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Role of GATM Gene Polymorphism in the Incidence of Myopathy in a Sample of Iraqi Patients Treated with Atorvastatin in Kerbala Province

A Thesis

Submitted to the Council of College of Pharmacy/University of Kerbala as a Partial Fulfillment Requirements for the Master Degree of Science in Pharmacology and Toxicology

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ﴾

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Dedication

To my son Ibrahim. . . .

The delight of my life, my inspiration, and my source of happiness.

Mohammed

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Abbreviations	**Full-Text**
ACC/AHA/NHLBI	American College of Cardiology/American Heart Association/National Heart, Lung, and Blood Institute
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BMI	Body mass index
CK	Creatine kinase
CVDs	Cardiovascular diseases
DMD	Duchenne muscular dystrophy
EDTA	Ethylene diamine tetra acetic
eQTLs	Expression quantitative trait loci
FKB12	FK506 binding protein 12
GAA	Guanidinoacetate
GATM	Glycine amidinotransferase gene
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
IBM	Inclusion body myositis
IPP	Isopentenyl-5-pyrophosphate
LCLs	Lymphoblastoid cell lines
LDL	Low density lipoprotein
MHC	Major histocompatibility molecule
mtDNA	Mitochondrial DNA
nDNA	Nuclear genome

NOD	New-onset diabetes
O_2^-	Superoxide anion
OATP	Organic-anion-transporting polypeptide
OXPHOS	Oxidative phosphorylation
PCr	Phosphocreatine
PCR	Polymerase Chain Reaction
ROS	Reactive oxygen species
RyR1	Ryanodine receptor 1
SAM	S-adenosyl-L-methionine
Scr	Serum creatinine
SERCA	Sarcoendoplasmic Reticulum Ca^{2+} ATPase
SNPs	Single nucleotide polymorphisms
SREBPs	Sterol regulatory element-binding proteins
SREs	Sterol binding elements
SRM	Statin related myopathy
T3	3,5,3'-triiodo-L-thyroxine
T4	L-thyroxine
TBE	Tries Borate EDTA
TG	Triglyceride
TSH	Thyroid stimulating hormone
TSH	Thyroid stimulating hormone
ULN	Upper limit of normal

Abstract

Background: Statins are a class of drugs that block the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase, preventing de novo cholesterol synthesis. They are the most commonly prescribed lipid-lowering drugs in the world for the primary and secondary prevention of cardiovascular disease. Statin-associated muscular symptoms are the most common side effects of statin therapy, forcing patients to discontinue their medication. The glycine amidinotransferase (GATM) gene codes for the mitochondrial enzyme L-arginine: glycine-amidinotransferase, a rate-limiting enzyme in creatine biosynthesis. Creatine is then necessary for normal muscle function. Statins have been shown to reduce GATM expression and thus creatine content in muscles, which may contribute to statin myopathy.

Aim of study: To investigate the relationship between GATM gene polymorphism rs9806699 G > A, C, T and statin-related myopathy (SRM) in patients taking atorvastatin 40 mg.

Patients and methods: Cross sectional study with a total of 150 participants, both male and female, ranging in age from 28 to 65 years old and receiving daily oral doses of 40 mg of atorvastatin as monotherapy for hyperlipidemia for at least one month were involved in the study. Biochemical tests were performed on each participant to determine their lipid profile (total cholesterol, triglycerides, low density lipoprotein and high-density lipoprotein), serum creatinine, creatine kinase (C.K) and thyroid stimulating hormone levels. The allele specific polymerase chain reaction technique was used to detect the rs9806699 G > A, C, T single nucleotide polymorphism (SNP).

Results: The genotypes distribution of rs9806699 G > A, C, T was 20 (13.3%), 57 (38.0%) and 73 (48.7%) for homozygous wild (GG), heterozygous (GA), and homozygous mutant (AA) respectively, with no allele frequency for C and T.

Despite a significant increase in mean creatine kinase in homo mutant (AA) patients compared to wild-type (GG) or heterozygous (GA) patients, there was no significant association between statin-related myopathy and GATM gene rs9806699 polymorphism.

Conclusion: According to the results of the currents study, the GATM gene polymorphism rs9806699 G > A, C, T is not associated with statin-related myopathy but it cannot be ruled out as one of the factors that contribute to myopathy since we observed that the A allele was found in more SRM patients than the G allele, with a significant increase in the mean creatine kinase level.

Chapter one

Introduction

1.1Myopathy

Myopathy implies muscular disease and is derived from the Greek words "myo" for muscle and "pathy" for pain. Myopathies are skeletal muscle illnesses characterized by a primary functional or structural dysfunction. The most prevalent signs and symptom include weakness, cramps, stiffness, and spasms(1). Myopathies can be acquired or hereditary, and they can develop at any age (2).

1.1.1 Hereditary Myopathy in General

Hereditary myopathies are skeletal muscle diseases that are passed down through the generations. Mutations in genes encoding proteins that play critical roles in muscle form and function cause these problems. In most inherited myopathies, skeletal muscle weakness and hypotonia are common clinical symptoms (3). There are several types of hereditary myopathy including, muscular dystrophies, ion channels myopathies, congenital myopathies, metabolic myopathies and mitochondrial myopathies (4).

1.1.1.1 Muscular Dystrophies

Mutations in a variety of genes cause muscular dystrophies, which are hereditary diseases. Dystrophy refers to abnormal growth and is derived from the Greek trophe, which means "nutrition"(5). These genetic mutations result in the malfunction or absence of proteins required for muscle cell stability, resulting in muscle cell death and weakening (6) . Duchenne and Becker muscular dystrophy are two examples of muscular dystrophy and are linked to mutation in dystrophin gene, Duchenne muscular dystrophy affects around one in every 3500 live male births and has an unstoppable deadly trajectory. Duchenne muscular dystrophy (DMD)

manifests clinically around the age of five years; most patients are wheelchair-bound by the time they reach adolescence and die of the illness by the time they reach early adulthood. Becker muscular dystrophy is a less prevalent and milder form of muscular dystrophy(7).

1.1.1.2 Ion Channels Myopathies

Mutations in genes encoding ion channel subunits produce primary ion channelopathies, which are uncommon disorders. The first ion channelopathy was discovered in hyperkalemic periodic paralysis, a genetic disease that affects only the skeletal muscles and is caused by mutations in the SCN4A gene, which codes for the alpha subunit of the voltage-gated sodium channel Nav1.4, whose expression is restricted to the fibers of the skeletal muscle(7,8). Myotonia or periodic paralysis are symptoms of ion channelopathies, which can lead to long-term disability (9,10).

1.1.1.3 Congenital Myopathy

Congenital myopathies are a clinically, histopathologically, and genetically diverse category of uncommon inherited muscle diseases marked by architectural defects in muscle fibers. Their frequency is estimated to be 1 in 26 000, and they account for 14% of all newborn hypotonia cases (11,12). The beginning of symptoms is generally from birth or infancy; however, a growing number of instances are being documented with symptom onset in adolescent or adults(13). Hypotonia ('floppy infant syndrome'), muscular weakness, hypotrophia, and/or missed motor milestones are common early clinical symptoms in congenital myopathies. A myopathic face, as well as ptosis and ophthalmoparesis, can appear in many people with congenital myopathies (14).

The most common structural pathological alteration on muscle biopsy divides congenital myopathies into five subgroups including nemaline myopathies, core myopathies, congenital fiber type disproportion myopathy, centronuclear myopathies and myosin storage myopathy. Electron microscopy, in addition to light microscopic examination, is frequently used in the histological diagnosis of congenital myopathies. The most common congenital myopathy subgroup is core myopathies, and mutations in the ryanodine receptor 1 gene (Responsible for the production of the ryanodine receptor, a protein that allows Ca^{2+} to be released from intracellular storage during excitation-contraction coupling) are the most common genetic cause of congenital myopathies to date (11,15).

1.1.1.4 Metabolic Myopathies

Metabolic myopathies are a set of uncommon disorders caused by abnormalities in glycogenolysis, glucose consumption (glycolysis), and fatty acid transport and oxidation(16,17). The latter include Carnitine transport system abnormalities or mitochondrial dehydrogenase enzyme deficits can both result in substantial fat buildup in myocytes (lipid myopathies) (18),While Glycogen storage diseases are caused by defects in enzymes that synthesize glycogen as well as those that interfere with glycogen breakdown and subsequent glucose mobilization(19).

These storge diseases might take the form of a systemic illness or a muscle-specific characteristic. Some of them are linked to long-term muscular injury and weakening. Others show up as recurrent episodes of significant muscle injury caused by activity or fasting, which is sometimes accompanied by acute renal failure and myoglobulinuria (rhabdomyolysis)(7).

1.1.1.5 Mitochondrial Myopathy

Mitochondrial myopathies are progressive muscle diseases characterized by oxidative phosphorylation (OXPHOS) dysfunction in the mitochondria. This results in a decrease in adenosine triphosphate (ATP) generation, which is particularly noticeable in skeletal muscle(20,21).

Mitochondrial function is controlled by two genomes: the mitochondrial genome (mtDNA) and the nuclear genome (nDNA); as a result, pathogenic genetic variations in either of these genomes can cause mitochondrial myopathy (22).
Mitochondrial myopathies often develop in early adulthood with proximal muscular weakness and, in some cases, substantial ocular musculature involvement (external ophthalmoplegia). Neurologic signs and symptoms, lactic acidosis, and cardiomyopathy are all possible(7,23).

1.1.2 Acquired Myopathy in General

Develop later in life and can be caused by infections, chemicals or drugs, endocrine disorders, or electrolyte imbalances, among other things (24).

1.1.2.1 Inflammatory Myopathies

Inflammatory myopathies, or myositis, are a diverse group of uncommon diseases that characterized by chronic inflammation and weakness of skeletal muscle, and also affect a variety of organs and systems, including lungs, skin, and joints(25) . Dermatomyositis, inclusion body myositis, and polymyositis are the most well-known inflammatory myopathies(26,27).

Dermatomyositis is most common in children and has distinct cutaneous manifestations and different degrees of systemic involvement(28). Patients may appear with skin disease alone, with accompanying muscle illness, or with

extracutaneous symptoms such as respiratory disease or malignancy(29).While the actual pathophysiology of Dermatomyositis is yet uncertain, it is thought to have an autoimmune basis(30). Unlike dermatomyositis, Polymyositis does not cause a rash and affect adults, while children are rarely affected(27).

Increased expression of major histocompatibility molecule (MHC) class I molecules on myofibers and primarily endomysial inflammatory infiltrates comprising CD8+ cytotoxic T lymphocytes are related with polymyositis. The autoimmune attack causes myofiber necrosis, which is followed by regeneration(31).

In case of inclusion body myositis, the development of muscular weakening symptoms in IBM patients usually begins between 45 and 70 years of age, and the disease progresses slowly(32), and has distinct clinical and pathological characteristics such as finger flexor and quadriceps weakness and the presence of invading cytotoxic T lymphocytes in muscle(33).

1.1.2.2 Endocrine Myopathy

Endocrine diseases, such as those affecting the thyroid, pituitary, adrenal glands, and parathyroid, can cause skeletal muscle abnormalities. Muscle dysfunction can be caused by an excess or deficit of hormones secreted by the glands, which can be rectified by initiating hormone replacement treatment(34).The incidence of neuromuscular disorders as a consequence of endocrine abnormalities is difficult to assess and is likely understated. Non-specific muscle symptoms such as myalgias, cramps, generalized or proximal weakness, and exertional discomfort may be part of the many clinical presentations of hyperparathyroidism, hypothyroidism, and hyperadrenocorticism(35).

1.1.2.3 Toxic Myopathy

Many chemicals, including routinely prescribed pharmaceuticals, might cause muscular damage(36). Alcohol, one of the oldest known chemicals, has been known to produce muscular weakness since the 19th century(37). Pharmaceutical side effects on muscles have largely been documented in the last 50 years. In recent years, cholesterol-lowering drugs, particularly statins, have been the most often prescribed treatments that have been linked to myopathy(38,39). Medications can damage the muscle in both direct and indirect ways. The direct impact might be localized, such as when a medicine is injected into tissue, or it can be generalized. Indirect harmful effects might occur if the chemical causes an electrolyte imbalance or triggers an immune response(40). Toxic myopathies can cause everything from muscular soreness to more significant muscle damage, which can lead to rhabdomyolysis(41,42).

1.2 Statins

1.2.1 Statins in General

Statins are a class of drugs that block the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase, which prevents de novo cholesterol synthesis, and they are the most widely prescribed lipid-lowering drugs in the world for the primary and secondary prevention of cardiovascular diseases (CVDs)(43,44), which are presently the leading cause of mortality worldwide(45). The development of cardiovascular diseases is caused by a number of known risk factors, including diabetes, high blood glucose, physical inactivity, cigarette smoking, low HDL cholesterol, and raised LDL cholesterol(46). As a result, treating and minimizing the risks associated with CVDs requires addressing hypertension and dyslipidemia.

Today, satins are the most effective oral medications for preventing and treating cardiovascular events caused by hypercholesterolemia (47).

Statins have been shown in several clinical trials to lower not just LDL-cholesterol and triglyceride levels(47), but also the morbidity and mortality associated with coronary heart disease, cerebrovascular disease, and peripheral arterial disease(44).

