
An allele frequency	47				
G Allele frequency	91				

The genotype frequencies of gene *ctla-4* SNP rs231775 exhibited not consistent with Hardy–Weinberg equilibrium in patient group

The genotype of SNP rs231775 in control and patient groups in codominant, dominant, over dominant, recessive and additive models were examined by multinomial logistic regression analysis (table 3-6) The analysis in regard with the co dominant, over dominant, recessive and additive models emphasized a significant (P= 0.003, 0.0008, 0.0003. 0.001 and 0.0032) respectively. In the homozygote genotype (GG) the OR=20.8, (95%CI: 2.735 to 158.72) P= 0.003 in Codominant model while have OR=15.63 (95%CI: 3.51-69.598), P= 0.0003 in recessive model that mean increase the folds of patient group more than 20.8 and 15.63 folds respectively. Heterozygous genotype (AG) the OR= 1.37 (95% CI: 0.308-6.063) P= 0.68 is not significant in control group and patient group.

Table (3.6) Genotype of *ctla-4* SNP rs231775 (+49A/G) in the studied groups of both the control and patients.

Table 3-6 Multinomial logistic regression analysis of *ctla-4* gene rs231775.

Model	**Control No. = 57**	**Patients No. = 69**	**OR (CI 95%)**	**P value**
Codominant				
AA (Wild Type)	5	3	1.00	
AG	50	41	1.37 (0.308-6.063)	0.68
GG	2	25	20.8 (2.735-158.72)	0.003
Dominant				
AG+GG	52	76	2.44 (0.558-10.64)	0.237
Over dominant				
AA+GG	7	28	1.00	
AG	50	41	0.21 (0.08-0517)	0.0008
Recessive				
AA+AG (Wild Type)	55	44	1.00	
GG	2	25	(3.51-69.598)	0.0003
Additive				
2AA+AG	60	47	1.00	
2GG+AG	54	91	2.15 (1.29-3.58)	0.0032

The results show that the GG genotype under the codominant and recessive models have a high OR at 95% CI (20.8 and 15.63 respectively) which means that the GG genotype is a considerable risk factor in the development of cardiovascular diseases.

This result is consistent with other were these studies concluded the GG genotype a risk factor of the studied CVD.

Inflammation and autoimmunity are a big risk factors in the development of cardiovascular diseases. The inflammatory process is a major contributor to the formation of atherosclerotic plaque.

Atherosclerosis is an inflammation of chronic nature and caused by dysfunction in immune system with lipids. In which a plaque formed due to interaction between immune and non- immune cell, lipids and inflammatory mediators).

Unnatural Continuous activation of T cell leads to formation of tumor and also inflammatory effects. CTLA -4 receptor act as an inhibitory regulator of the T-cells responses. CTLA-4 down regulate T-cell activation, thus the mutation in this regulatory molecule results in autoimmune disorders

The study concludes the same as the previous studies that the 49 A>G polymorphism in the *ctla-4* gene is a risk factor for the cardiovascular diseases, and GG homozygous genotype is associated with the increase risk of CVD. [3-5,47]

3.4 Serum CTLA-4 Concentration

In this part of study, a total of 90 subject were included ,45 of them were healthy subjects and 45 subjects with CVD. The comparison of CTLA-4 levels between

cardiovascular patients and healthy subjects showed no significant variation between both groups ($p > 0.05$) as showed in table 3.7.

Table 3-7 Serum CTLA-4 concentration in CVD and control groups

Group	No.	CTLA-4 (ng/L) ±SD	P value
Patient Group	45	1.38±1.03	0.474
Control Group	45	1.23±0.09	

SD = Standard deviation

3.4.1 The Cytotoxic T Lymphocyte Antigen-4 Concentrations in Healthy Subjects and Cardiovascular Disease Patients According to Gender

Table (3-2) shows increase concentration of serum CTLA-4 in of the patients in both female and male subjects but the increase was statically insignificant between mean differences between CVD patients and control group with p value of (0.56).

Table 3-8: The mean difference in CTLA-4 between female and male groups

Gender	Group	No.	Mean ± SD	P value
Female	Control	26	1.29 ± 0.82	0.46
	Patients	20	1.54 ± 1.2	
Male	Control	19	1.06 ± 1.1	0.44
	Patients	25	1.31 ± 0.88	

The CTLA-4 concentration of each genotype was analyzed. there was no significant difference in CTLA-4 levels between the genotypes.

3.4.2 Concentration of CTLA-4 in CVD Types in Patient Group

Analysis of patient group according to the cardiovascular disease type (MI and HF) showed no difference statically with a P value of 0.1.

Table (3-9): The difference of concentration of CTLA-4 between CVD types.

Type	No.	Mean ± SD	P value
Myocardial infraction	13	1.7 ± 0.7	0.12
Heart failure	32	1.2 ± 0.8	
Total	45	1.4 ± 1.01	

This study also shows no association between serum CTLA-4 concentration and the risk of CVDs.

The 49 A>G mutation in the *ctla-4* gene cause alteration the ctla-4 protein structure by replacing on of the amino acids with another. and thus, might lead to dysfunction of the ctla-4 receptor, this change in the protein produced occur due to substitution of alanine instead threonine at position 17. This change causes total energy of the protein

to decrease when threonine switched to alanine also the threonine is a non-charged polar hydrophilic amino acid while alanine is a non-polar hydrophobic amino acid. These changes in structure might be the cause of the dysfunction of CTLA-4 receptor even though the serum levels aren't different between patient and control group.

The protein was studies using online servers to determine if there are structural changes between the wild type and mutated protein and their effect on insufficiency of CTLA-4 in inhibiting the T cell activation.

3.5 Protein Modeling

3.5.1 3D Construction of Homo Sapiens CTLA-4 Protein (GI:83700231)

In order to construct a 3D model, the first step of protein modeling include obtaining of amino acid sequence (protein sequence) from NCBI server. Then, the obtained sequence has been submitted to I-TASSER modeling server (https://zhanggroup.org/I-TASSER/) with a job id (S635560) and the result predicted top five 3D models starting from the structure templates identified by LOMETS (meta server alignment) from the PDB library depending thousand template protein alignments. Based on the quality parameters that available in that server, first model was predicted to be more favorable than other models and built depending ten templates. The confidence of quality estimation of predicted CTLA-4 model by I-TASSER was expressed as C-score (C-Score =-1.90), while the measuring standard structural similarity between two structures which measure the accuracy of structure modeling –score was expressed as TM-score (TM= 0.49±0.15).

