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Fetuin -A- Gene Polymorphism (rs4918) As Predictor Marker for Type 2 Diabetic Patients

A Thesis
Submitted to the Council of College of Medicine, University of Kerbala, in Partial Fulfilment of the Requirements for the Degree of Master in Clinical chemistry

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بسم الله الرحمن الرحيم

(فَفَهمْنَاهَا سُلَيْمَانَ وَكُلاً اتَيْنَا حُكْماً

وَعِلماً وَسَخرْنَا مَعَ دَاوُودَ الْجِبَالَ

يُسَبحْنَ وَ الطيْرَ وَ كُنا فَاعِلِينَ)

الأنبياء 79

Supervisor Certification

We certify that this thesis was prepared under our supervision at the College of Medicine /University of Kerbala, as a partial fulfillment for the requirement for the degree of Master in Clinical Chemistry

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We the examining committee, certify that we have read this M. Sc thesis entitle; ***(Fetuin -A- Gene Polymorphism (rs4918) As Predictor Marker for Type 2 Diabetic Patient)*** *and we have examined the student* ***(Huusein Saad Mohammed Ali)*** *in its content, at our opinion it is adequate with "Excellent" as a thesis for the degree of Master in Clinical Chemistry.*

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Dedication

To whom Allah sent as mercy to the worlds ...

Prophet Mohammed

To my family and friends for their encouragement during the period of study ...

To my dear wife for her patience and encouragement during the period of study ...

To my daughter the beautiful flowers and Precious gift...

I Dedicate This Thesis with Love ...

Hussein Saad

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Hussein Saad

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Abbreviations	***Descriptions***
A	Absorbance
A_{Sample}	Absorption of sample
$A_{Standard}$	Absorption of standard
ADA	American Diabetic Association.
AHSG	α-2-heremans Schmid glycoprotein
BMI	Body Mass Index
bp	Base Pair
$C_{Standard}$	Concentration of standard
CAD	Coronary Artery Disease.
CC	Mutation genotype
CI 95%	Central Interval 95%
CM	Chylomicrons
C^o	Degree centigrade
CVD	Cardiovascular Disease.
dl	Deciliter
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
EPIC	The European Prospective Investigation into Cancer & Nutrition
FBS	Fasting Blood Sugar
FFAs	Free Fatty Acids
GC	heterozygous genotype
GG	Wild genotype
HbA1c	Glycosylated hemoglobin

HDL-c	High Density Lipoprotein
HWE	Hardy - Weinberg equilibrium
IDL-c	Intermediate Density Lipoprotein
IR	Insulin Resistance
Kg/m^2	Kilogram per square meter ^{2}
LDL-c	Low Density Lipoprotein
Lp	Lipoprotein
M ± SD	Mean ± Standard Deviation
MGB	Minor Groove Binding
MI	Myocardial Infarction
NCBI	National Center for Biotechnology Information
NFW	Nuclease free water
nm	NanoMeter
OR	Odds Ration
P value	Probability value
PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR
R	Ratio
SNP	Single Nucleotide Polymorphism
T2DM	Type 2 diabetic mellitus.
TC	Total Cholesterol
TG	Triglyceride
TLR4	Toll-like receptor 4
VLDL-c	Very Low Density lipoprotein
WHO	World Health Organization.
µL	Micro Liter

Abstract

Background; Diabetes Mellitus (DM) remains the first killer and a common silent disease in the world. Many environmental and genetic risk factor are prominent mechanism in the pathogenesis of DM. In the last years, DM is increasing and accounts for a high proportion among other diseases in Iraq. Many studies have shown that a single nucleotide polymorphism (SNP) within α-2 heremans scemid glycoprotein (AHSG) rs4918 at 766 (C/G) are associated with DM and this SNP can cause variation in the fetuin -A- function.

Aim; to study the association of AHSG rs4918 at 766 (C/G) gene polymorphism with DM in Kerbala population.

Materials and Methods; This case-control study comprised from 200 subjects about 100 of them are with type 2 diabetes mellitus (T2DM) as a cases and 100 of them are apparently healthy as control groups. Phenotypic data included BMI, level of fasting blood sugar (FBS), Glycosylated hemoglobin (HbA1c), and lipid profile. Detection of AHSG (rs4918) polymorphism was carried out by TaqMan polymerase chain reaction. DNA was extracted from whole blood and genotyping was achieved with specific primers and probes designed to AHSG gene by ALPHA company. Various statistical analyses were applied to analyze the research data. While the genotype and allele frequencies were examined under the co-dominant, dominant and recessive models with the use of multinomial logistic regression analysis.