1.2.2 Development of Statins

In the 1970s, Japanese researcher Akiro Endo developed compactin or mevastatin (Figure 1.1) from Penicillim citrinum cultures, which became the first HMG-CoA reductase inhibitor. The compound has the potential to block cholesterol production in various cultured mammalian cells such as monkeys, and dogs, and in individuals with a history of hypercholesterolemia (48).

Compactin was never marketed due to its lethality in dogs. The next isolated statin was lovastatin, that extracted from Aspergillus terreus and was licensed for use in 1987(49).A semisynthetic statin, simvastatin, was developed in 1988 by methylating lovastatin(50).

Cerivastatin, Fluvastatin, rosuvastatin, and atorvastatin, the next generation of statins, are totally synthetic statins with entirely distinct structures from statins generated from fungal compounds(51). Cerivastatin was taken off the market on August 9, 2001, after 52 people died from rhabdomyolysis induced by drug, which resulted in renal failure(52,53).

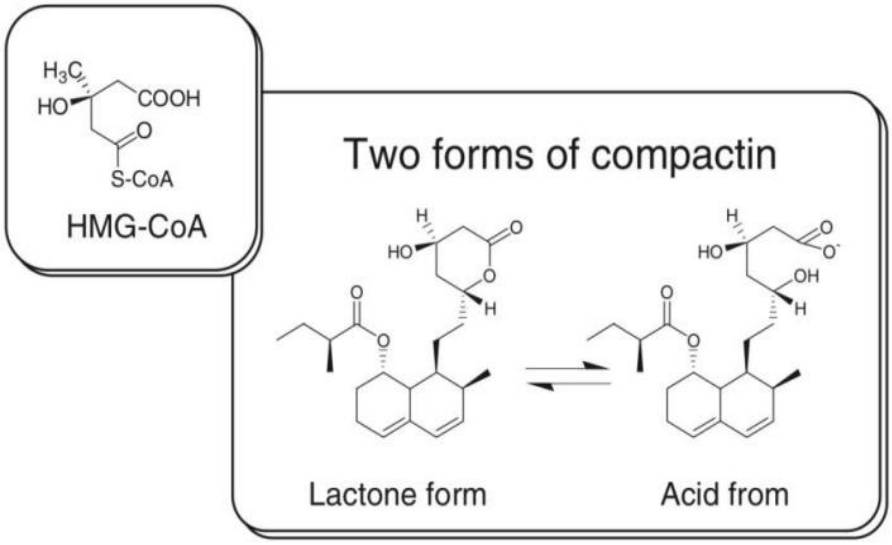


Figure 1.1: Structure of HMG-CO and Compactin(48).

1.2.3 Mechanism of Action

The rate-limiting enzyme in the cholesterol production pathway, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase that converts HMG-CoA to mevalonate, is competitively inhibited by statins (Figure 1.2). Due to their similarities to the substrate, statins occupy the catalytic region of the enzyme, and interfere with the binding of HMG-CoA to the reductase active site. The effect of this inhibition is a reduction cellular production of cholesterol(54,55).

The primary site of cholesterol biosynthesis is the liver, where statins have their pharmacological effects on hepatocytes(56). The effectiveness of statins is based on two distinct mechanisms: a reduction in hepatic cholesterol production and an increase in the clearance of LDL cholesterol from the circulation (57) (figure 1.3).

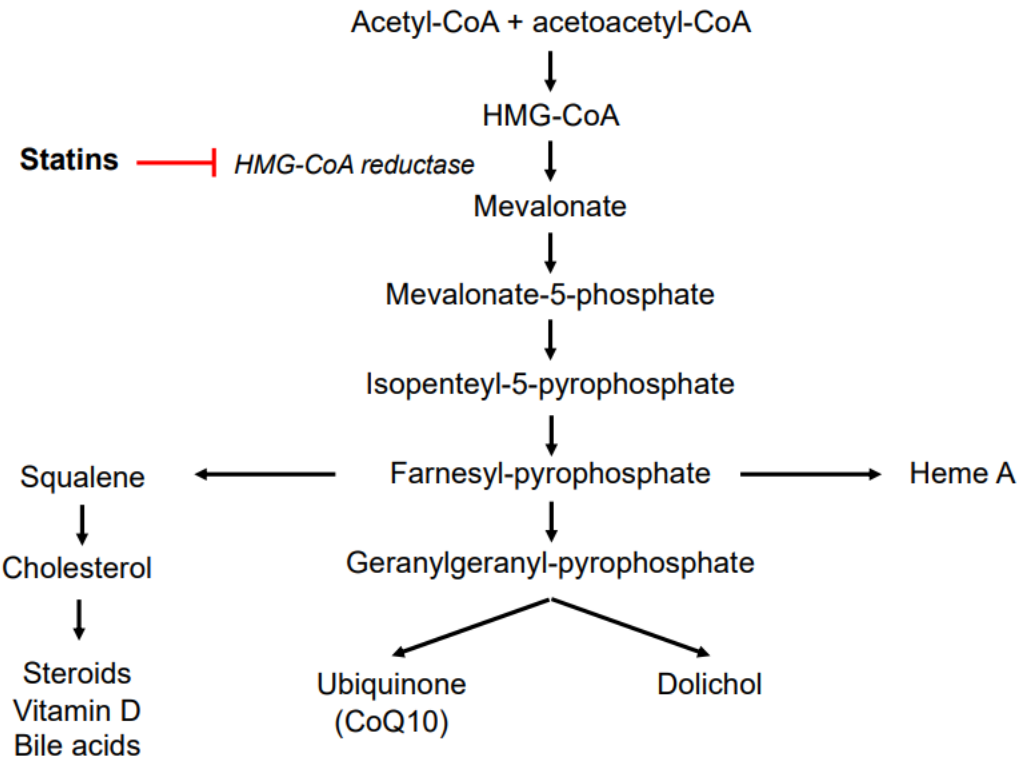


Figure 1.2 Pathway of cholesterol biosynthesis(58).

Indeed, a feedback regulatory mechanism that is mediated by a family of transcription factors known as sterol regulatory element-binding proteins (SREBPs) regulates intracellular levels of fatty acids and cholesterol. SREBPs are produced as inactive precursors that are attached to endoplasmic reticulum membranes(59,60).

Statins can activate sterol regulatory element-binding protein by lowering intracellular cholesterol levels and as a result NH2-terminal fragments of SREBPs are released from membranes by proteolysis and move to the nucleus where they bind to sterol binding elements (SREs) in the promoters of the target genes and stimulate isoprenoids and sterol resupply in the cells. SREBPs also promote LDL

cholesterol receptor expression, which increases the removal of LDL-cholesterol from the circulation(61–63).

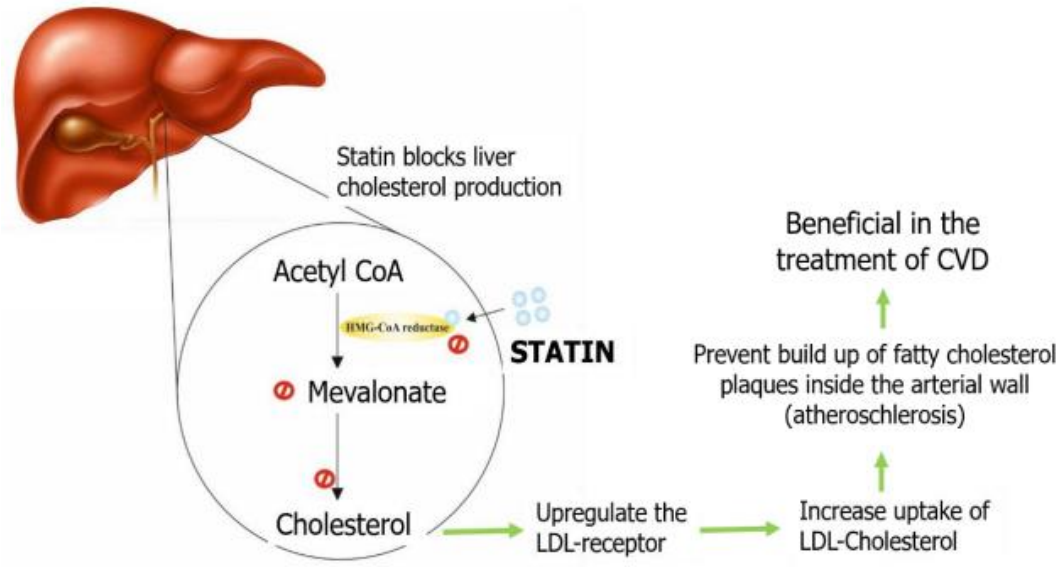


Figure 1.3 Mechanisms of action of statins (64)

Furthermore, apolipoprotein-B100 synthesis is inhibited by statins, and the liver's production of triglyceride-rich lipoproteins is decreased(65). Powerful statin (simvastatin, rosuvastatin, atorvastatin) decreases triglycerides by up to 15-30% while increasing high-density lipoprotein (HDL) cholesterol(66).

1.2.4 Pharmacokinetics of Atorvastatin

Statins are chemicals that are amphiphilic. Atorvastatin is lipid soluble, can enter cells via penetrating the lipid bilayer and rapidly absorbed following oral administration(67). Nevertheless, atorvastatin is extensively first-pass metabolized in the gut as well as the liver and about 12-14 % of atorvastatin is bioavailable(68).

Atorvastatin is taken orally as an acidic calcium salt and is transformed to the lactone form by an enzymatic process involving acyl glucuronide intermediates and non-enzymatic lactonization of the intestine(69). It has been demonstrated that a number of UDP-glucuronosyltransferases catalyze the glucuronidation of atorvastatin(70).

The majority of atorvastatin metabolism is carried out by cytochromes P450 in the liver. CYP3A4 was estimated to be responsible for 85% of atorvastatin metabolism(71,72). The family of organic-anion-transporting polypeptide (OATP) solute carrier transporters Polypeptide (OATP1B1, OATP1B3, and OATP2B1) and the sodium taurocholate co-transporting polypeptide mediates the transport of statins into liver cells(73–75) and this transport is the rate limiting step in the elimination of the drug in vivo(76). Genetic defects and polymorphisms are known to reduce the function of these transporters, which can result in reduced hepatic uptake and statin buildup. As a result, the occurrence of statin-induced adverse effects may increase(77).

1.2.5 Pleiotropic Effects of Atorvastatin

The majority of atorvastatin's positive effects are related to its ability to reduce systemic LDL cholesterol levels. However, atorvastatin has positive effects that are independent of cholesterol, primarily for the cardiovascular system(78). Because isoprenoids synthesis is impaired, atorvastatin maintains endothelial function by boosting nitric oxide availability (vasodilator), decreasing activating leukocytes and vascular smooth cell proliferation. Furthermore, atorvastatin has an anti-inflammatory effect, which reduces macrophage proliferation(79). Other beneficial effects of atorvastatin include anti-oxidant action and immune system regulation owing to decreased activity of small GTP-binding proteins(80,81). Together, these

pleiotropic effects decrease the susceptibility of atherosclerotic plaques and preserve the functioning of the vascular system and other bodily systems(57).

1.2.6 Adverse Effects of Atorvastatin

Statins (including atorvastatin) are generally well-tolerated and safe(82). Additionally, the benefits of lowering cholesterol and other pleiotropic effects outweigh the drawbacks of atorvastatin(77). However, atorvastatin can induce several side effects most of them being rare(83). Hepatotoxicity with elevation of transaminases has been documented(84). However, pharmacovigilance researches have revealed that the incidence of severe hepatotoxicity is modest, affecting around one patient per million each year(85). Other negative consequences include reduced renal function, insomnia, nasopharyngitis, tendon rupture, arthralgia, and low testosterone levels(83,86).

Observational researches and meta-analyses of data from randomized clinical trials show that statin therapy is associated with a 10–12% higher risk of new-onset diabetes (NOD); this risk is higher with intensive treatment regimens and in people with metabolic syndrome or prediabetes(87–90). There is no doubt about atorvastatin's effectiveness in avoiding cardiovascular events, the fact that atorvastatin significantly exceeds the risk of plasma glucose elevation should comfort patients that taking it will help them avoid developing cardiovascular disease (CVD)(90).

Indeed, the risk of muscular effects associated with statin usage has garnered the most attention since the unexpected case of cerivastatin when taken off the market on August 9, 2001, after 52 people died from rhabdomyolysis (47,52).

1.2.7 Statin Myotoxicity

The most prevalent side effects of statin therapy are statin-associated muscular symptoms. All statins can cause myotoxicity, which is dose-dependent and can force patients to stop taking their medication(91).

Statin associated myotoxicity symptoms might appear after 4–6 weeks although they might also appear after several years of therapy(92). Interestingly, myotoxicity is more common in physically active people(93). Other predisposing risk factors that may increase the likelihood risk of skeletal muscle side effects with statin are summarized in table 1.1. Several muscular symptoms are observed such as cramps, fatigue, tenderness, pain, stiffness, and heaviness. The most frequent symptoms are those involving the legs (calf muscles, thighs), however back, neck, shoulder, and more generalized muscular problems have also been reported(94–96). Approximately 40% of statin-associated myotoxicity patients report a probable trigger, most typically extraordinary physical effort or the start of a new treatment. Three-quarters of patients with statin-associated myotoxicity experience muscle pain intermittently, and one-quarter experience it continuously(97).