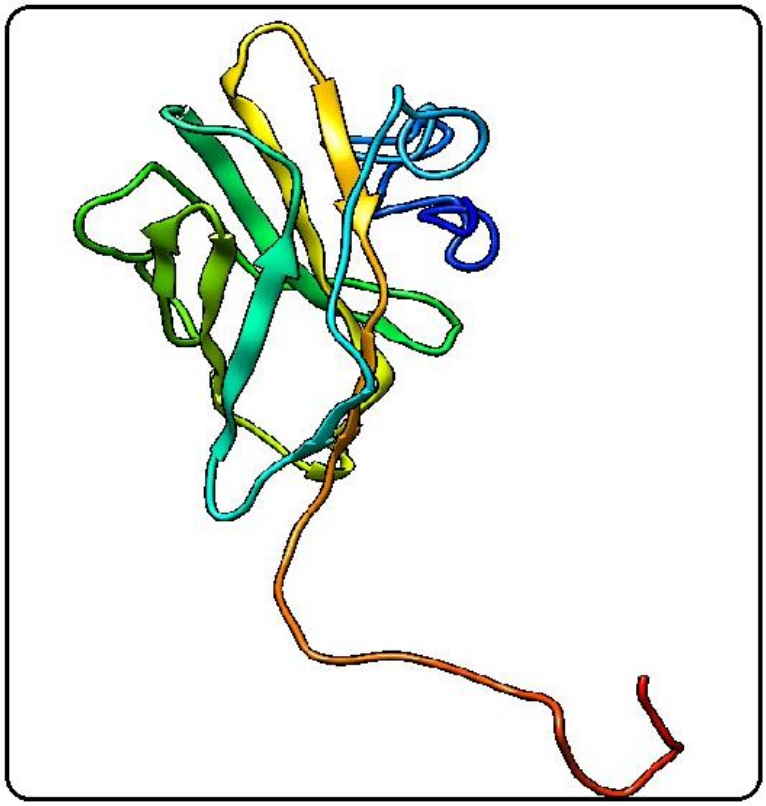


Figure 3-3: Predicted model of wild type human CTLA-4 protein (3D) according to I-TASSER server.

Results confirmation of the predicted models from I-TASSER modeling server, the obtained models have been submitted to Ramachandran plot server. The result comes identical to identified predicted I-TASSER modeling protein. The green dots distribution in the gray color demonstrates the stability of amino acids and represent (77.483%) and 117 out 174 amino acids. In the other hand, only (5.96%) and 9 out 174 amino acids were not preferred (red dots) and the remaining represents the allowed observation (brown dots), figure bellow.

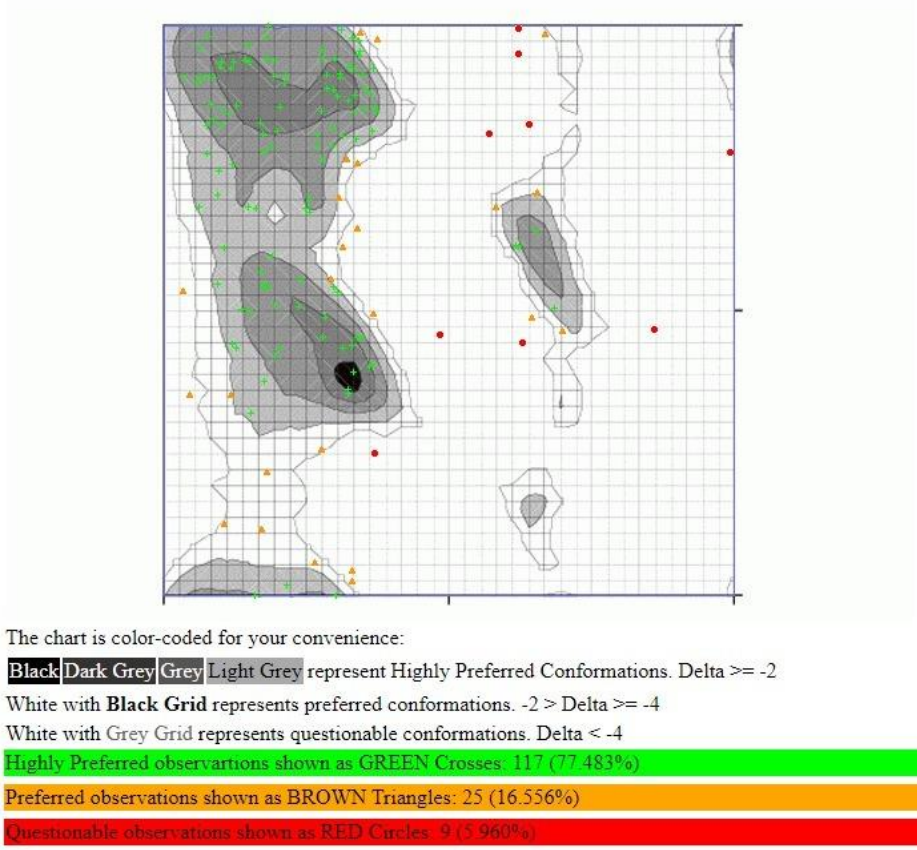


Figure 3-4: Ramachandran plot for model 1 showing the most favored regions (black and dark gray) and allowed regions but rare (light gray) for the torsion angles in alpha helices and beta sheets. Regions in white are not possible due to strict collision.

In the 3D construction, crystal model had been released in June 2021 in PDB server (7elx) involving build a crystal model of the same protein. The constructed model in the current study showed a sequence identity about 95% with the crystal model. (96,95)

3.5.2 Construction of a Mutant Model of CTLA-4

The predicted model of CTLA-4 from I-TASSER server (model 1) has been submitted to UCSF Chimera software in order to build a mutant model of the wildtype protein. Due to the details from dbSNP/NCBI, Threonine substituted with alanine at position 17. Then the differences between two models have been analyzed

(hydrophobic, Size, and Stability). The 3D variants of predicted CTLA-4 with wild type threonine amino acid, and substitution alanine instead it (mutant) as present in figure bellow. (97)

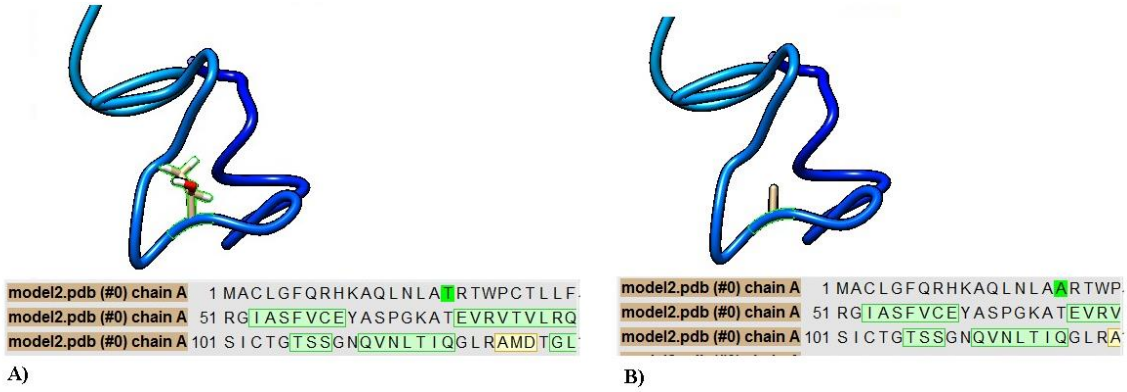


Figure 3-5: 3D variants modeling of predicted CTLA-4, **A)** Represents wild type CTLA-4 protein with threonine amino acid, **B)** Represents 3D modeling of predicted CTLA-4 protein with replaced alanine amino acid at position 17.

3.5.3 Computational Analysis of the Effect of SNP(+49A>G) on *ctla-4* gene

Several significant methods that applied in a number of subdisciplines will be helps to give an idea about the toxicological and deleterious effect (99). They are not limited to a certain field, but may be extended to carcinogenicity, cardio toxicology, genetic toxicology, and involved in the simulation of xenobiotic metabolism (100,101). In silico modeling have important and elegant effects “in omics” sciences that giving a specific vigilance in the computational analysis availability (101). The dealing with multi omics-suffix, like genomics, proteomics, metabolomics, and transcriptomic in biological system rely mainly on the advanced computer platforms for simulation, analysis, and presentation of data (102). CTLA4 is an output receptor protein of *ctla-4*

gene expressed on the surface of CD4+ T cells, which plays main effectors cells of chronic valve illness in heart disease (103). T-cell function regulated negatively through CTLA-4 by transferring and competing an inhibitory signal and with the stimulatory CD28 protein respectively [104]. *ctla-4* gene (rs231775 A>G) polymorphism has been associated with idiopathic dilated cardio myopathy (IDCM) [105]. However, +49 A>G SNP is associated with several diseases like T1DM [106], and Rheumatoid Arthritis [107]. The overall computational servers' tools and platforms that used were analyzing the effect of this SNP on protein function, structure, stability and related with disease and the figure below summarized the servers that used in the current study.