Result; The distribution of the genotype of the SNP (rs4918) was found to be consistent with Hardy–Weinberg equilibrium, patients with the heterozygous genotype (GC) were evident to be significantly (chi-square (X^2)= 9.78, probability value (P) =0.001, odds Ratio (OR) = 2.58, Confidence Interval (CI) 95%= 1.41 – 4.71,) and homozygous genotype (CC) were evident to be not significantly (X^2= 1.95, P value =0.162, OR= 1.99, CI 95%=0.74 – 5.28). The

major allele frequency (G) was (0.74) in DM patient and (0.62) in control group, while the minor allele frequency (C) was (0.26) in DM patient and (0.38) in control group. The co-dominant model in patients with GC were evident to be significantly (OR = 0.38, CI 95%= 0.21 – 0.7, P value =0.001) and CC were evident to be not significantly (OR= 0.5, CI 95%=0.18 - 1.33, P =0.167) higher than those of the control. Under the dominant model, the present study were found that DM patients with the (CC+GC) genotypes have significant difference (OR =0.4, CI 95% = 0.22 – 0.71, P = 0.001) higher than those of the control group. Major allele frequency (C) was not significantly (P= 0.08) higher in patient with DM versus control group. The biochemical parameters of the DM patients was examine under the co-dominant, and the FBS, HDL/LDH, and HbA1c were highly significant (p value < 0.001) with the GG, GC and CC genotypes, while the HDL-C with GG and GC genotype, the LDL-C with GC genotype, the VLDL-C and TG with GG genotype. As the HDL-C and LDL-C was significant (P value 0.001) with the CC genotype, while the LDL-C was significant (P value = 0.037) with the GG genotype, the VLDL-C and TG was (p value = 0.004) with the GC genotype, the non-HDL (p value = 0.028) with the CC genotype all parameter and genotype was comparing between the DM patients and the control group.

***Conclusion*:** The AHSG gene polymorphism (rs4918) is significantly association with DM. In addition this SNP plays a role in FBS, HbA1c, and lipid profile changes of the diabetic patients, as there significant association between them.

Introduction

People with type 2 diabetes mellitus (T2DM) have a higher cardiovascular morbidity and mortality risk, and are disproportionately affected by cardiovascular disease (CVD) compared with non-diabetic subjects (American Diabetic Association (ADA) 2014).

Fetuin -A- is a negative acute phase glycoprotein that synthesis and secreted from the liver (Li M, *et al.,* 2010). It have effect on insulin receptor, where fetuin -A- binds reversibly to the insulin receptor tyrosine kinase in peripheral tissues and inhibit the insulin induced intracellular signal cascade (Fatima, *et al*., 2013),

The changes in the fetuin -A- level may influences the body defense mechanisms such as inflammatory regulation. The reduction of the fetuin -A- level are linked to many disease such as T2DM (Stefan *et al*, 2014), fatty liver (Stefan and Haring, 2013), adipocyte dysfunction (Dasgupta *et al*, 2010), obesity (Reinehr and Roth, 2008), and metabolic syndrome (Ix *et al*, 2006). Ix JH *et al.*, (2012) has reported that higher level of fetuin-A was associated with higher triglycerides, low density lipoproteins (LDL-C), body mass index (BMI), and insulin resistance (IR).

Fetuin -A- is encoded by α2- Heremans-Schmid glycoprotein (AHSG) gene, which located on chromosome 3q27 (Rizzu and Baldini, 1995). The most common single polymorphism nucleated (SPN) is (rs4918) at 766 (C/G) in exon7, which is a susceptible locus for metabolic syndrome specially the diabetes mellitus (Shanshan, *et al* 2013).

Literature Review

1. Diabetes mellitus

Diabetes mellitus (DM) is a major health problem that is growing rapidly worldwide, DM may be caused by decrease the level or action of insulin, where the Insulin is effected by abnormal carbohydrate, fat and protein metabolism caused by liver abnormalities which lead to hyperglycemia (Boles *et al.*. 2017).

1.1. Diagnosis of diabetes mellitus;

The criteria of the World Health Organization (WHO) to diagnosis the diabetic patient is when the Glycosylated hemoglobin (HbA1c) > 6.5%, fasting plasma glucose (FPG) (110-140 mg/dL)**,** random blood glucose (RBG) ≥ 200 mg/dL, Two-hour plasma glucose ≥ 200 mg/dL after a 75 g oral glucose tolerance test (OGTT) (ADA 2018)

1.2. Epidemiology of diabetes mellitus;

Diabetes is a large global health, and it may lead to death in many countries especially if it's not diagnosis, which may be a 30-80% are undiagnosed. Number of people with diabetes (20-79 years) in 2017 (415 million), in 2045 (642 million) (International Diabetes Federation, 2015) as shown in figure (1- 1). The diabetes prevalence has rising more rapidly in middle and low income countries (WHO, 2016).