The absence of established terminology and phenotypic definitions has, to some extent, impeded research in statin associated myotoxicity. The American College of Cardiology/American Heart Association/National Heart, Lung, and Blood Institute (ACC/AHA/NHLBI) developed terminology used to categorize statin-related muscle adverse effects including myalgia, asymptomatic increases in creatine kinase (C.K), myositis, and rhabdomyolysis(2), as summarized in table 1.2.

Table 1.1 Risk factors for statin related myopathy(98).

Endogenous risk	**Exogenous risk**
Advanced age (>75)	Major surgery or trauma
Low body mass index	Alcohol consumption
Female sex	Vigorous exercise
Kidney or liver disease and untreated hypothyroidism	Drug interactions (nicotinic acid, fibrate)
Genetic polymorphism of cytochrome p 450 isoenzymes or drug transporters, hereditary muscle metabolism defects, traits that influence fatty acid oxidative metabolism	Excessive grape fruit consumption (inhibit cytochrome p450)
Family history	pharmacokinetics

Symptoms and/or biochemical abnormalities commonly begin to manifest early after the start of therapy, disappear after stopping it, and return days to weeks later after restarting the statin(99,100). Creatine kinase (CK), a muscle injury biomarker, is routinely used to identify and assess the severity of skeletal muscle damage. However, in symptomatic patients using statins, CK values are usually normal, but in asymptomatic patients, CK value may be elevated(101). Due to the lack of additional specialized laboratory tests, CK is currently employed in the assessment of SRM even though it is a non-sensitive biomarker for statin-induced myopathy(102).

Table 1.2 Different terms used by the ACC/AHA/NHLBI to describe skeletal muscles disorders(2).

Condition	Definition
Myopathy	A collective term for any skeletal muscle-related side effects.
Asymptomatic	Elevation of creatine kinase (CK) without muscular symptoms.
Myalgia	Weakness or pain in muscle without creatine kinase (CK) elevation.
Myositis	Weakness or pain in muscle associated with creatine kinase elevation (CK).
Rhabdomyolysis	Muscle symptoms associated with significant creatine kinase (CK) elevation (>10 times of upper limit of normal) and myoglobinuria as a result of muscular destruction.

CK: Creatine kinase.

1.2.8 Mechanism of Statin Related Myopathy (SRM)

To date, the mechanisms behind statin-related myopathies, which are dose-dependent and tissue-specific, affecting only skeletal muscle and not cardiac or smooth muscle, are unknown. (103). Statin related myopathy (SRM) is thought to be caused by statin buildup in the skeletal muscle in relation to risk factors (age, genetics, ethnicity, gender)(104,105). Furthermore, certain statins' physicochemical characteristics enhance their accumulation in extrahepatic tissues. It is a prevalent

assumption that lipophilic statins, such as atorvastatin, might exacerbate myotoxicity because, in contrast to hydrophilic ones, they can diffuse non-selectively through the cell membranes of extra-hepatic tissues(106,107). To comprehend the origins of the myopathy brought on by statins, several hypotheses have been put up.

1.2.8.1 Inhibition of Cholesterol and Isoprenoid Biosynthesis

1.2.8.1.A Elucidation of the Mevalonate Pathway

At the crossroads of several metabolic activities, the cholesterol biosynthesis pathway, also known as the mevalonate pathway, is a crucial pathway for appropriate cell function, development, and survival. As a result, it is a therapeutic target for the treatment of several such as cardiovascular disease, cancer, and hyperlipidemia(108–110). In eukaryotic cells, the mevalonate pathway is the only one that leads to the formation of farnesyl pyrophosphate and geranyl pyrophosphate(111,112). The mevalonate pathway reactions occur in the cytoplasm of cells. Acetyl-CoA from beta-oxidation and glycolysis, as well as ATP and NADPH, are used to synthesize sterol (cholesterol) and non-sterol compounds (dolichols, geranylgeranyl pyrophosphate, farnesyl pyrophosphate)(113). The enzymatic synthesis of mevalonate from acetyl-CoA requires three stages. The HMG-CoA reductase's conversion of HMG-CoA to mevalonate is the rate-limiting step. Isopentenyl-5-pyrophosphate (IPP) and farnesyl pyrophosphate are produced in subsequent processes. The pathway then splits, with one side producing dolichol, geranylgeranyl pyrophosphate, and ubiquinone, and the other side producing cholesterol (figure 1.2)(114,115). Dolichol is required for the endoplasmic reticulum's N-linked glycosylation of proteins(116,117). Ubiquinone is required for electron transport in the mitochondria during ATP synthesis(118,119). Cholesterol

is required for the fluidity of cell membranes, the integrity of lipid rafts, the generation of steroid hormones, bile acids, and vitamins(120,121).

1.2.8.1.B Effect of Cholesterol Synthesis Inhibition in Regard of SRM

When statins' systemic concentration is high enough, they can suppress cholesterol synthesis not just in the liver but also in other tissues including skeletal muscle(122). As a result, reduced cholesterol availability alters cell membrane fluidity and lipid raft integrity, making cells more susceptible to lysis(123,124). Furthermore, because ion channels and transporters are embedded within the membrane, changing the structure of the skeletal cell membrane may disrupt ion conductance and reduce muscle membrane excitability(125).

1.2.9.1.C Inhibition of Prenylation

Isoprenoids synthesized in the mevalonate pathway are essential for cellular signaling and homeostasis. Cell death can result from a shortage of these non-sterol intermediates. Isoprenylation is the addition of lipid adducts (farnesyl or geranylgeranyl) to proteins after they have been translated(126). Proteins that have been prenylated migrate from the cytosol to the cellular membranes to perform their functions and signal transduction(127). Defects in isoprenylation disrupt protein attachment to membranes and cause loss of activity(128). Small GTP-binding proteins, including as Ras, Rab, Rho and nuclear lamins, are the most common prenylation substrates. These protein families trigger a variety of signaling pathways, including G-protein receptors and tyrosine kinase receptors(126). Statins, which reduce isoprenoid synthesis, block prenylation of these GTP-binding proteins, resulting in lower amounts of prenylated forms. Dysprenylation of these small

GTPases causes myofiber vacuolation, organelle degradation and swelling, and eventually apoptosis(129,130).

1.2.8.2 Impairment of Calcium Signaling

Calcium release and uptake are essential for muscle cell activity. According to the findings of certain studies, statins may cause myopathy through impairing calcium signaling(131). In skeletal muscle, statin therapy caused the sarcoplasmic reticulum's ryanodine receptor 1 (RyR1) to disassociate from its stabilizing protein, FKB12 (FK506 binding protein 12)(132). Ryanodine receptor 1 (RyR1) is an intracellular channel with a large conductance that regulates intracellular calcium homeostasis in myocytes(133). When RyR's constitutive cytoplasmic partner FKBP12 is lost, the channels become more sensitive and the cytoplasmic Ca^{2+} level rises (Ca^{2+} sparks) which causes protein degradation and apoptosis(134,135).

1.2.8.3 Impairment of Mitochondrial Function

Energy supply for the vast majority of cells is mostly determined by mitochondrial ATP synthesis(136). Ubiquinone, commonly known as coenzyme Q10, is produced by the mevalonate pathway and is an important factor of the mitochondrial electron transport chain. It is also an antioxidant molecule that aids in membrane stabilization(137). By inhibiting HMG-CoA reductase and hence mevalonate pathway, Statins decrease ubiquinone biosynthesis(138). In this respect, statins' inhibitory effect on the mevalonate pathway may result in a 16–54% reduction in blood CoQ10 levels(139), and as a result mitochondrial dysfunction which is manifested by the inhibition of mitochondrial electron transport chain complexes, decrease in the copy number of mitochondrial DNA (mtDNA),

disruption of mitochondrial membrane potential, interference with oxidative phosphorylation, swelling of the mitochondria, and release of cytochrome c (140). Concerning the existence of mitochondrial impairment, it has been demonstrated in the muscles of statin-treated patients a defect in calcium homeostasis and mitochondrial malfunction, explaining the observed myopathy(141).

1.2.8.4 Statin Induced Apoptosis and Atrophy

In human skeletal cell lines, statins cause apoptosis(142). Apoptosis can occur as a result of mitochondrial damage, cytochrome c release, or Akt pathway modulation. It has been reported that rats treated with atorvastatin have an increased Bax/Bcl-2 ratio and caspase-3 activation in glycolytic muscle, as well as increased caspases 3, 9, and PARP cleavage in C2C12 myotubes and gastrocnemius of mice treated with simvastatin(143,144).

Atrophy is a process that causes cell resorption and shrinkage. Apoptosis is observed simultaneously with tissue breakdown and wasting away during atrophy. Skeletal muscle cell atrophy could indicate statins' ability to induce the expression of atrogin-1, which is in charge of destroying some muscle proteins(145,146).

1.2.8.5 Statin Induced Oxidative Stress

The imbalance between the production of reactive oxygen species (ROS) and their proper handling by the antioxidant system is referred to as oxidative stress. Aerobic organisms continuously produce ROS. When produced in appropriate amount, they are beneficial for cellular signaling(147,148); however, increased ROS production causes several lipid, protein, and DNA damage, which contributes to ROS-induced cell senescence and cell death(149).

Quite apart from their effects on apoptosis, ROS modify and regulate transcription factors that encourage the elicitation of atrophy, such as the FoXO transcription factors(150,151). Mitochondrial ATP production causes electron leakage from complexes I and III. Superoxide (O_2^-) is formed when these electrons react with oxygen. The mitochondrial superoxide dismutase converts superoxide anion to hydrogen peroxide (H_2O_2). In muscle cells, hydrogen peroxide can diffuse outside of the mitochondria, leading to oxidative stress and redox imbalance(152). According to reports, ROS have been linked to oxidative stress and cell death in human skeletal muscle(153). Furthermore, Bouitbir et al. proposed a deeper understanding of the role of ROS in statin-induced myotoxicity. They discovered that atorvastatin causes an increase in H_2O_2 production in the glycolytic muscle of rats, which can be prevented by supplementing with the antioxidant quercetin(154).

1.2.9 Pharmacogenetics of Statin Related Myopathy

Since the 1950s, it has become clear that genetic traits or enzyme deficiencies may be the underlying cause of the pathogenesis of drug hyper- and hypo-susceptibility.

Later, Friedrich Vogel used the term "pharmacogenetics" to describe the process by which "an individual's genetic inheritance affects the body's response to drug"(77). Its main goal is to understand why certain polymorphisms indicate that a person has a higher or lower risk of experiencing adverse effects from the same drug, allowing doctors to use the safest drug for each patient from the beginning, as a result, compliance increases. Another of its primary goals is to understand why the presence of certain polymorphisms causes a drug's metabolism to be faster or slower in different patients, resulting in decreased or increased effectiveness. The United

States Food and Drug Administration (US FDA) defines it as the study of variations in DNA sequence as they relate to drug response(155,156).

The same for atorvastatin, several side effects, including rhabdomyolysis, are more common in certain genetic settings. So far, candidate gene studies have not been conclusive because they produce low odds ratio values(157), most likely due to the multiple pathways that may affect statin pharmacokinetics and pharmacodynamics. Overall, atorvastatin genetic side effects can be divided into those associated with impaired pharmacokinetics in the liver, which could result in increased plasma levels of atorvastatin, and those associated with changes in specific genes in the muscles(77). Various candidate genes, including CYP3A4, which is involved in the metabolism of some statins (including atorvastatin), genes encoding organic anion-transporting polypeptides, some of which are associated with statin elimination, and genes involved in ubiquinone (coenzyme Q10) deficiency have all been examined in previous studies for their relevance to myopathy(158,159). However, the association of single nucleotide polymorphisms (SNPs) in other genes, such as the glycine amidinotransferase gene (GATM), and the risk of SRM has piqued the interest of researchers.

1.3 Glycine Amidinotransferase Gene (GATM)

The glycine amidinotransferase gene (GATM) is located on chromosome 15q15.3. The enzyme L-arginine: glycine-amidinotransferase (AGAT or GATM) is encoded by this gene and is the rate-limiting enzyme in the biosynthesis of creatine. This is accomplished by converting arginine and glycine to ornithine and guanidinoacetate (GAA) (160). A mutation in the GATM gene, which has a length of 41,203 base pairs (bp), results in hereditary creatine deficiency syndromes, which

are also characterized by severe mental retardation, speech delay, epilepsy, autism, and hypotonia(161).

1.3.1 Creatine Synthesis

Creatine is a naturally occurring nitrogen-containing amino acid compound that belongs to the guanidine phosphagens family(162,163). Creatine is primarily produced in the kidney and liver, and to a lesser extent in the pancreas, from three amino acids: glycine, arginine, and methionine. In humans, the main route of creatine biosynthesis involves the formation of guanidinoacetate (GAA) in the kidney. Amidino group transfer from L-arginine to glycine results in the formation of guanidinoacetate, which is then converted to creatine. Glycine amidinotransferase (GATM) catalyzes this rate-limiting step. Guanidinoacetate is transported by blood to the liver and methylated to creatine. This involves the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to guanidinoacetate, which is catalyzed by guanidinoacetate N-methyltransferase (GAMT)(164–166). Creatine travels through the bloodstream and is taken up and concentrated in tissues with high creatine kinase activity, such as muscle and nerve tissue. The sodium- and chloride-dependent creatine transporter 1, a specific Na+-dependent creatine transporter system, is responsible for uptake(167). Creatine kinase phosphorylates creatine to form a pool of N-phosphocreatine (PCr). During times of high energy demand, the N-phosphocreatine pool stores energy for ATP replenishment(168). Creatine can undergo spontaneous, irreversible, non-enzymatic cyclization in vivo, resulting in creatinine, which is excreted in urine (figure 1.4)(169).