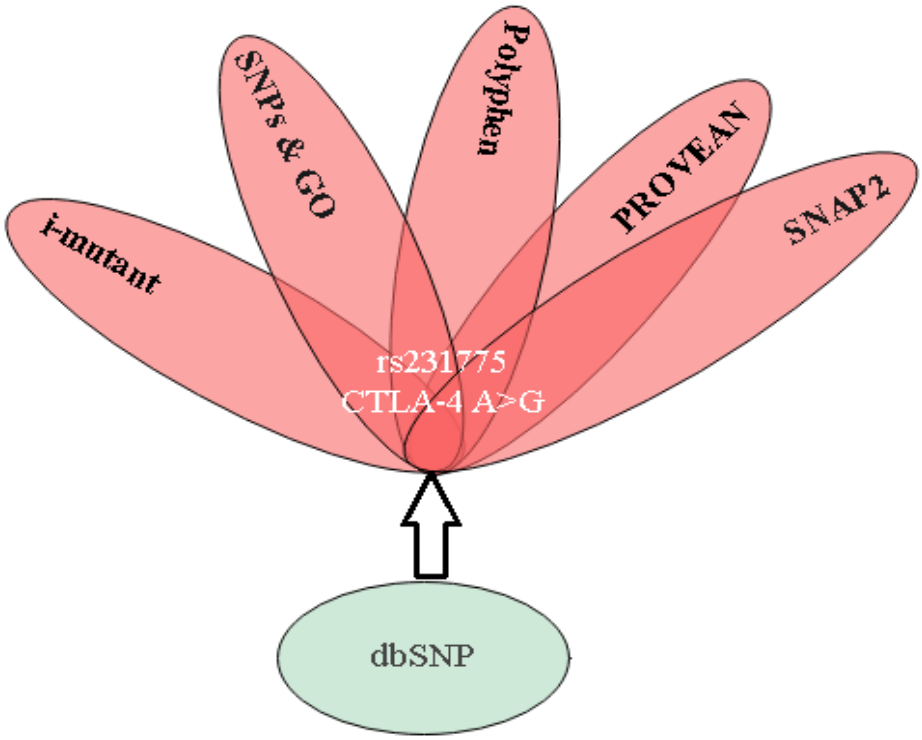
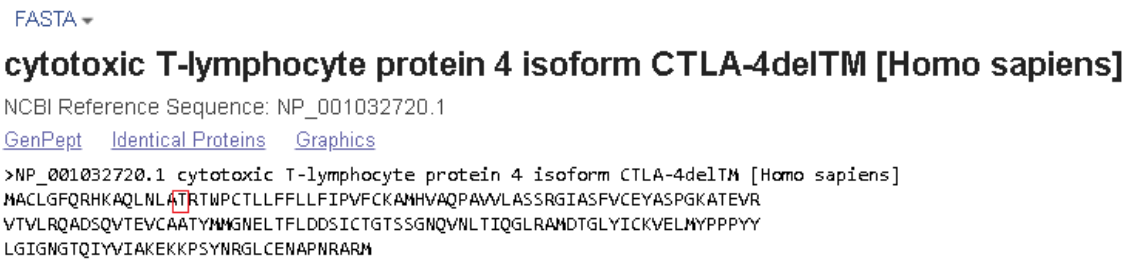


Figure 3-6: Overall computational tools that used in assessment of CTLA-4 protein depending nsSNP (rs231775 A>G).

The computational assessment of single nucleotide polymorphism for the structural and functional effects of the identified rs231775 Non-synonymous single nucleotide polymorphisms (nsSNP) was built on several in silico servers, involving dbSNP (https://www.ncbi.nlm.nih.gov/snp/), PolyPhen-2 (Polymorphism Pheno-typing v2) (http://genetics.bwh.harvard.edu/pph2/), PROVEAN (Protein Variation Effect Analyzer) (http://provean.jcvi.org/index.php). The collecting amino acids sequence of protein was retrieved from the nsSNPs of the *ctla-4* gene to be analyzed depending dbSNP (https://www.ncbi.nlm.nih.gov/protein/NP_001032720.1?report=fasta) as in figure bellow.


FASTA

cytotoxic T-lymphocyte protein 4 isoform CTLA-4delTM [Homo sapiens]

NCBI Reference Sequence: NP_001032720.1

GenPept Identical Proteins Graphics

>NP_001032720.1 cytotoxic T-lymphocyte protein 4 isoform CTLA-4delTM [Homo sapiens]
MACLGFQRHKAQLNLATRTWPCTLLFFLLFIPVFCKAMHVAQPAVVLASSRGIASFVCEYASPGKATEVR
VTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYY
LGIGNGTQIYVIAKEKKPSYNRGLCENAPNRARM

Figure 3-7: Represents the amino acids sequences of protein CTLA-4, retrieved from NCBI, red box represents wild type threonine (T) amino acid at 17 positions with ACC codon.

Structural and functional effect of rs231775 by Polyphen-2 was applied. The possible impact of an amino acid substitution in the original protein structure by Polyphen-2 server was used in addition to function of the studied protein by means of multiple sequence alignment. The output of this server illustrates “probably damaging”, “possibly damaging”, and “possibly benign” respectively rely on the range score (0-1) as in figure bellow.

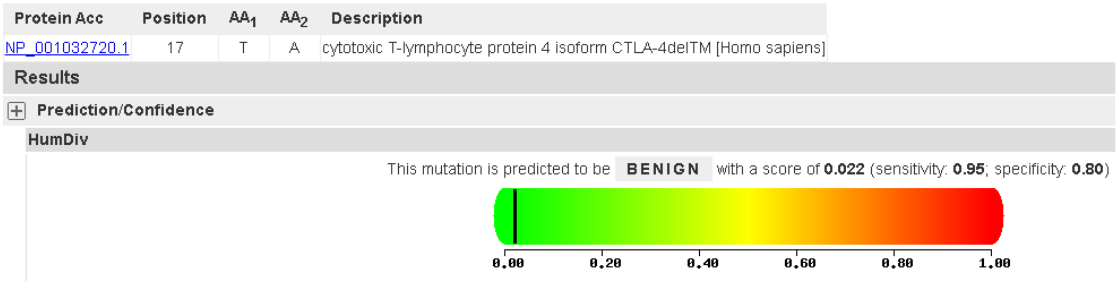


Figure 3-8: Represent Polyphen-2 output result and the benign effect of the substitution with sensitivity 96%, specificity 80%, and 0.022 p value.

The studied SNP (rs231775) was validated deleterious using PROVEAN server to monitoring biological consequences through discrimination among the neutral concerning amino acids and harmful amino acids, depending on the score of threshold prediction -2.5 (default), while the variants with a score ≤-2.5 considered deleterious, whereas>-2.5 are considered neutral, as explained in figure bellow.