Endogenous creatine synthesis meets roughly half of the daily creatine requirement(165). The remaining amount of creatine required to maintain normal levels of creatine is acquired from red meat and fish(170,171), as well as

dietary supplements(172,173). The majority of creatine is stored in muscle (about 95%), with the remainder found in other tissues such as the brain, heart, and testes(174). About two-thirds of this creatine is bound with inorganic phosphate (Pi) and stored as phosphocreatine (PCr), with the rest stored as free creatine (Cr)(175).

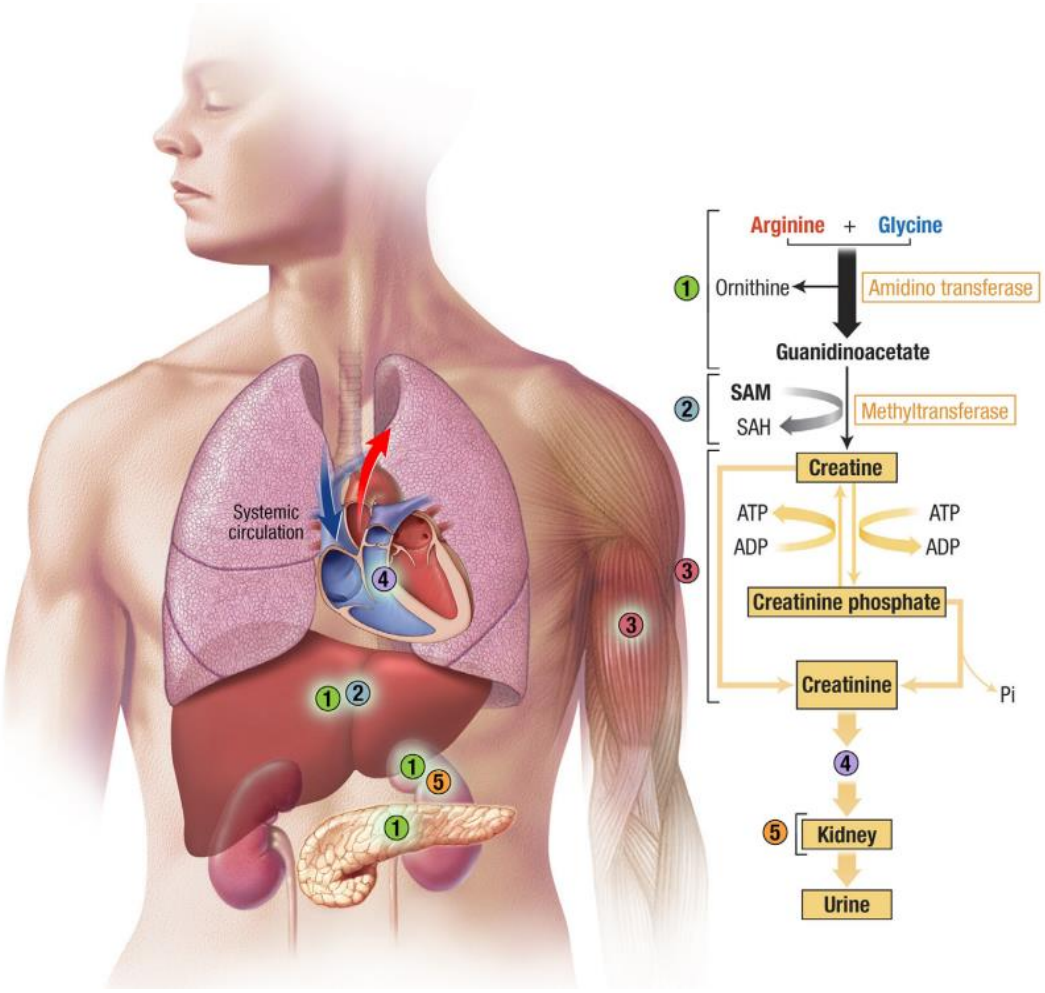


Figure 1.4: Pathway of creatine synthesis(169).

1.3.2 Role of Creatine in Muscle

Creatine is required for normal muscle function. Creatine is phosphorylated to phosphocreatine within the muscle (PCr). The reaction is reversible, and the two molecules are always in balance. When phosphocreatine reverted back to creatine, the phosphate bond is broken, releasing 45 kJ/mol of free energy. In comparison, the phosphate bond that is broken during the conversion of adenosine triphosphate to adenosine diphosphate has a free energy of only 31.8 kJ/mol. Thus, in order to resynthesize ATP, phosphocreatine can transfer its phosphate group to adenosine diphosphate (ADP), a reaction catalyzed by the creatine-kinase enzyme(176,177). The muscle takes advantage of the creatine-phosphocreatine system's unique properties, in which phosphocreatine rapidly re-synthesizes ATP near the ATPase enzymes that are using it under normal conditions. In addition to the plasma Na/K-ATPase, which maintains the cell membrane resting potential(178), two other ATPase enzymes are active in the muscle: myosin and the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA). Myosin uses ATP to contract muscles(179), while SERCA uses it to relax muscles (by removing calcium ions from the cytosol, pumping it into the lumen of the sarcoplasmic reticulum)(180). While all three ATPase enzymes are required for muscle function, phosphocreatine is required for their smooth functioning by providing a ready, nearby source of high-energy phosphate capable of rapidly regenerating ATP when used(181). Furthermore, when ATP is synthesized in the mitochondria, the creatine-phosphocreatine system takes up the phosphate group. It then rapidly moves through the cytoplasm to the periphery, where ATP must be synthesized and used. There, it gives ADP its phosphate group in order to create ATP. Because creatine actually transports the high-energy phosphate from the mitochondrion to the peripheral ATPase, this process is known as the "shuttle" function of creatine(179). It should be noted that

creatine and phosphocreatine are smaller molecules with a lower negative charge than ATP and ADP, and thus move faster through the cytoplasm. They offer a much more efficient means of transporting energy from the mitochondria to the periphery(182).

The creatine-phosphocreatine system also contributes to muscle contraction by supplying extra ATP during periods of high exertion, such as when the blood supply of oxygen and glucose is insufficient to synthesize the rapidly depleting ATP. Phosphocreatine acts as an energy buffer in these conditions by offering a ready supply of extra phosphate that enables rapid re-synthesis of ATP without the need for oxygen or glucose(183). Furthermore, creatine plays an important role in muscular physiology by favoring the differentiation of precursor cells into muscle cells, thereby facilitating the maintenance and recovery of muscle trophism(184,185). Creatine supplementation has been shown to improve the symptoms of a variety of muscle pathologies, including muscular dystrophies, inflammatory myopathies, mitochondrial cytopathies, and others(186).

1.3.3 GATM Gene Polymorphism and Statin Related Myopathy

Mangravite *et al*. reported the first association between GATM rs9806699 G > A polymorphism and SRM in a case control study with 72 SRM cases in 2013. The A allele was linked to a decrease in GATM expression, which resulted in a decrease in creatine synthesis. Reduced creatine availability may affect energy metabolism in skeletal muscle cells, contributing to the pathogenesis of myopathy induced by statin(187). However, the following studies on the effect of rs9806699 G > A on SRM produced contradictory results(188–191). As a result, the role of GATM polymorphism in SRM remains a hotly debated topic to this day.

1.4 Aims of Study

The aim of this study is designed to investigate:

1. The prevalence of GATM gene polymorphism rs9806699 G > A, C, T in the Kerbala province population.
2. The prevalence of myopathy in Kerbala province patients that receiving daily oral doses of 40 mg of atorvastatin.
3. The relation between GATM gene polymorphism rs9806699 G > A, C, T and statin-related myopathy in patients that receiving daily oral doses of 40 mg of atorvastatin

Chapter Two

Patients, Materials, and Methods

2. Patients, Materials, and Methods

2.1 Patients

Cross-sectional observational study was carried out in Imam Al-Hussein Medical City in Kerbala between November 2021 and April 2022. The Scientific and Ethical Committee of Pharmacy College/Kerbala University approved the study's protocol, and each participant signed a consent form after being informed of the nature and purpose of the study. A total of 150 participants, both male and female, ranging in age from 28 to 65 years old and receiving daily oral doses of 40 mg of atorvastatin as monotherapy for hyperlipidemia for at least one month were involved in the study.

The classification system described by American College of Cardiology/American Heart Association/National Heart, Lung, and Blood Institute (ACC/AHA/NHLBI) was used in this study to define statin-related myopathy (SRM) as described in table (1.2) (2).

Table 1.2: Different terms used by the ACC/AHA/NHLBI to describe skeletal muscles disorders(2).

Condition	Definition
Myopathy	A collective term for any skeletal muscle-related side effects.
Asymptomatic	Elevation of creatine kinase (CK) without muscular symptoms.
Myalgia	Weakness or pain in muscle without creatine kinase (CK) elevation.

Myositis	Weakness or pain in muscle associated with creatine kinase elevation (CK).
Rhabdomyolysis	Muscle symptoms associated with significant creatine kinase (CK) elevation (>10 times of upper limit of normal) and myoglobinuria as a result of muscular destruction.

CK: Creatine kinase.

2.1.1 Patients Criteria

2.1.1.1 Inclusion Criteria

The inclusion criteria were that all patients (both male and female) had been taking daily oral doses of 40 mg of atorvastatin as monotherapy for hyperlipidemia for at least one month prior to the start of the study, and that they had no serious disease.

2.1.1.2 Exclusion Criteria

1. Untreated hypo or hyperthyroidism
2. Advanced age (>65).
3. Recent surgery or trauma.
4. Using drugs known to interact with atorvastatin (such as fibrates, nicotinic acid).
5. Vigorous exercise.
6. Patients suffering from severe renal, hepatic, or cardiac dysfunction.
7. Taking dietary supplements containing creatine monohydrate.

2.1.2 Collection of Clinical Data

At the time of blood sample collection, patients were asked if they were taking any other drugs that could interfere with the metabolism of atorvastatin. The following data was obtained from the medical records of patients who gave their informed consent as well as directly from the patients themselves: age, weight, height, education level, type of myopathy, smoking status, duration of treatment, any adverse effects caused by the drug, other diseases, and any additional drugs taken.

2.1.3 Samples Collection

Following an overnight fast, blood samples were collected from patients and divided into two portions: the first (3ml) was placed in a gel tube for subsequent serum isolation for hormonal and biochemical analyses, and the second (2ml) was placed in an ethylene diamine tetra acetic acid (EDTA) tube for DNA extraction. After collection, ice-filled packaging was used to keep the samples at the right temperature before being transported to the laboratory.

2.1.4 Determination of Body Mass Index

It was computed by dividing the patient's weight (in kilos) by the square of his or her height (in meters)(192).

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

2.2 Materials

2.2.1 Instruments

All instruments used in this study, along with the manufacturer and country of origin are listed in table (2.1).

Table 2.1: List of instruments, manufacturer and country of origin.

Instrument	Company	Country
Centrifuge	SIGMA	Germany
Digital camera	Canon	England
Electrophoresis apparatus	Techinme	England
Hood	Lab Tech	Korea
Hotplate Stirrer	LabTEch	Korea
Micropipettes	SLAMED	Japan
PCR- thermocycler	TECHNE	England
Sensitive balance	AND	Taiwan
UV-trans illuminator	Syngene	England
Vortex mixer	Human twist	Germany

2.2.2 Chemicals and Kits

All chemicals and kits used in this study, along with the manufacturer and country of origin are listed in Table (2.2).

Table 2.2: List of all Chemicals, kits, manufacturer and country of origin.

Kits and chemicals	Company	Country
Absolute ethanol	Systerm	Malaysia
Agarose	Intron	Korea
Cholesterol Kit	Mindray	China
Creatine kinase kit	Mindray	China
DNA extraction kit	Geneaid	Taiwan
DNA ladder	Bioneer	Korea
Ethidium Bromide	Intron	Korea
HDL kit	Mindray	China
LDL kit	BioBase	China
PCR PreMix Kit	Bioneer	Korea
Serum creatinine kit	Mindray	China
TBE buffer	Bioneer	Korea
Thyroid stimulating hormone kit	Snibe	China
Triglyceride kit	Mindray	China

2.3 Methods

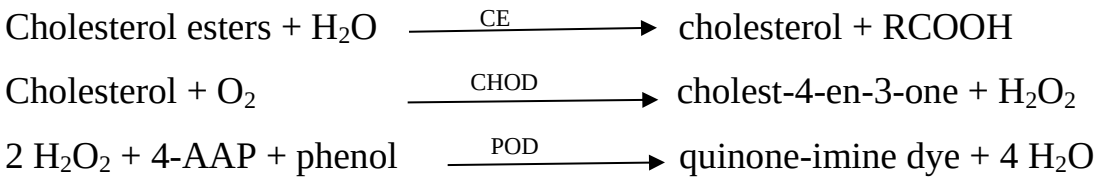
2.3.1 Hormonal and Biochemical Assay Methods

2.3.1.1 Determination of Lipid Profile

2.3.1.1.A Estimation of Total Cholesterol

Total cholesterol (TC) in serum was determined in vitro using the enzymatic colorimetric technique. Cholesterol esters are broken to free cholesterol and fatty acids by the action of the cholesterol esterase enzyme (CE). The enzyme cholesterol

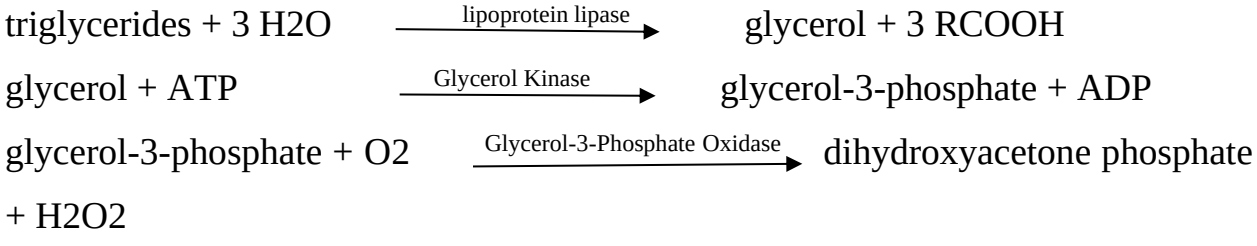
oxidase (CHOD) then oxidizes cholesterol to cholest -4-en-3-one and hydrogen peroxide. Due to the action of the peroxidase enzyme (POD), the hydrogen peroxide affects the oxidative coupling of phenol and 4-aminoantipyrin (4-AAP) to produce red quinone-imine dye.



The content of cholesterol is exactly proportional to the color intensity of the dye produced, as assessed by an increase in absorbance at 512 nm (193).

2.3.1.1.B Estimation of Serum Triglyceride

In vitro triglyceride (TG) estimation was performed using the enzymatic quantitative colorimetric method. Triglycerides are catalyzed to yield H_2O_2 through a series of enzymatic catalysis steps by lipase, glycerol kinase, and Glycerol-3-Phosphate Oxidase, which oxidizes 4-aminoantipyrinel to yield a colored dye of quinoneimine. The increase in absorbency is proportional to the concentration of triglycerides(194).



H2O2 + 4-aminoantipyrine + 4-chlorophenol $\xrightarrow{\text{peroxidase}}$ 4-(p-benzoquinone-monoimino)-phenazone + 2 H2O + HCl

2.3.1.1.C Estimation of Serum High-Density Lipoprotein (HDL)

High density lipoprotein (HDL) levels are determined directly in serum. The method's fundamental principle is as follows. Under assay conditions, the apo-B containing lipoproteins in the specimen are reacted with a blocking reagent, rendering them non-reactive with the enzymatic cholesterol reagent. As a result, apo-B containing lipoproteins are totally excluded from the assay, and only HDL-cholesterol is identified under the conditions of the assay. For the HDL-cholesterol measurement, sulfated alpha-cyclodextrin in the presence of Mg^{+2} forms complexes with apo-B containing lipoproteins, and polyethylene glycol-coupled cholesteryl esterase and cholesterol oxidase(195).

Apo-B containing lipoproteins + α-cyclodextrin + Mg+2 + dextran SO4 $\longrightarrow$ soluble non-reactive complexes with apo-B containing lipoproteins

HDL-cholesterol esters + H_2O $\xrightarrow{\text{PEG-cholesterol esterase}}$ HDL-cholesterol + RCOOH

HDL-cholesterol + O_2 $\xrightarrow{\text{PEG-cholesterol oxidase}}$ Δ4-cholestenone + H_2O_2

H_2O_2 + 5-aminophenazone+ N-ethyl-N-(3-methylphenyl)-N-succinyl ethylene diamine + H+ + H2O $\xrightarrow{\text{peroxidase}}$ purple blue pigment (quinoneimine) + H_2O

The amount of HDL cholesterol present is proportionally correlated with the amount of blue quinoneimine dye produced.

2.3.1.1.D Estimation of Serum Low-Density Lipoprotein (LDL-C)

The concentration of LDL-C was estimated indirectly using Friedewald and colleagues' equation, which has become the more prevalent method in clinical laboratories(196).

LDL-C mmol/L = [Total-chol.] - [HDL-C] - [TG/5]

2.3.1.2 Estimation of Thyroid Stimulating Hormone (TSH)

Thyroid stimulating hormone is measured using a sandwich chemiluminescence immunoassay. The sample, ABEI (N-(4-aminobutyl)-N-ethylisoluminol) labeled with anti-TSH monoclonal antibody, and magnetic microbead coated with another anti-TSH monoclonal antibody are thoroughly mixed and incubated at °C, forming an immune-complex sandwich. Following precipitation in a magnetic field, the supernatant is decanted and a wash cycle is performed. The starter reagents are then added, and a flash chemiluminescent reaction is started. Within 3 seconds, a photomultiplier measures the light signal as a relative light unit RLU, which is proportional to the concentration of TSH in the samples(197).

2.3.1.3 Estimation of Serum Creatinine

Creatinine measurements are employed in the diagnosis and treatment of renal disorders, in the monitoring of renal dialysis, and in the computation of other urine analytes. Enzymatic approach is a preferable clinical alternative for precise creatinine measurement, particularly in newborns, pediatrics, and hematology departments. The enzymatic assay for creatinine involves a series of coupled enzymatic reactions, including creatininase enzymatic conversion of creatinine into

creatine, which is then converted to sarcosine by creatinase, followed by sarcosine oxidase (SOD) producing hydrogen peroxide.

The Increase In absorbency at 546 nm of the product quinonimine is proportional to the concentration of creatinine(198).

$$\text{Creatinine} + H_2O \xrightarrow{\text{Creatininase}} \text{Creatinine}$$

$$\text{Creatine} + H_2O \xrightarrow{\text{Creatinase}} \text{Sarcosine}$$

$$\text{Sarcosine} + O_2 + H_2O \xrightarrow{\text{SOD}} \text{Glycin} + HCHO + H_2O_2$$

$$2H_2O + O_2 + \text{4-aminoantipyrin} + \text{ESMPT} \xrightarrow{\text{Catalase}} \text{quininimine} + 4H_2O_2$$

2.3.1.4 Estimation of Creatine Kinase

Creatine kinase (CK) activity in plasma and serum is quantitatively determined in vitro using the International Federation of Clinical Chemistry (IFCC) method on photometric systems. In the presence of creatine phosphate, creatine kinase catalyzes the phosphorylation of ADP to produce ATP and creatine. The catalytic concentration is calculated by measuring the rate of NADPH formation at 340 nm using hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PD) coupled reactions(199).

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{Glucose-6-phasphate}$$

$$\text{Glucose-6-phasphate} + NADP^+ \xrightarrow{\text{G6PD}} \text{Glucose-6-phasphate} + H^+ + \text{NADPH}$$

2.3.2 Genetic Analysis

2.3.2.1 Extraction of Genomic DNA from Blood Sample

The DNA extraction was conducted at the College of Pharmacy Laboratory / University of Kerbela. Genomic DNA was extracted from blood samples according to the protocol of the gSYNC DNA Extraction Kit which is designed for the purification of genomic, mitochondrial, and viral DNA from whole blood (fresh and frozen blood), tissue, amniotic fluid, and sperm in a single convenient kit. Proteinase K and chaotropic salt are used in this DNA extraction kit to lyse cells and breakdown protein, allowing DNA to adhere to the glass fiber matrix of the spin column. A Wash Buffer is used to remove contaminants, and the purified genomic DNA is eluted with a low salt Elution Buffer or water. The purified DNA (about 20-30 kb) can be used in PCR or other enzymatic reactions. The following steps demonstrate the principle of DNA extraction from blood:

1.Sample Preparation

Fill a 1.5 ml microcentrifuge tube with up to 200 µl of whole blood. With Phosphate-buffered saline (PBS), adjust the volume to 200 µl. Pipette 20 µl of Proteinase K into the mixture. Incubate for 5 minutes at 60^{o}C.

2. Cell Lysis

Add 200 µl of Gel Sample Buffer (GSB), then vigorously shake to mix. Every two minutes, invert the tube during the five minutes of incubation at 60 °C.

3. DNA Binding

The sample lysate should immediately be mixed with 200 µl of absolute ethanol by vigorously shaking for 10 seconds. If precipitate appears, use a pipette to break it up as much as you can.

In a 2 ml collection Tube, place a GS column. Transfer the entire mixture including any precipitate that is insoluble to the GS column. 1 minute of centrifuging at 14–16,000 x g. If the mixture did not flow through the GS column membrane after centrifugation, extend the centrifuge time until it does. Transfer the GS column to a fresh 2 ml collection tube after discarding the flow-through-containing 2 ml collection tube.

4.Washing

400 µl of W1 buffer should be added to the GS column. Centrifuge for 30 seconds at 14-16,000 x g, then discard the flow-through. Return the GS column to the 2 ml collection tube. Fill the GS column with 600 µl of wash buffer. Centrifuge for 30 seconds at 14-16,000 x g, then discard the flow-through. Return the GS column to the 2 ml collection Tube. To dry the column matrix, centrifuge it for 3 minutes at 14-16,000 x g.

5.Elution

In a clean 1.5 ml microcentrifuge tube, transfer the dried GS column. 100 µl of pre-heated elution buffer should be added to the column matrix's center. Allow at least 3 minutes for elution buffer to be completely absorbed. To elute the purified DNA, centrifuge at 14-16,000 x g for 30 seconds.

2.3.2.2 Quantitation of DNA by Spectrophotometric Method

The nano-spectrophotometric method (Nanodrop)was used to determine the purity and concentration of extracted DNA samples. The absorbance measurements were taken at 260 and 280 nm. The DNA absorbs light most strongly at 260 nm, while the protein absorbs light most strongly at 280 nm. The A260/A280 ratio was

used to determine DNA purity. The A260/A280 ratio of 1.8-2.0 is widely accepted as indicating a high-quality DNA. After cleaning the micro detector with deionized water and performing a blank measurement with 1 µL of buffer onto the lower optical surface, pipette 1 µL of nucleic acid sample onto the lower optical surface, and close the lever arm. In the application software, select "Measure." The nucleic acid concentration and purity ratios will be calculated automatically by the software(200).

2.3.2.3 Allele Specific Polymerase Chain Reaction (AS-PCR)

The allele-specific PCR also called as an amplification refractory mutation system–polymerase chain reaction (ARMS-PCR) or PCR amplification of specific alleles (AS-PCR) used to detect the GATM gene rs9806699 G > A, C, T (201).

2.3.3.3.A Primers Preparation

Using specific primers, Polymerase Chain Reaction (PCR) was utilized to amplify the GATM gene rs9806699 G > A, C, T. Assistant Professor Dr. Hassan Mahmood Mousa Abo Almaali designed the primers for this study using primer-blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and purchased them as lyophilized products from Alpha DNA, Canada, in various picomole concentrations. Lyophilized forward and reverse primers were dissolved in specific volumes of nuclease-free water to achieve a concentration of 100 pmol/ µl (stock solution) (table 2.3)(202). To make 2.5 pmol/ µl of primer working solution, 2.5µl of each primer (stock solution) was diluted with 97.5 µl of nuclease free water. The primers were stored at -20 degrees Celsius until further use. The primers used to amplify the gene alleles are shown in Tables (2.4).

Table 2.3: The volume of nuclease free water added to each Primers of GATM rs9806699 G > A, C, T to obtain 100 Pmol/µL.

Primers of GATM rs9806699 G > A, C, T	Volume of nuclease water added (µL)
M-ALLELE G	1026
M-ALLELE A	1210
M-ALLELE T	1113
M-ALLELE C	1271
M-Reverse primer	1589

Table 2.4: Primers sequences of GATM rs9806699 G > A, C, T.

ALLELES	Sequence (5'->3')	Time (seconds)	Product length
M-ALLELE G	AATGTCACCATGCCCCAGAGC	63.02	194
M-ALLELE A	AATGTCACCATGCCCCAGAGT	61.75	
M-ALLELE T	AATGTCACCATGCCCCAGAGA	61.47	
M-ALLELE C	AATGTCACCATGCCCCAGAGG	62.43	
M-Reverse primer	TGCGCCTTCCTGGTGTTCAT	62.41	

2.3.3.3.B Optimizing the Conditions for Polymerase Chain Reaction

Polymerase chain reaction optimization was accomplished through several attempts to establish the optimal annealing temperature, concentration of both DNA and primers and number of amplification cycles. The tables (2.5) and (2.6) detail the PCR components for all of the amplified fragments and the optimized PCR programs respectively.

2.3.3.3.C Running the Polymerase Chain Reaction

As can be seen in the tables that follow, the PCR was prepared by mixing the reaction components with the optimized concentrations, and then running the PCR according to the optimized programs(203).

Table 2.5: PCR mix reaction for genotyping of GATM rs9806699 G > A / G > C / G >T.