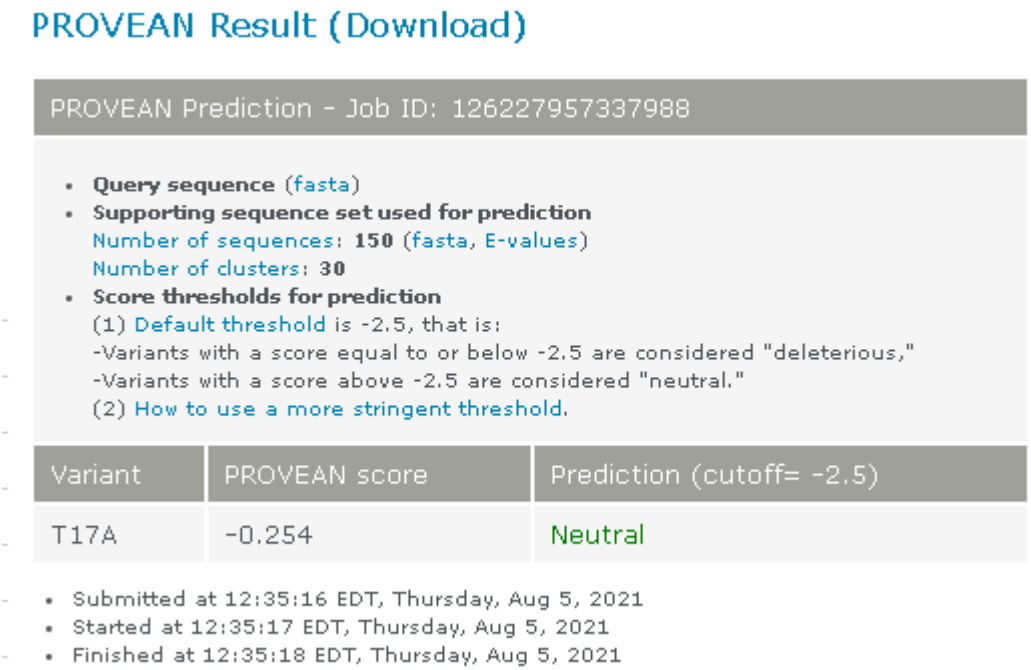


Figure 3-9: PROVEAN output result for (rs321775) SNP of CTLA-4.

Stability of the protein with amino acid substitution was used through using i-mutant (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi) server to verify the impact of amino acid substitution on homo sapiens CTLA-4 protein stability. The substitutions were examined and the result showed that the SNP (rs321775) of *ctla-4* gene decrease the protein stability with ddG score -1.04 in pH=7, and temperature 37.

To predict the human associated disease related mutation in CTLA-4 of single point mutation (codon involved) and follow the diseases progression SNPs & Go server (https://snps-and-go.biocomp. unibo.it/snps-and-go/) was used. A specific effective mutation related to disease when it scored >0.05, as in the figure bellow.

SNPs&GO Prediction

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RESULTS

SEQ File: P16410

Position	WT	NEW	Effect	RI
17	T	A	Neutral	9

BP: GO:0006955
No GO Term available

WT: Residue in Wild-Type Protein
NEW: Mutated Residue
RI: Reliability Index
Effect:
Neutral: Neutral Polymorphism
Disease: Disease-related Polymorphism
BP: Biological Process GO term
MF: Molecular Function GO term
CC: Cellular Component GO term

Figure 3-10: SNPs & Go server result, showed neutral effect of rs231775 A>G of *ctla-4* gene polymorphism on protein with substitution alanine instead threonine at position 17. (93)

To clarify functional consequences of the point mutation in its corresponding potion in the whole protein and the effect of substitution of alanine instead threonine amino acid at protein sequence at 17 positions through using SNAP2 platform to predict the damaging protein effect from no effect of whole protein as a net result. The scored effect results between (100) to (-100), with neutral effect when have a positive result and vice versa as in figure bellow.

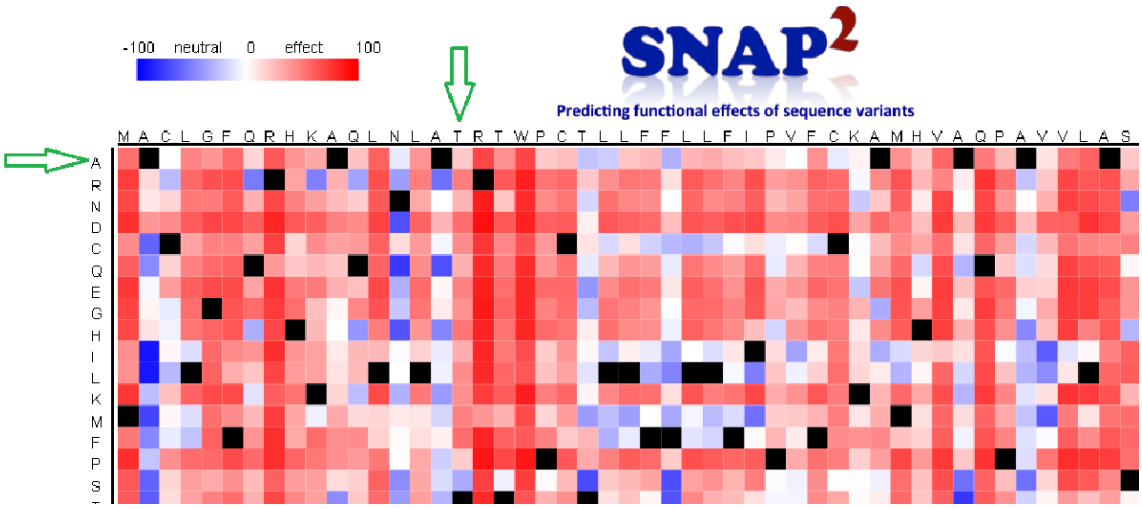

Figure 3-11: SNAP2 result explained the mild or neutral effect of substitutional alanine instead threonine at position 17 of CTLA-4.

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When the SNP has been analyzed by Polyphen, Provean, i-mutant, SNP & Go, and SNAP2, the only one server who gave a significant result was I-Mutant which showed that the total energy of the protein has been decrease when Threonine switched to alanine as mentioned earlier. These data were supported when the 3D model of mutant protein has been generated. It was shown that threonine has bigger size than alanine, another

physical property is threonine is a non-charged polar hydrophilic amino acid while alanine is a non-polar hydrophobic amino acid. Thus, the final model might have differences when compared mutant protein with the wild type model (94)

The three dimension constructed predicted model of CTLA-4 of the non-synonymous polymorphism of (+49A>G) in this current study provide evidence coming with similarity of the crystal model which released in 2021.the hydrophobicity, stability and size were assessed in both wild type and mutant protein with substitution of threonine with alanine in various online software (Imutant, SNPs and GO, Polyphen and SNAP2) that used to analyze the mutant protein indicated that neutral to little effect of substitutional alanine instead of threonine.

Conclusions

conclusion

conclusion

1- Gene Polymorphism of *ctla-4* +49A>G SNP is a risk for developing CVD.

2 – The risk of *ctla-4* + 49 A>G most likely derived from GG allele variant.

3- Minimal effect of both serum CTLA-4 levels and predicted structural changes in development of CVD.

RECOMMENDATIONS

Recommendations

1. More study of the relationship of the various SNPs of *ctla-4* gene and their association with autoimmune and inflammatory diseases and with CVDs in particular.