Components	Volume (µL)
Forward primer	1 µL (2.5 pmol/ µl)
Reverse primer	1 µL (2.5 pmol/ µl)
DNA	3 µL
Nuclease free water	15 µL
PCR premix	5 µL
Total volume	25 µL

Table 2.6: PCR condition for genotyping of GATM rs9806699 G > A, C, T.

step	Temperature / °C	Time	Number of cycles
Denature template	95	5 minutes	1
Initial denaturation	95	20 seconds	30
Annealing	61.5	10 seconds	
Extension	72	15 seconds	
Final extension	72	5 minutes	1

2.3.3.4 Agarose Gel Electrophoresis

- In order to make agarose gel, 1.5 g of agarose powder was dissolved in 100 ml of 1x TBE (Tries Borate EDTA) buffer (pH 8), and after that, the mixture was heated to boiling with a heater until all of the gel particles were dissolved.
- The solution was stirred to mix it and avoid bubbles until the gel solution appeared clear and pure.
- The solution was cooled to 50-60 °C.
- 3 µL of stock Ethidium Bromide was added to the gel.
- The comb was fixed to one end of the tray to form wells for the PCR product samples to be loaded.
- Gently pouring the agarose into the tray, allowing it to solidify at room temperature for 30 minutes, and then carefully removing the comb from the gel.
- A gel electrophoresis tank was used to install the gel. The tank was filled with 1X TBE- electrophoresis buffer until it reached 3-5 mm above the gel's surface.
- One agarose gel well was loaded with 5 µL of DNA ladder, while the other wells were loaded with 5 µL of each PCR product.
- The electrophoresis apparatus's voltage was set to ensure an electrical field of 5 v.cm-1 for the distance between the cathode and anode.
- At the end of the run, a UV trans-illuminator set to 320-336 nm was utilized to identify bands.
- A digital camera was used to photograph the gel (204).

2.4 Statistical Analysis

The data of the present study was entered and analyzed through the Statistical Package for the Social Sciences (SPSS version 24). The data were presented as frequencies and percentages or mean and standard deviation in appropriate tables and graphs. Chi square test, Fisher's exact test, ANOVA test, T test and post hoc analysis were used where is appropriate to find out the possible association between the related variables of the current study. Statistical association was considered significant when p value equal or less than 0.05 (P value ≤ 0.05).

Chapter Three

Results

3.1 Socio-demographic and Some Related Characteristics of Patients

The age of the included patients (N=150) ranged from 28 to 65 years with a mean of 50.9±9.2 years. Female to male ratio was 1.2:1. More than two thirds of the patients of the present study reported using atorvastatin 40 mg for at least one year. Less than one thirds of the patients of the study reported development of muscle pain. More than one half of the patients (56%) had no statin related myopathy as shown in table (3.1).

Table 3.1: Socio-demographic and some related characteristics of the included participants.

Characteristics		Total=150 No. (%)
Age (in years)	mean ±SD	50.9±9.2
	Range	28- 65
Age groups (years)	< 40	22 (14.7)
	40- 50	49 (32.7)
	51-60	59 (39.3)
	>60	20 (13.3)
Gender	Female	81 (54)
	Male	69 (46)
BMI	Normal weight	42 (28)
	Overweight	70 (46.7)
	Obese	38 (25.3)
Duration of treatment (months)	1-11	44 (29.3)
	12-23	41 (27.3)
	24-36	40 (26.7)
	>36	25 (16.7)
Smoking	Yes	41 (27.3)
	No	109 (72.7)
Diabetics	Yes	50 (33.3)
	No	100 (66.6)
Muscle pain	Yes	47 (31.3)
	No	103 (68.7)
SRM	Asymptomatic	19 (12.7)
	Myalgia	29 (19.3)
	Myalgia with mild C.K elevation	18 (12)
	Normal	84 (56)

BMI: Body mass index, N: Numbers of the Study participant, SD: Standard deviation, SRM: statin related myopathy, CK creatine kinase.

3.2 Biochemical Parameters

3.2.1 Mean of Biochemical Parameters

The table that follows displays the mean of the biochemical parameters of the participants who were included in the study.

Table 3.2: Mean of biochemical parameters of the included participants.

Biochemical parameters	Mean	Normal range	± SD	Minimum	Maximum
TSH	2.99	(0.27-5) mlU/L	11.27	0.01	5.36
Cholesterol	164.88	(50-200) mg/Dl	50.06	52	293
TG	164.29	(35-160) mg/Dl	92.66	27	621
LDL	90.94	(0-140) mg/dL	40.18	23	238
HDL	40.92	(35-65) mg/dL	11.23	18	75
SCr	0.84	(0.3-1.1) mg/dL	0.48	0.27	4.57
CK	145.75	(24-170) U/L	81.44	41	521

TSH: thyroid stimulating hormone, TG: triglycerides, LDL: low density lipoprotein, HDL: high-density lipoprotein, SCr: serum creatinine, CK: creatine kinase.

3.2.2 Effect of Duration of Treatment on Mean of Biochemical Parameters

Table 3.3 clarify that the mean difference of biochemical parameters in relation to duration of treatment with atorvastatin was statistically significant only in regard to triglyceride ($p<0.05$).

Table3.3: Association of mean biochemical parameters and duration of treatment.

Variables	Duration of treatment (months)								P value
	<12		12-23		24-36		>36		
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	
TSH	2.68	4.07	1.88	0.91	5.13	21.41	1.97	1.42	0.562
Chol	171.73	45.08	165.43	54.94	157.40	52.57	163.88	47.07	0.634
TG	186.86	115.85	142.17	54.71	145.45	82.58	190.96	100.43	0.032*
LDL	95.05	31.72	97.32	49.54	86.56	41.78	80.26	32.29	0.293
HDL	41.37	8.055	39.36	10.77	41.05	13.51	42.48	13.06	0.723
SCr	0.767	0.37	0.85	0.63	0.88	0.54	0.91	0.24	0.622
CK	145.61	96.02	136.27	52.06	145.30	89.88	162.24	81.07	0.668

*ANOVA test was used with a significant P value of less than 0.05.
TSH: thyroid stimulating hormone, TG: triglycerides, LDL: low density lipoprotein, HDL: high-density lipoprotein, SCr: serum creatinine, CK: creatine kinase.

3.3 Genotyping of rs9806699 G > A, C, T Genetic Polymorphism

3.3.1 Results of Amplification Reaction

The gene polymorphism rs9806699 G > A, C, T produced a clear band with a molecular size of 194 bps. (Figure 3.1). The size of the amplicon was estimated by comparing it to a 100-1000 bp DNA ladder.

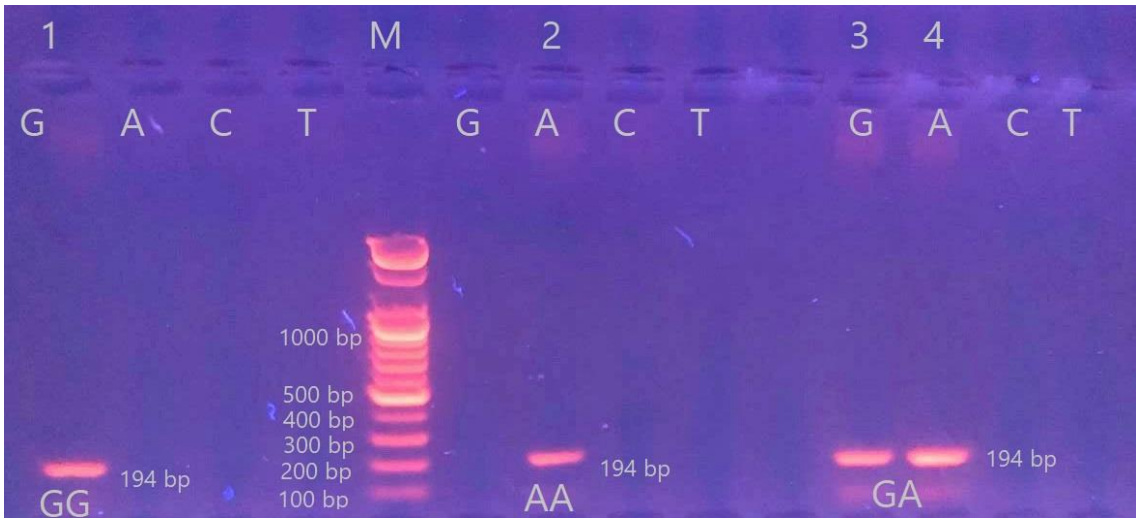


Figure 3.1: Genotyping of rs9806699 genetic polymorphism showed: lane M represent DNA ladder 100 – 1500 bp, lane 1 represent GG genotype (wild), lane 2 represent AA genotype (homozygous mutant) and 3 and 4 lanes represent GA genotype (heterozygous).

3.3.2 Distribution of Allele Frequencies of GATM Gene Polymorphism (G>A, C, T)

For the purpose of this study, the participants were categorized according to one of three genotypes for the GATM gene rs9806699 (G>A, C, T) genetic polymorphism: homozygous for the G allele (GG) wild type, heterozygous (GA), and homozygous for the A allele (AA) mutant type. According to table 3.4 and figure 3.2, there were 73 AA genotypes (48.7%), 57 GA genotypes (38%), and 20 GG genotypes (13.3%) among 150 patients. There was no allele frequency found for C or T alleles in the population of Kerbala province.

Table 3.4: Allele frequencies of GATM rs9806699 (G>A, C, T) gene polymorphism.

SNP	Genotypes	Frequency	percentage
rs9806699	GG	20	13.3%
	GA	57	38.0%
	AA	73	48.7%
	GC	0	0.0%
	CC	0	0.0%
	GT	0	0.0%
	TT	0	0.0%
Total		150	100%

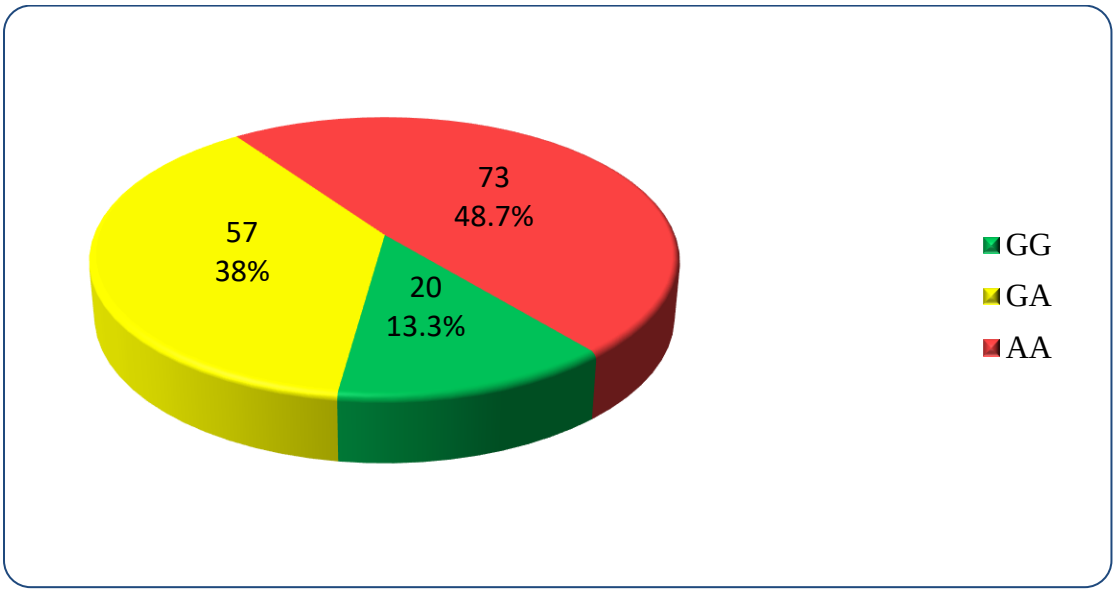


Figure3.2: Distribution of gene polymorphism SNP among study patients.

3.3.3 Association of Socio-demographic and Biochemical Parameters with Genotype

As can be seen in table 3.5, the associations between the socio-demographic and some other characteristics of the included participants and their genotype were not statistically significant (p>0.05).

Table3.5: Association of socio-demographic and some characteristics with genotype.

Variables	Categories	Genotype: No. (%)			P value
		GG	GA	AA	
		(n=20)	(n=57)	(n=73)	
Age (years)	Mean ±SD	51.2±9.45	49.81±9.04	50.82±9.28	0.769
Gender	Female	14(17.3)	26(32.1)	41(50.6)	0.149
	Male	6(8.7)	31(44.9)	32(46.4)	
Smoking	No	15(13.8)	44(40.4)	50(45.9)	0.527
	Yes	5(12.2)	13(31.7)	23(56.1)	
Muscle pain	No pain	14(13.6)	41(39.8)	48(46.6)	0.746
	Pain	6(12.8)	16(34)	25(53.2)	
SRM	Asymptomatic	0	6(31.6)	13(68.4)	0.236
	Myalgia	5(17.2)	10(34.5)	14(48.3)	
	Myalgia with mild C.K elevation	1(5.6)	6(33.3)	11(61.1)	
	Normal	14(16.6)	35(41.7)	35(41.7)	

*Chi-square test and ANOVA test was used with a significant p value of less than 0.05.
N: Numbers of the study participant, SRM: statin related myopathy.

The analysis of the results of the present study revealed that there was statistically significant difference of mean creatine kinase in relation to genotype; post hoc analysis concluded that AA allele responsible for this difference as

illustrated in table 3.6, whereas there were no significant statistical differences of mean lipid profile parameters in relation to genotype (table 3.7 below).

Table3.6: Effect of genotype on the mean of biochemical parameters.

Variables	Genotype: (mean ±SD)			P value
	GG	GA	AA	
	(n=20)	(n=57)	(n=73)	
TSH	1.71±0.88	1.95±1.56	4.16±16.07	0.468
SCr	0.89±0.89	0.80±0.30	0.87±0.45	0.657
CK	112.70±74.34	134.81±66.80	163.34±89.89	0.02*

*ANOVA test was used with a significant P value of less than 0.05.
TSH: thyroid stimulating hormone, SCr: serum creatinine, CK: creatine kinase.