2. The study of CTLA-4 protein defects and post translational abnormalities.

3. Using DNA microarray technique for genotyping.

4. Study a larger sample size to get more accurate results.

References:

1- WHO. Cardiovascular diseases (CVDs). 2021. Available at: http://www.who.int/mediacentre/factsheets/fs317/en/ (accessed 10 March 2021).

2- Behradmanesh S, Nasri P,J Renal Inj Prev. Serum cholesterol and LDL-C in association with level of diastolic blood pressure in type 2 diabetic patients. 2012; 1(1):23-6

3- Weber C, Noels H,Nat Med. Atherosclerosis: current pathogenesis and therapeutic options. 2011 Nov 7; 17(11):1410-22.

4- McCoy KD, Hermans IF, Fraser JH, Le Gros G, Ronchese F. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) can regulate dendritic cell-induced activation and cytotoxicity of CD8(+) T cells independently of CD4(+) T cell help. J Exp Med. 1999 Apr 5;189(7):1157-62.

5- Rowshanravan B, Halliday N, Sansom DM. CTLA-4: a moving target in immunotherapy. Blood. 2018;131(1):58-67.

6- Saverino D, Simone R, Bagnasco M, Pesce G. The soluble CTLA-4 receptor and its role in autoimmune diseases: an update. Auto Immun Highlights. 2010;1(2):73-81.

7- Saverino D, Brizzolara R, Simone R, Chiappori A, Milintenda-Floriani F, Pesce G, Bagnasco M,Clin Immunol. Soluble CTLA-4 in autoimmune thyroid diseases: relationship with clinical status and possible role in the immune response dysregulation. 2007 May; 123(2):190-8.

8- Simone R, Brizzolara R, Chiappori A, Milintenda-Floriani F, Natale C, Greco L, Schiavo M, Bagnasco M, Pesce G, Saverino D,Int Immunol. A functional soluble form of CTLA-4 is present in the serum of celiac patients and correlates with mucosal injury. 2009 Sep; 21(9):1037-45.

9- Yokosuka T, Kobayashi W, Takamatsu M, Sakata-Sogawa K, Zeng H, Hashimoto-Tane A, Yagita H, Tokunaga M, Saito T .Spatiotemporal basis of CTLA-4 costimulatory molecule-mediated negative regulation of T cell activation.Immunity. 2010 Sep 24; 33(3):326-39.

10- Podlesnykh, S.V.; Abramova, K.E.; Gordeeva, A.; Khlebnikov, A.I.; Chapoval, A.I. Peptide Blocking CTLA-4 and B7-1 Interaction. Molecules 2021, 26, 253.

11- Simone R, Pesce G, Antola P, et al. The soluble form of CTLA-4 from serum of patients with autoimmune diseases regulates T-cell responses. Biomed Res Int. 2014;2014:215763.

12- Olvera Lopez E, Ballard BD, Jan A. Cardiovascular Disease. [Updated 2021 Aug 11]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK535419/

13- Ruan, Y., Guo, Y., Zheng, Y. et al. cardiovascular disease (CVD) and associated risk factors among older adults in six low-and middle-income countries: results from SAGE Wave 1. BMC Public Health (2018) 18, 778.

14- Pownall HJ, Gotto AM Jr. Lipids and Cardiovascular Disease: Putting It All Together. Methodist Debakey Cardiovasc J. 2019;15(1):5-8.

15- Orozco-Beltran D, Gil-Guillen VF, Redon J, Martin-Moreno JM, Pallares-Carratala V, Navarro-Perez J, et al. Lipid profile, cardiovascular disease and mortality in a Mediterranean high-risk population: The ESCARVAL-RISK study. PLoS ONE(2017) 12(10): e0186196.

16- John R. Petrie, Tomasz J. Guzik, Rhian M. Touyz,Diabetes, Hypertension, and Cardiovascular Disease: Clinical Insights and Vascular Mechanisms,Canadian Journal of Cardiology.(2018) 34(5)75-584.

17- WHO fact sheets - cardiovascular diseases ,11 June 2021. https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)

18- Charles D A Wolfe, The impact of stroke, BRITISH MEDICAL BULLETIN, Volume 56, Issue 2, 2000, Pages 275–286,

19- Johnson W, Onuma O, Owolabi M, Sachdev S. Stroke: a global response is needed. Bull World Health Organ. 2016;94(9):634-634A.

20- Lo, E., Dalkara, T. & Moskowitz, M. Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci (2003) 4, 399–414.

21- Gorelick PB. Stroke Prevention. ARCH NEUROL. 1995;52(4):347–355.

22- Khoshnam, S.E., Winlow, W., Farzaneh, M. et al. Pathogenic mechanisms following ischemic stroke. Neurol Sci (2017) 38, 1167–1186 .

23- Samaniego EA, Shaban A, Ortega-Gutierrez S, et al.Stroke mechanisms and outcomes of isolated symptomatic basilar artery stenosis,Stroke and Vascular Neurology 2019;4 116-186.

24- Institute of Medicine (US) Committee on Social Security Cardiovascular Disability Criteria. Cardiovascular Disability: Updating the Social Security Listings. Washington (DC): National Academies Press (US); 2010. 7, Ischemic Heart Disease. Available from: https://www.ncbi.nlm.nih.gov/books/NBK209964/

25- Johannes Siegrist, Karin Siegrist, Ingbert Weber,Sociological concepts in the etiology of chronic disease: The case of ischemic heart disease,Social Science & Medicine,Volume 22, Issue 2,1986,247-253,0277-9536,

26- Mosterd A, Hoes AW,Clinical epidemiology of heart failure.Heart 2007;93:1137-1146.

27- Mudd, J., Kass, D. Tackling heart failure in the twenty-first century. Nature (2008) 451, 919–928.

28- Refining Classification of Heart Failure Based on Ejection Fraction.Gregg C. Fonarow,J Am Coll Cardiol HF. 2017 Nov, 5 (11) 808–809

29- Detels, Martin Gulliford, Quarraisha Abdool Karim, and Chorh Chuan Tan. chapter: Epidemiology and prevention of cardiovascular disease edited by Nathan D. Wong, Feb 2015)

30- Rodgers JL, Jones J, Bolleddu SI, et al. Cardiovascular Risks Associated with Gender and Aging. J Cardiovasc Dev Dis. 2019;6(2):19.

31- GBD 2015 Eastern Mediterranean Region Cardiovascular Disease Collaborators. Burden of cardiovascular diseases in the Eastern Mediterranean Region, 1990–2015: findings from the Global Burden of Disease 2015 study. Int J Public Health .(2018) 63, 137–149 .

32- Wu CY, Hu HY, Chou YJ, Huang N, Chou YC, Li CP. High Blood Pressure and All-Cause and Cardiovascular Disease Mortalities in Community-Dwelling Older Adults. Medicine (Baltimore). 2015;94(47):e2160.

33- Drozdz D, Kawecka-Jaszcz K. Cardiovascular changes during chronic hypertensive states. Pediatr Nephrol. 2014;29(9):1507-1516.

34- Nelson RH. Hyperlipidemia as a risk factor for cardiovascular disease. Prim Care. 2013;40(1):195-211. doi:10.1016/j.pop.2012.11.003 Oxford Textbook of Global Public Health (6 ed.)

35- Zhang J, Chen L, Delzell E, Muntner P, Hillegass WB, Safford MM, et al. The association between inflammatory markers, serum lipids and the risk of cardiovascular events in patients with rheumatoid arthritis. Ann Rheum Dis. 2014;73:1301–1308.

36- Teo KK, Ounpuu S, Hawken S, Pandey MR, Valentin V, Hunt D, et al. Tobacco use and risk of myocardial infarction in 52 countries in the interheart study: A case-control study. Lancet. 2006;368:647–658.

37- Roy A, Rawal I, Jabbour S, et al. Tobacco and Cardiovascular Disease: A Summary of Evidence. In: Prabhakaran D, Anand S, Gaziano TA, et al. Cardiovascular, Respiratory, and Related Disorders. 3rd edition. 2017 17- 4.

38- Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the interheart study): Case-control study. Lancet. 2004;364:937–952.

39- Akil L, Ahmad HA. Relationships between obesity and cardiovascular diseases in four southern states and Colorado. J Health Care Poor Underserved. 2011;22(4 Suppl):61-72.

40- Danik JS, Manson JE. Vitamin d and cardiovascular disease. Curr Treat Options Cardiovasc Med. 2012;14(4):414-424. doi:10.1007/s11936-012-0183-8

41- Chiva-Blanch G, Badimon L. Benefits and Risks of Moderate Alcohol Consumption on Cardiovascular Disease: Current Findings and Controversies. Nutrients. 2020; 12(1):108.

42- Kubota Y, Evenson KR, Maclehose RF, Roetker NS, Joshu CE, Folsom AR. Physical Activity and Lifetime Risk of Cardiovascular Disease and Cancer. Med Sci Sports Exerc. 2017;49(8):1599-1605.

43- Rychter AM, Ratajczak AE, Zawada A, Dobrowolska A, Krela-Kaźmierczak I. Non-Systematic Review of Diet and Nutritional Risk Factors of Cardiovascular Disease in Obesity. Nutrients. 2020; 12(3):814.

44- Dong Y, Wang X, Zhang L for the China CVD study investigators, et alHigh-sensitivity C reactive protein and risk of cardiovascular disease in China-CVD study,J Epidemiol Community Health 2019;73:188-192.

45- Karakas, M., Koenig, W. CRP in Cardiovascular Disease. Herz (2009) 34, 607–613 .

46- Petrie, John R. et al .Diabetes, Hypertension, and Cardiovascular Disease: Clinical Insights and Vascular Mechanisms.Canadian Journal of Cardiology,(2018) 575 – 584.

47- Gistera A, Hansson GK. The immunology of atherosclerosis. Nat Rev Nephrol. (2017) 13:368–80.

48- Milioti N, Bermudez-Fajardo A, Penichet ML, Oviedo-Orta E. Antigen-induced immunomodulation in the pathogenesis of 318sclerosis. Clin Dev Immunol. (2008) 2008:723539. doi: 10.1155/2008/723539

49- Döring Y, Drechsler M, Soehnlein O, Weber C. Neutrophils in atherosclerosis from mice to man. Arterioscler Thromb Vasc Biol. (2015) 35:288–95.

50- Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, et al. Natural regulatory T cells control the development of atherosclerosis in mice. Nat Med. (2006) 12:178–80. doi: 10.1038/nm1343

51- Basatemur GL, Jørgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. (2019) 16:727–44.

52- 13. Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol. (2005) 25:29–38

53- Mallat Z, Taleb S, Ait-Oufella H, Tedgui A. The role of adaptive T cell immunity in atherosclerosis. J Lipid Res. (2009) 50:S364–9.

54- Albany Caraugh J., Trevelin Silvia C., Giganti Giulio, Lombardi Giovanna, Scottà Cristiano. Getting to the Heart of the Matter: The Role of Regulatory T-Cells (Tregs) in Cardiovascular Disease (CVD) and Atherosclerosis . Frontiers in Immunology,(2019) 10:2795 , 1664-3224

55- de Boer OJ, van der Meer JJ, Teeling P, van der Loos CM, van der Wal AC. Low numbers of FOXP3 positive regulatory T cells are present in all developmental stages of human atherosclerotic lesions. PLoS ONE. (2007) 2-779.

56- Arce-Sillas A, Álvarez-Luquín DD, Tamaya-Domínguez B, Gomez-Fuentes S, Trejo-García A, Melo-Salas M, et al. Regulatory T cells: molecular actions on effector cells in immune regulation. J Immunol Res. (2016) 2016:1720827.

57- Olejarz W, Łacheta D, Kubiak-Tomaszewska G. Matrix Metalloproteinases as Biomarkers of Atherosclerotic Plaque Instability. Int J Mol Sci. 2020;21(11):3946.

58- Shah PK, Chyu KY, Dimayuga PC, Nilsson J. Vaccine for atherosclerosis. J Am Coll Cardiol. (2014) 64:2779–91.

59- Yazdani MR, Khosropanah S, Doroudchi M. Interleukin-17 production by CD4+CD45RO+Foxp3+ T cells in peripheral blood of patients with atherosclerosis. Arch Med Sci Atheroscler Dis. 2019;4:e215-e224.

60- Wang YM, Ghali J, Zhang GY, Hu M, Wang Y, Sawyer A, et al. Development and function of Foxp3(+) regulatory T cells. Nephrology. (2016) 21:81–5.

61- Libby P. Atherosclerosis: disease biology affecting the coronary vasculature. Am J Cardiol. 2006; 98: 3Q–9Q.

62- Liu W, Putnam AL, Xu-yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, de St. Groth BF, Clayberger C, Soper DM, Ziegler SF, Bluestone JA. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med. 2006; 203: 1701–1711

63- Inamdar AA, Inamdar AC. Heart Failure: Diagnosis, Management and Utilization. J Clin Med. 2016;5(7):62

64- Nishiga, M., Wang, D.W., Han, Y. et al. COVID-19 and cardiovascular disease: from basic mechanisms to clinical perspectives. Nat Rev Cardiol (2020) 17, 543–558

65- Polonsky, T., Greenland, P. CVD screening in low-risk, asymptomatic adults: clinical trials needed. Nat Rev Cardiol (2012) 9, 599–604 .

66- Chen, S., Shauer, A., Zwas, D.R., Lotan, C., Keren, A. and Gotsman, I. The effect of thyroid function on clinical outcome in patients with heart failure. Eur J Heart Fail.(2014), 16: 217-226.

67- Karen K. Davis, Jerilyn K. Allen,Identifying cognitive impairment in heart failure: A review of screening measures,Heart & Lung, 2013, 42:292-97, 0147-9563

68- Karl Swedberg, John Cleland, Henry Dargie, Helmut Drexler, Ferenc Follath, Michel Komajda, Luigi Tavazzi, Otto A. Smiseth, Antonello Gavazzi, Axel Haverich, Arno Hoes, Tiny Jaarsma, Jerzy Korewicki, Samuel Lévy, Cecilia Linde, José-Luis Lopez-Sendon, Markku S. Nieminen, Luc Piérard, Willem J. Remme, Authors/Task Force Members, Guidelines for the diagnosis and treatment of chronic heart failure: executive summary (update 2005): The

Task Force for the Diagnosis and Treatment of Chronic Heart Failure of the European Society of Cardiology, European Heart Journal, Volume 26, Issue 11, June 2005, Pages 1115–1140

69- ERIKSSON, H., Heart failure: a growing public health problem. Journal of Internal Medicine, (1995), 237: 135-141.

70- Ting W-H, Chien M-N, Lo F-S, Wang C-H, Huang C-Y, Lin C-L, et al. Association of Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA4) Gene Polymorphisms with Autoimmune Thyroid Disease in Children and Adults: Case-Control Study. PLoS ONE (2016) 11(4): e0154394.