Table 3.7: Effect of genotype on the mean of lipid profile parameters.

Variables	Genotype: (mean ±SD)			P value
	GG	GA	AA	
	(n=20)	(n=57)	(n=73)	
Cholesterol	173.95±56.62	159.51±46.84	166.59±50.84	0.5
TG	178.90±92.04	156.25±90.48	166.56±95.13	0.619
LDL	96.30±49.16	90.89±38.35	89.51±39.36	0.801
HDL	41.40±14.60	40.02±8.94	41.50±11.91	0.744

*ANOVA test was used with a significant P value of less than 0.05.
TG: triglycerides, LDL: low density lipoprotein, HDL: high-density lipoprotein.

3.4 Association of Muscle Pain and Creatine Kinase Level

As can be seen in table 3.8, the statistical analysis revealed that patients who experienced muscle pain had a significantly higher mean creatine kinase level compared to patients who did not experience muscle pain ($p<0.05$).

Table 3.8: Association of muscle pain with mean Creatine kinase.

Variables	Muscle pain		P value
	No pain	Pain	
CK (mean ±SD)	129.59±61.04	181.15±106.56	0.0001*

*Independent Samples t test was used with a significant P value of less than 0.05.
CK: creatine kinase.

3.5 Effects of Atorvastatin Treatment on Muscle

3.5.1 Effect of Duration of Treatment on Muscle Pain

According to the data presented in table 3.9 below, there was no statistically significant association of muscle pain in relation to the duration of treatment with atorvastatin ($p>0.05$).

Table 3.9: Association of muscles pain with duration of treatment.

Variables	Categories	Muscle pain		P value
		No pain (%)	Pain (%)	
Duration of treatment (months)	<12	30(68.2)	14(31.8)	0.654
	12-23	28(68.3)	13(31.7)	
	24-36	30(75)	10(25)	
	>36	15(60)	10(40)	

*Chi-square test was used with a significant p value of less than 0.05.

3.5.2 Effect of Duration of Treatment and Muscle Pain on Creatine Kinase Level

Further analysis concluded that, patients taking atorvastatin for a period of 24 - 36 months who experienced muscle pain had a statistically significant higher mean creatine kinase level than patients who did not experience muscle pain ($p<0.05$), as shown in table 3.10 below.

Table 3.10: Association of duration of treatment and muscle pain with mean Creatine kinase.

Duration of treatment (months)	Muscle pain	CK		P value
		Mean	SD	
<12	No pain	126.60	66.75	0.053
	Pain	186.36	133.92	
12-23	No pain	130	55.80	0.263
	Pain	149.77	41.72	
24-36	No pain	123.67	48.84	0.007*
	Pain	210.20	145.18	
>36	No pain	146.67	81.02	0.248
	Pain	185.60	79.41	

*ANOVA test was used with a significant P value of less than 0.05.

3.5.3 Effect of Patient's age and Muscle pain on Creatine kinase Level

Further analysis also concluded that patients on treatment of atorvastatin with age group 51 to 60 years with muscle pain had statistically significant higher mean creatine kinase level than patients with no muscle pain ($p=0.003$) as shown in table 3.11.

Table 3.11: Association of patient's age and muscle pain with mean Creatine kinase.

Age groups (years)	Muscle pain	CK		P value
		Mean	SD	
<40	No pain	130.12	71.92	0.640
	Pain	113.40	57.38	
40-50	No pain	128.90	57.76	0.077
	Pain	170.09	92.23	
51-60	No pain	132.74	62.37	0.003*
	Pain	207.24	123.61	
>60	No pain	119.40	56.27	0.143
	Pain	172.40	93.77	

*ANOVA test was used with a significant P value of less than 0.05.
SD: standard deviation, CK: creatine kinase.

3.6 Association of Socio-demographic Characteristics with SRM Risk

The analysis of the current data demonstrated that male gender had significantly higher proportion of SRM than female (p=0.028) as illustrated in table 3.12.

Table 3.12: Association of socio-demographic and some related characteristics with SRM.

Variables	Categories	SRM: No. (%)		P value
		Normal	SRM	
		(n=84)	(n=66)	
Age groups	<40	14(63.6)	8(36.4)	0.269
	40-50	31(63.3)	18(36.7)	
	51-60	31(52.5)	28(47.5)	
	>60	8(40)	12(60)	

BMI	Normal weight	22(52.4)	20(47.6)	0.653
	Overweight	42(60)	28(40)	
	Obesity	20(52.6)	18(47.4)	
Gender	Female	52(64.2)	29(35.8)	0.028*
	Male	32(46.4)	37(53.6)	
Smoking	No	61(56)	48(44)	0.988
	Yes	23(56.1)	18(43.9)	

*Chi-square test was used with a significant p value of less than 0.05.
SRM: statin related myopathy, BMI: body mass index.

3.7 Association of Mean Biochemical Parameters with SRM

The analysis of mean biochemical parameters revealed that patients with SRM had significantly higher mean creatine kinase level than patients with no SRM as illustrated in table 3.13 below.

Table 3.13: Association of mean biochemical parameters with SRM

Variables	SRM	Mean	SD	P value
TSH	Normal	3.70	14.79	0.391
	SRM	2.10	3.27	
Cholesterol	Normal	164.43	48.39	0.901
	SRM	165.46	52.47	
TG	Normal	154.49	85.50	0.145
	SRM	176.76	100.32	
LDL	Normal	93.51	40.74	0.379
	SRM	87.67	39.52	
HDL	Normal	41.39	11.62	0.565
	SRM	40.32	10.78	
SCr	Normal	0.85	0.57	0.843
	SRM	0.834	0.35	

CK	Normal	108.27	41.18	0.0001*
	SRM	193.44	94.40	
Duration of treatment in months	Normal	21.98	18.42	0.397
	SRM	24.86	23.22	

*Independent Samples t test was used with a significant P value of less than 0.05.
SRM: statin related myopathy, TSH: thyroid stimulating hormone, TG: triglycerides, LDL: low density lipoprotein, HDL: high-density lipoprotein, SCr: serum creatinine, CK: creatine kinase.

3.8 Estimation of Risk in GATM Gene rs9806699 SNP in Relation to SRM

According to the analysis of the data, which is displayed in tables 3.14 and 3.15, there was no statistically significant difference between patients with SRM

Table 3.14: Association of SRM with genotype GG and GA.

SRM	Genotype: No. (%)		Subtotal	P value	Odds Ratio (95% C.I)
	GG	GA			
No SRM	14(28.6)	35(71.4))	49	0.492	1.467 (0.491-4.384)
SRM	6(21.4)	22(78.6)	28		

*Chi-square test was used with a significant p value of less than 0.05.
SRM: statin related myopathy.

Table 3.15: Association of SRM with genotype GG and AA.

SRM	Genotype: No. (%)		Subtotal	P value	Odds Ratio (95% C.I)
	GG	AA			
No SRM	14(28.6)	35(71.4))	49	0.08	2.533 (0.877-7.319)
SRM	6(13.6)	38(86.4)	44		

*Chi-square test was used with a significant p value of less than 0.05.
SRM: statin related myopathy.

Chapter Four

Discussion

4. Discussion

The most prevalent side effects of statin therapy are statin-related myopathy (SRM). All statins can cause myotoxicity, which is dose-dependent and can force patients to stop taking their medication(91). Statin-related myopathy (SRM) are reported by 10% to 33% of people taking statins. An internet survey of former statin users revealed that 60% had SRM and 62% had discontinued statin therapy due to side effects(83).

The L-arginine: glycine-amidinotransferase (AGAT or GATM) gene encodes the mitochondrial enzyme L-arginine: glycine-amidinotransferase (GATM), which is a rate-limiting enzyme involved in creatine biosynthesis (160). Creatine is then needed for proper muscular function (177). Statin exposure has been demonstrated to reduce GATM expression twofold higher in cells from rs9806699 carriers than in cells from noncarriers. As a result, decreased GATM expression should result in decreased creatine biosynthesis(205).

Because genetic variations are one of the most important factors in determining whether a person is at a higher or lower risk of having side effects from the same drug(77), it seemed reasonable to investigate the impact of this polymorphism on statin-related myopathy.

This is the first study of its kind to investigate the effect of the GATM gene polymorphism rs9806699 G > A, C, T in an Iraqi population taking atorvastatin.

4.1 Demographic Data

The ages of the 150 patients who participated in the study ranged from 28 to 65 years with a mean of 50.9±9.2 years. More than 70% of the patients are either overweight or obese. The definition of statin myopathy is controversial. Different definitions of statin-related muscle effects have been put out by the American

Heart Association (AHA), American College of Cardiology (ACC), National Heart, Lung, and Blood Institute (NHLBI), National Heart, and FDA. The ACC/AHA/NHLBI definitions, however, are the ones that are most frequently used in the researches (table 1.2). The lack of agreement in the definition of SRM makes exact assessment of their real incidence difficult(206). Furthermore, the risk of statin myopathy in clinical trials (1.5-5%) is much lower than in observational studies (10-33%)(207).

According to our findings, the total prevalence of statin-related myopathy was 44% which is approximately the same percentage as a cross-sectional study conducted on 700 Iranian patients taking atorvastatin treatment, which showed that 44.3% of patients experienced atorvastatin-associated muscular symptoms (208). Only 31.3% of them reported muscular pain with or without creatine kinase (CK) elevation, whereas only 12.7% reported CK elevation without muscle pain. This study backs up prior studies that statins induce myopathy. The unusually high prevalence of SRM identified in our population may suggest to the relevance of genetic factors, cultural differences in reporting drug side effects, and/or potential interactions between dietary factors and statins.

Aging (>75 years) is one of many predisposing risk factors that may increase the likelihood risk of skeletal muscle side effects with statin(98). Sarcopenia, or muscular mass loss, has been linked to aging. This loss of muscle tissue occurs around the age of 50, but gets more pronounced after the age of 65. Muscular mass loss in the elderly leads to decreased muscle function(209). As a result of aging process, SRM may be more severe in the elderly population who takes statins to treat cardiovascular disease(210). This study deviates from previous researches, which found no association between age groups and statin-related myopathy, while agreeing with(211). However, there is a modest increase in SRM associated with older patient age, despite the fact that this difference does not reach statistical

significance. Statin-related myopathy (SRM) was discovered in 36.4% of participants aged <40, 36.6% of participants aged 40-50, and 47% of participants aged 51-60 years. It is remarkable that SRM affects approximately 60% of participants over the age of 60, which is a higher percentage than the rest of the age groups. Because our study was limited to individuals under the age of 65 and the effect of statins on muscle becomes more pronounced after the age of 65, and, we were unable to accurately determine the effect of statins on the elderly.

In addition to age, this study also shows that there was no association between the patient's BMI and SRM which concur with(212).

The gender distribution was 54% female and 46% male, resulting in a female to male ratio of 1.2:1. Our data showed that males had a significantly higher proportion of SRM than females ($p<0.05$), which contradicts the findings of Skiliving *et al*.(213), who found that female had a higher frequency of SRM than male and matched with(214). Interestingly, SRM are more common in physically active people(93).This finding may have been influenced, in part, by the fact that male engage in greater physical activity than female do, making them more susceptible to SRM.

4.2 Biochemical Finding

In this study, 150 patients had their lipid profile, thyroid stimulating hormone (TSH), serum creatinine, and creatine kinase (CK) levels tested.

Thyroid stimulating hormone (TSH) was assessed to remove patients at risk of hyperthyroid or hypothyroid myopathy, separating patients with non-pharmacological muscular symptoms from those with true SRM.

Creatine kinase (CK), a muscle injury biomarker, is routinely used to identify and assess the severity of skeletal muscle damage. However, in symptomatic patients using statins, CK values are usually normal, but in asymptomatic patients, CK

value may be elevated(101). Due to the lack of additional specialized laboratory tests, CK is currently employed in predicting the degree of SRM even though it is a non-sensitive biomarker for statin-induced myopathy(102). Based on the results obtained, creatine kinase (CK) levels are significantly higher in patients with muscular pain compared to patients without muscular pain ($P<0.0001$) as shown in Table 3.9. This finding indicates the role of C.K in predicting the severity of SRM. Among the mechanisms of creatine kinase (CK) elevation in response to statins are increased muscular membrane fragility caused by decreased cholesterol content, suppression of isoprenoid formation (a critical step in the biosynthesis of membrane proteins), and ubiquinone depletion resulting in mitochondrial dysfunction(215).

A systematic review of 35 randomized, placebo-controlled studies of the six FDA-approved statins found no significant differences in CK elevations between statin-treated and placebo patients(216). The previous study is consistent with our study as average of creatine kinase show no significant increase with duration of treatment groups as shown in table 3.3. Parker et al. (2013), on the other hand, found that atorvastatin caused a significant increase in creatine kinase average($P<0.0001$)(217).

Atorvastatin has been found to reduce cholesterol levels effectively(47). The same was confirmed in our study (table 3.3), where the mean of cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels after <12, 12-36 and >36 months of atorvastatin 40mg treatment are within normal range, indicating the role of atorvastatin in correcting cholesterol levels in patients with hyperlipidemia.