71- Deichmann K, Heinzmann A, Bruggenolte E, Forster J, Kuehr J. An Mse I RFLP in the human CTLA4 promotor. Biochem Biophys Res Commun 1996; 225: 817–8. pmid:8780695

72- https://www.genecards.org/cgi-bin/carddisp.pl?gene= 2505

73- Marquez Pete, N.; Maldonado Montoro, M.d.M.; Pérez Ramírez, C.; Sánchez Martín, A.; Martínez de la Plata, J.E.; Martínez Martínez, F.; Caliz Caliz, R.; Daddaoua, A.; Ramírez Tortosa, M.d.C.; Jiménez Morales, A. Impact of Single-Nucleotide Polymorphisms of CTLA-4, CD80 and CD86 on the Effectiveness of Abatacept in Patients with Rheumatoid Arthritis . J Pers Med. 2020 Nov 11;10(4):220

74- Chen, Y, Chen, S, Gu, Y. (2018) CTLA-4 +49 G/A, a functional T1D risk SNP, affects CTLA-4 level in Treg subsets and IA-2A positivity, but not beta-cell function. Scientific Reports 8(1): 10074

75- Lindsten T, Lee KP, Harris ES, Petryniak B, Craighead N, Reynolds PJ, Lombard DB, Freeman GJ, Nadler LM, Gray GS, et al. Characterization of

CTLA-4 structure and expression on human T cells. J Immunol. 1993 Oct 1;151(7):3489-99. PMID: 8397258.

76- Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet, (2013).7 - 7.20

77- Zhao Y, Gu Z, Zhao X, Wang L, Gao M. Association of CTLA4 and NFKB1 polymorphisms with chronic heart failure: a case-control study in the Chinese population. European Journal of Inflammation. January 2019.

78- .. Muhadasi Tuerxunyiming, Muhetaer Muhemaiti , Weiya Niu , Qifeng Li: Relationship between CTLA4 + 49 A/G polymorphism and susceptibility to type 1 diabetes mellitus: a meta-analysis, Int J Clin Exp Med 2019;12(9):11149-11159

79- Pedro M. Azevedo , Tony R. Merrimanb , Ruth K. Topless , Nigel J. Wilson , Sue Crengle , Diana R. Lennon: Association study involving polymorphisms in IL-6, IL-1RA, and CTLA4 genes and rheumatic heart disease in New Zealand population of Ma¯ori and Pacific ancestry, Cytokine, 2016. 2016 Sep;85:201-6.

80- Zhao Liu, Zhao Song1, Jing Sun , Fenglei Sun , Chuanzhi Li , Jiuzheng Sun , Liyou Xu: Association between CTLA-4 rs231775 polymorphism and hepatocellular carcinoma susceptibility, Int J Clin Exp Pathol 2015;8(11):15118-15122.

81- Buchbinder EI, Desai A. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. American Journal of Clinical Oncology. 2016 Feb;39(1):98-106.

82- Rudd CE, Taylor A, Schneider H. CD28 and CTLA-4 coreceptor expression and signal transduction. Immunological Reviews. 2009 May;229(1):12-26.

83- Chao Yu, Andreas F.-P. Sonnen. Roger George,Benoit H Dessailly. Rigid-body Ligand Recognition Drives Cytotoxic T-lymphocyte Antigen 4 (CTLA-4) Receptor Triggering. Journal of Biological Chemistry (2011) 286(8):6685-96.

84- Petro TM, Chen SS, Panther RB. Effect of CD80 and CD86 on T cell cytokine production. Immunol Invest. 1995 Nov;24(6):965-76.

85- Wang, X., Zhao, X., Giscombe, R. et al. A CTLA-4 gene polymorphism at position −318 in the promoter region affects the expression of protein. Genes Immun (2002) 3, 233–234.

86- Zhou C, Gao S, Yuan X, et al. Association between CTLA-4 gene polymorphism and risk of rheumatoid arthritis: a meta-analysis. Aging (Albany NY). 2021;13(15):19397-19414.

87- Overbergh L, Giulietti A, Valckx D, Decallonne R, Bouillon R, Mathieu C. The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. J Biomol Tech. 2003;14(1):33-43.

88- John M. Butler,DNA Quantitation,Advanced Topics in Forensic DNA Typing: Methodology,Academic Press. (2012) 49-67,9780123745132.

89- V.H.J. van der Velden, T. Szczepański, J.J.M. van Dongen.Polymerase Chain Reaction, Real-Time Quantitative,ncyclopedia of Genetics.(2001)1503-1506

90- Sri Harsha Kanuri, Rolf P. Kreutz. Micro RNA sequencing for myocardial infarction screening. Academic Press. 2020,187-198

91- Meier, A., & Söding, J. Automatic Prediction of Protein 3D Structures by Probabilistic Multi-template Homology Modeling. PLoS computational biology. (2015)11(10), e1004343.

92- Hecht M, Bromberg Y & Rost B. Better prediction of functional effects for sequence variant. BMC Genomics. 2015; 16(Suppl 8):S1 .

93- Choi Y, Sims GE, Murphy S, Miller JR, Chan AP Predicting the Functional Effect of Amino Acid Substitutions and Indels. PLoS ONE (2012) 7(10): e4668.

94- Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet, (2013) 7:7.20.

95- Bava KA, Gromiha MM, Uedaira H, Kitajima K, Sarai A. ProTherm, version 4.0: thermodynamic database for proteins and mutants. Nucleic Acids Res. (2004). 32, D120-D121

96- Montanucci L, Capriotti E, Frank Y, Ben-Tal N, Fariselli P. DDGun: an untrained method for the prediction of protein stability changes upon single and multiple point variations. BMC Bioinformatics. 2019;20(Suppl 14):335.

97- Ruppert V, Meyer T, Struwe C, et al. Evidence for CTLA4 as a susceptibility gene for dilated cardiomyopathy. Eur J Hum Genet. 2010;18(6):694-699.

98- Zhao Y, Gu Z, Zhao X, Wang L, Gao M. Association of CTLA4 and NFKB1 polymorphisms with chronic heart failure: a case-control study in the Chinese population. European Journal of Inflammation. January 2019. 17 - 10.1177.

99- . Lee, Vannajan Sanghiran; Chong, Wei Lim; Sukumaran, Sri Devi; Nimmanpipug, Pivarat; Letchumanan, Vengadesh; Goh, Bey Hing; Lee, Learn-Han; Md. Zain, Sharifuddin; Abd Rahman, Noorsaadah (2020). "Computational screening and identifying binding interaction of anti-viral and anti-malarial drugs: Toward the potential cure for SARS-CoV-2". Progress in Drug Discovery & Biomedical Science. 3.

100- Esteves, F.; Rueff, J.; Kranendonk, M. The Central Role of Cytochrome P450 in Xenobiotic Metabolism—A Brief Review on a Fascinating Enzyme Family. J. Xenobiot. 2021, 11, 94–114.

101- Abdelsalam NA, Ramadan AT, ElRakaiby MT and Aziz RK Toxicomicrobiomics: The Human Microbiome vs. Pharmaceutical, Dietary, and Environmental Xenobiotics. Front. Pharmacol. (2020) 11:390 .

102- Pinu FR, Beale DJ, Paten AM, et al. Systems Biology and Multi-Omics Integration: Viewpoints from the Metabolomics Research Community. Metabolites. 2019;9(4):76

103- Azevedo PM, Merriman TR, Topless RK, Wilson NJ, Crengle S, Lennon DR. Association study involving polymorphisms in IL-6, IL-1RA, and CTLA4 genes and rheumatic heart disease in New Zealand population of Māori and Pacific ancestry. Cytokine. 2016 Sep;85:201-6.

104- Spencer C. Wei, Roshan Sharma, Nana-Ama A.S. Anang, ..., Jing Wang, Dana Pe'er, James P. Allison: Negative Co-Stimulation Constrains T Cell Differentiation by Imposing Boundaries on Possible Cell States, Immunity, 2019, Immunity 50, 1084–1098.

105- Zhao Liu, Zhao Song1, Jing Sun , Fenglei Sun , Chuanzhi Li , Jiuzheng Sun , Liyou Xu: Association between CTLA-4 rs231775 polymorphism and hepatocellular carcinoma susceptibility, Int J Clin Exp Pathol 2015;8(11):15118-15122.

106- Muhadasi Tuerxunyiming, Muhetaer Muhemaiti , Weiya Niu , Qifeng Li: Relationship between CTLA4 + 49 A/G polymorphism and susceptibility to type 1 diabetes mellitus: a meta-analysis, Int J Clin Exp Med 2019;12(9):11149-11159

107- Mousavi MJ, Shayesteh MRH, Jamalzehi S, et al. Association of the genetic polymorphisms in inhibiting and activating molecules of immune system with rheumatoid arthritis: A systematic review and meta-analysis. J Res Med Sci. 2021;26:22. Published 2021 Mar 31. doi:10.4103/jrms.JRMS_567_20

108- Sanne Abeln, Klaas Anton Feenstra and Jaap Heringa, in Encyclopedia of Bioinformatics and Computational Biology, 2019, Pages 497-511

Appendix

Questionaire

Patient's name :

Age:

Weight:

Height:

BMI :

Gender : Male ——— Female ———

Patient's address :

1 - Does the patient have diabetes ? Yes ——— No ———

2- Does the patient have any kidney disorders ? Yes ——— No ———

3- Does the patient have any liver disorders? Yes ——— No ———

4 – Does the patient have any bones disorders ? Yes ——— No ———

5 – Does the patient have high blood pressure ? Yes ——— No ———

6– Does the patient smoke cigarettes ? Yes ——— N0 ———

——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ———

Does the patient take any medications ? Yes ——— No ———

If yes, what are the medications? ———————————————————————————

——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ———

Does the patient have high cholesterol levels ? Yes ——— No ———

Last cholesterol test result : Total cholesterol ——— HDL ——— LDL ——— Triglycerides ———

——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ———

Troponin test results if available : ————

D-dimer test results if available : ————

——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ——— ———

For how long the patient is having the cardiovascular disease ? ————

Which CVD the patient is diagnosed with? ——————————

خلاصة

أمراض القلب والأوعية الدموية أو الأمراض القلبية الوعائية مصطلح يستخدم لوصف العديد من الأمراض التي تصيب القلب والأوعية الدموية وهي سبب حوالي ٣١٪ من الوفيات في جميع أنحاء العالم. هي السبب لموت حوالي ١٧,٩ مليون شخص على مستوى العالم في عام ٢٠١٩. مستضد الخلايا الليمفاوية التائية السامة ٤ (CTLA-4) المعروف أيضًا باسم CD152 هو مستقبل يوجد على سطح خلايا التائية CD4 + و CD8 +. و يوجد بأعلى المستويات في الأنسجة اللمفاوية.CTLA-4 هو مستقبل مثبط يعمل كمنظم سلبي رئيسي لاستجابة الخلايا التائية. ألفة مستقبل CTLA4 مع روابط عائلة B7، CD80 و CD86 ، أقوى بكثير من ألفة مستقبل CD28 المسؤول عن تحفير استجابة الخلايا اللمفاوية. تهدف الدراسة الحالية إلى تحديد دور تعدد الأشكال الجيني لجين CTLA-4 في الموقع +٤٩ A> G كعامل خطورةللإصابة بأمراض القلب والأوعية الدموية. تم تضمين ١٢٦ شخصاً في هذه الدراسة ، ٦٩ منهم يعانون أمراض القلب والأوعية الدموية ،أعمارهم بمتوسط ± إنحراف معياري 7.9 ± 52.18) (SD)]). و ٥٧ شخصً سليم كمجموعة تحكم بمتوسط عمر ± إنحراف معياري 6.4 ± 49.5) SD).

هذه الدراسة أجريت في مختبرالكيمياء في قسم الكيمياء الحيياتية ، كلية الطب ، جامعة بابل. تم جمع العينات خلال الفترة من ٢٠ أكتوبر ٢٠٢٠ حتى ٢٠ يناير ٢٠٢١ ، وتم جمع العينات من مستشفى مرجان التعليمي و مستشفى الإمام الصادق التعليمي بمحافظة بابل. تم استخلاص الحمض النووي من الدم ، ثم تم إجراء التنميط الجيني لـ SNPs (rs231775) بواسطةتفاعل البوليميراز المتسلسل في الوقت الحقيقي باستخدام بادئات ومسابير خاصة. تم قياس CTLA-4 في المصل باستخدام تقنية الساندويتش ELISA. في مجموعة المرضى ، وجدت الأنماط الجينية AA و AG و GG بتكرار ١٩ (٤,٣٪) و ١١ (٥٩,٤٪) و أمراض القلب والأوعية الدموية أو الأمراض القلبية الوعائية مصطلح يستخدم لوصف العديد من الأمراض التي تصيب القلب والأوعية الدموية وهي سبب حوالي ٣١٪ من الوفيات في جميع أنحاء العالم. هي السبب لموت حوالي ١٧,٩ مليون شخص على مستوى العالم في عام ٢٠١٩. مستضد الخلايا الليمفاوية التائية السامة ٤ (CTLA-4) المعروف أيضًا باسم CD152 هو مستقبل يوجد على سطح خلايا التائية CD4 + و CD8 +. و يوجد بأعلى المستويات

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أظهرت النتائج الحالية عدم وجود فرق في تركيز CTLA-4 في مصل الدم بين مجموعة المرضى (١,٣٨ ± ١,٠٣ نغ / مل) ومجموعة التحكم (١,٢٤ ± ٠,٩ نغ / مل).

أظهرت هذه الدراسة أن تعدد الأشكال لجينCTLA4 في الموقع + ٤٩A>G يشكل خطرا للإصابة بأمراض القلب والأوعية الدموية وحيث يزيد وجود النمط الجيني GG من خطر الإصابة بالأمراض القلبية الوعائية.

لم يكن هناك ارتباط بين تركيز CTLA-4 في مصل الدم وخطر الإصابة بأمراض القلب والأوعية الدموية.

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وزارة التعليم العالي

و البحث العلمي

جامعة بابل

كلية الطب

دراسة مخاطر التغاير الجيني ل CTLA-4 مع التغايرات البروتينية في تطور امراض القلب و الاوعية الدموية

رسالة

مقدمة الى مجلس كلية الطب في جامعة بابل و هي جزء من متطلبات نيل درجة الماجستير في العلوم/ الكيمياء الحياتية السريرية.

من قبل

محمد حيدر محمد راضي

بكالوريوس تحليلات مرضية /الكلية التقنيات الصحية و الطبية/جامعة الفرات الاوسط (٢٠١٦)

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كلية الصيدلة/ جامعة الكوفة

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