The results were not the same for triglycerides (TG), our data show that the mean TG level significantly higher when duration of treatment <12 months or >36 months compared to duration of treatment 12-36 months. In large cross-sectional observational study found that dyslipidemia is widespread among patients

with type 2 DM in the United Kingdom and TG remain high after statin monotherapy. The main conclusion was that Persistent hypertriglyceridemia was frequent, affecting half of those taking statins alone(218). Complex mechanisms are involved in this process, but they can be summarized into three pathways. First, lipolysis of triglycerides in adipocytes and myocytes is unchecked in insulin-resistant patients, resulting in a flood of fatty acids returning to the liver. The increased return of fatty acids to the liver stimulates the liver to produce more VLDL. Second, insulin resistance indirectly causes an increase in the production of apolipoprotein B and VLDL by preventing the liver from degrading apolipoprotein B. Thirdly, there is evidence that elevated apolipoprotein CIII expression in the presence of insulin resistance contributes to the overproduction of VLDL(219). The previous studies may explain the particular increase in triglycerides since 50 patients in this study had type 2 diabetes mellitus, and more than half of them (33 patients) had hypertriglyceridemia, with the majority of those patients falling into the <12 and >36 months duration of treatment groups. In addition, our data did not take into account a baseline triglyceride value before starting treatment to estimate the extent of the triglyceride reduction.

4.3 Molecular Study

4.3.1 Glycine Amidinotransferase Gene Polymorphism

The GATM gene rs9806699 G > A, C, and T was amplified using Polymerase Chain Reaction (PCR), and the findings showed that the A allele was the major allele and the G allele was the minor allele in our population. These findings were roughly in line with those observed in the Chinese population(190).

4.3.2 Role of GATM Gene Polymorphism in the Occurrence of SRM

In the first place, Mangravite *et al*. discovered a potential genetic marker associated with a reduced risk of SRM. They found an association between expression quantitative trait loci (eQTLs) for the GATM (rs9806699 G > A) and simvastatin exposure using gene expression profiling of lymphoblastoid cell lines (LCLs). The rate-limiting enzyme in creatine production is glycine amidinotransferase. Creatine is primarily synthesized in the liver and kidneys and is then transferred to skeletal muscle, where it serves as a vital source of cellular energy. Simvastatin-exposed LCLs with the A allele showed a higher decline in GATM RNA expression than non-exposed control LCLs. As a result, decreased GATM expression should result in decreased creatine biosynthesis and creatine phosphate storage. They hypothesize that decreased creatine phosphate storage alters skeletal muscle cellular energy pathways, making them less susceptible to statin myopathy, and that the A allele is significantly associated with a lower risk of SRM(187). On the contrary, later research did not corroborate this conclusion. According to Carr *et al*., there was no significant different in the minor allele frequency between controls (n = 587) and myopathy patients (n = 150)(188). Moreover, utilizing a case-control study of SRM in 715 Caucasians, Luzum *et al*. were unable to replicate the protective role of GATM described by Mangravite et al(191). Other study was conducted in Japanese populations had similarly insignificant results(220). This study supports earlier studies by demonstrating that the GATM gene polymorphism (rs9806699) has no statistically significant impact on SRM (p=0.08). Despite the fact that our results did not meet the criteria for statistical significance most likely due to the multiple pathways that may affect atorvastatin pharmacokinetics and pharmacodynamics, we discovered that more patients with SRM had the A allele than the G allele. A statistically significant increase in the mean Creatine kinase was also seen in homozygous mutant (AA)

patients compared to wild-type (GG) or heterozygous (GA) patients, as shown in table (3.6), which summarizes the data. As a result, patients with the A allele may be at higher risk of SRM.

Statins, as previously stated, reduce GATM expression and hence creatine content in muscles(187). Several studies found that GATM deficiency was associated with a myopathy that was improved by oral creatine therapy. These findings suggest that a decrease in intramuscular creatine does not protect against myopathy but rather causes it. (221).

Furthermore, in ten patients with statin myalgia, over the counter creatine supplementation appeared to minimize statin-associated muscular symptoms(222). Since creatine serves as the ultimate acceptor of the phosphate group of ATP at the ending of mitochondrial oxidative phosphorylation, a statin-induced drop in the intracellular concentration of creatine may result in mitochondrial dysfunction. Reduced ATP production by cells would come next. As a result, cells treated with statins produce less ATP. In vitro, statin-induced apoptosis is halted by creatine supplementation through preventing the opening of the mitochondrial permeability transition pore(179).

The role of creatine in sustaining appropriate muscular function is also supported by the observation that muscles in mice deficient the enzyme GATM had atrophy and lower strength. These animals had essentially little creatine in their muscles and many metabolic anomalies for example, their inorganic phosphate/-ATP ratio was fourfold higher, indicating reduced phosphate consumption in ATP production. These mice's muscles also exhibited morphological changes, including lipid droplets and unusual crystal formations in the mitochondria, along with a 70% reduction in muscle volume. Functionally, mice had a more than 70% reduction in muscle strength and were hypotonic.

When creatine was added to the diet, the aforementioned abnormalities nearly returned to normal(223).

4.4 Conclusions

1. Glycine amidinotransferase gene was detected with different genotypes and variable frequencies in Iraqi patients that taking atorvastatin. The homozygous mutant type (AA) of GATM gene polymorphism rs9806699 G > A, C, T is more predominant than GG and GA genotype, with no allele frequency for C and T.
2. The GATM gene SNP that was detected in Iraqi patients that taking atorvastatin was noted to be non-significantly associated with statin-related myopathy but it cannot be ruled out as one of the factors that contribute to myopathy since we observed that the A allele was found in more SRM patients than the G allele, with a significant increase in the mean creatine kinase level.
3. Muscle adverse reactions as a result of treatment with atorvastatin were relatively common, and it's likely that they're far more common than was previously reported. Myopathy was found in 44% of Kerbala province patients taking atorvastatin and males are more likely to develop statin-related myopathy than females. Only 31.3% of patients reported muscle pain with or without creatine kinase (CK) elevation, while only 12.7% reported CK elevation without muscle pain. According to the findings, males are at a greater risk of developing statin-related myopathy than females.

4.5 Recommendations for Future Work

1. Additional clinical trials with a large number of patients should be conducted in the goal of providing more clear information on the role of GATM gene polymorphism and statin myopathy risk, as well as the benefit of creatine supplementation in statin myopathy.
2. Study of another GATM gene SNPs on atorvastatin-induced myopathy.
3. Study the impact of GATM gene polymorphism on occurrence of myopathy by another type of commonly used statins (e.g., rosuvastatin).
4. Investigate the genetic variations in drug transporters and metabolizing enzymes that may contribute to individual variations in statin related myopathy.

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Appendices

جامعة كربلاء
الموافقة على أن تكون مشاركاً في البحث

عنوان الدراسة: دور تعدد الأشكال الجيني GATM في حدوث اعتلال عضلي لدى عينة المرضى العراقيين المعالجين بالأتورفاستاتين في محافظة كربلاء

اسم الباحث: محمد شهيد ساجت

هذه دراسة بحثية طبية ، وليس عليك المشاركة فيها. سيشرح لك الباحث والدكتور حسن مرتضى الكتبي هذه الدراسة. إذا كان لديك أي أسئلة ، يمكنك أن تسألني أو تسأل الطبيب.
يُطلب منك المشاركة في هذه الدراسة لأنك كنت تتناول جرعة فموية يوميه 40 ملغ من عقار الأتورفاستاتين كعلاج وحيد لفرط شحميات الدم لمدة شهر على الأقل. في هذه الدراسة ، يقوم الباحث بجمع عينات دم منك لمعرفة المزيد حول ارتباط تعدد الأشكال الجيني لجين GATM بالاعتلال العضلي المرتبط بالستاتين.
إذا وافقت على المشاركة في هذه الدراسة ، فستذهب إلى المختبر وتعطي عينة دم لمرة واحدة فقط. سيتم سحب الدم عن طريق إدخال إبرة في الوريد الذي في ذراعك. سيتم أخذ أنبوب دم صغير و سيستغرق هذا حوالي خمس دقائق.

المخاطر؟
قد تؤلم عصا الإبرة وهناك خطر ضئيل للإصابة بكدمات وإغماء ، وخطر نادر للإصابة بالعدوى.

هل ستبقى معلوماتي الطبية سرية؟

سنبذل قصارى جهدنا لحماية المعلومات التي نجمعها منك ومن سجلك الطبي. ستبقى المعلومات التي تحدد هويتك آمنة ومقيدة. إذا تم نشر معلومات من هذا البحث أو تقديمها في اجتماعات علمية ، فلن يتم استخدام اسمك والمعرفات الأخرى. سيتم إتلاف المعلومات التي تحدد هويتك عند اكتمال هذا البحث و إعطاؤك نسخًا من نموذج الموافقة هذا للاحتفاظ بها.

الموافقة:

إذا كنت ترغب في أن تكون في هذه الدراسة ، يرجى التوقيع في الاسفل .

اسم المشترك:
التاريخ:
توقيع المشترك:

Questionnaire for Patients taking statin therapy

Demographic characterization

الاسم: رقم الهاتف:

الوزن: الطول:

Parameters	variable	Notes
Age		
Gender		
Smoking	Yes No	
BMI		
Education	Primary Secondary Collage	
Symptoms	Myalgia Weakness	
Duration of treatment of statin		
Other Drug side effects		
Other diseases		
Other medication		

الاستنتاجات: على الرغم من أن rs9806699 SNP لجين GATM لا يرتبط بالاعتلال العضلي المرتبط بالستاتين ، إلا أنه لا يمكن استبعاده بأعتباره واحداً من العوامل التي تساهم في اعتلال عضلي نظرًا للنسبة الملحوضه لتواجد الأليل نوع A في مرضى SRM أكثر من الأليل نوع G ، مع الزيادة الكبيرة في متوسط مستوى انزيم الكرياتين كيناز.

الخلاصة

المقدمة: تعد الستاتينات من الأدوية التي تمنع عمل إنزيم 3-هيدروكسي-3-ميثيل جلوتاريل- (كو-أي) المختزل ، مما يمنع تخليق الكوليسترول. حيث ان الستاتينات من الأدوية الخافضة للدهون الأكثر شيوعًا في العالم للوقاية من أمراض القلب والأوعية الدموية بشقيها الأولي والثانوي. لقد وجد ان الأعراض العضلية المرتبطة بالستاتين هي الآثار الجانبية الأكثر شيوعًا للعلاج بالعقاقير المخفضة للكوليسترول ، مما يجبر المرضى في التوقف عن تناولها. يشفر جين (GATM) إنزيم الميتوكوندريا غلايسين امدينو ترانسفيريز ، وهو الإنزيم المسؤول عن الخطوة الأولى في التخليق الحيوي للكرياتين. و يؤدي الأخير دوراً ضروريًا في وظيفة العضلات الطبيعية. لقد ثبت أن العقاقير المخفضة للكوليسترول تقلل من تعبير جين (GATM) وبالتالي محتوى الكرياتين في العضلات ، مما قد يساهم في الاعتلال العضلي للستاتين.

هدف الدراسة: هدفت الدراسة لمعرفة العلاقة بين تعدد الأشكال الجيني GATM rs9806699 G> A, C, T والاعتلال العضلي المرتبط بالستاتين (SRM) في المرضى الذين يتناولون عقار أتورفاستاتين 40 ملغ يومياً.

المرضى والأساليب: دراسة مقطعية مع إجمالي 150 مشاركًا ، من الذكور والإناث ، تتراوح أعمارهم من 28 إلى 65 عامًا ويتلقون جرعة فموية يوميه 40 ملغ من عقار الأتورفاستاتين لمدة شهر واحد على الأقل. أُوجريت الاختبارات البيوكيميائية لكل المشاركين لتحديد مستوى الدهون (الكوليسترول الكلي ، الدهون الثلاثية ، البروتين الدهني منخفض الكثافة والبروتين الدهني عالي الكثافة) ، الكرياتينين في الدم ، الكرياتين كيناز (CK) ومستويات الهرمون المنبه للغدة الدرقية, حيث أُستخدم تقنية تفاعل البوليميراز المتسلسل النوعي للأليل للكشف عن تعدد أشكال النوكليوتيدات المفردة GATM rs9806699 G> A, C, T.

النتائج: أضهرت الدراسة بأن توزيع الأنماط الجينية لـ GATM rs9806699 G> A, C, T كان بنسبة 13.3٪ للنمط الوراثي المتماثل الزيجة (GG) وبنسبة 38.0٪ للنمط الوراثي متغاير الزيجة (GA) وبنسبة 48.7٪ للنمط الوراثي متماثل الزيجة الطافر(AA) مع عدم وجود الأليلات C و T. على الرغم من الزيادة الكبيرة في متوسط انزيم الكرياتين كيناز في مرضى النمط الوراثي متماثل الزيجة الطافر(AA) مقارنةً بالمرضى من النوع (GG) أو (GA) ، لم يكن هنالك ارتباط كبير بين الاعتلال العضلي المرتبط بالستاتين و تعدد الأشكال الجيني . GATM rs9806699 G> A, C, T

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية الصيدلة
فرع الادويه والسموم

دور تعدد الاشكال الجيني (GATM) في حدوث الاعتلال العضلي لدى عينة من المرضى العراقيين المعالجين بالأتورفاستاتين في محافظة كربلاء

رسالة

مقدمة الى مجلس كلية الصيدلة / جامعة كربلاء

كجزء من متطلبات نيل درجة الماجستير في علم الادوية والسموم

بواسطة

محمد شهيد ساجت

بكالوريوس صيدلة (جامعة كربلاء – 2015)

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2023 ميلادي

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