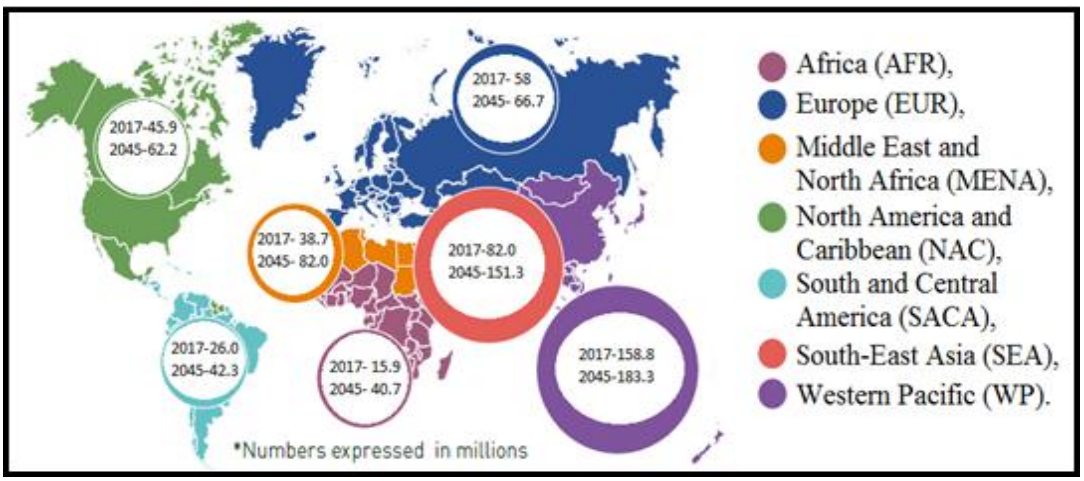

Figure (1.1): Prevalence of diabetes in world (International Diabetes Federation, 2015).

1.3. Classification

The widely accepted classification of diabetes is type 1 diabetes (T1DM), type 2 diabetes (T2DM), and gestational diabetes (GDM). Monogenic diabetes (ADA 2018).

1.3.1. Type 1 diabetes (TIDM)

It is caused by an autoimmune reaction where the body's immune system attacks the insulin-producing β- cells in the islets of the pancreas gland. As a result, the body produces none to very little insulin with a relative or absolute deficiency of insulin. It's may be due to a genetic susceptibility or environmental triggers as viral infection or toxins (Haffner *et al*, 1998). It's mostly occur in the children. The patient need the daily insulin treatment and a healthy diet and lifestyle otherwise they cannot control the hyperglycemia. Figure (1.2) show the symptom of TIDM;

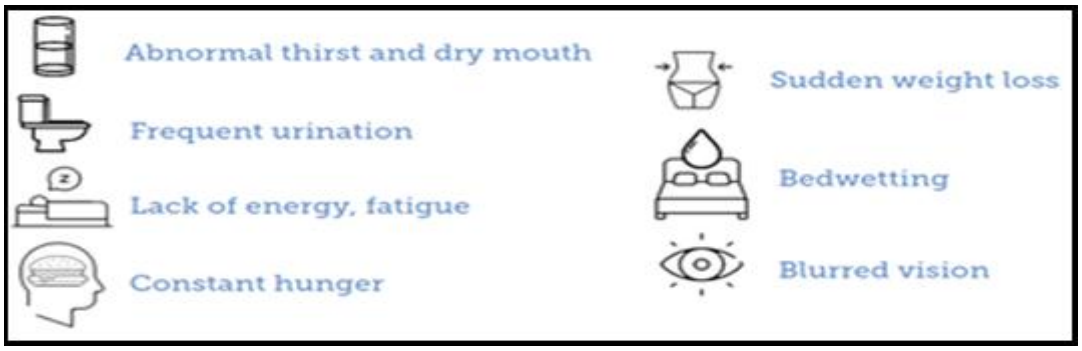

Figure (1.2); the symptom of TIDM (IDF, 2017a).

1.3.2. Type 2 diabetes (T2DM)

It is the most common type of diabetes (90% of diabetes cases) (Holman *et al*, 2015). The hyperglycemia is caused by genetic or environmental, such as; obesity, physical inactivity, poor diet, increasing age as well as ethnicity and family history (Imamura *et al.*, 2015), which effect the β-cell of pancreases and lead to decreases the level of insulin secretion or secrete inefficient insulin (DeFronzo *et al*., 2015). Figure (1.3) show the symptom of T2DM;

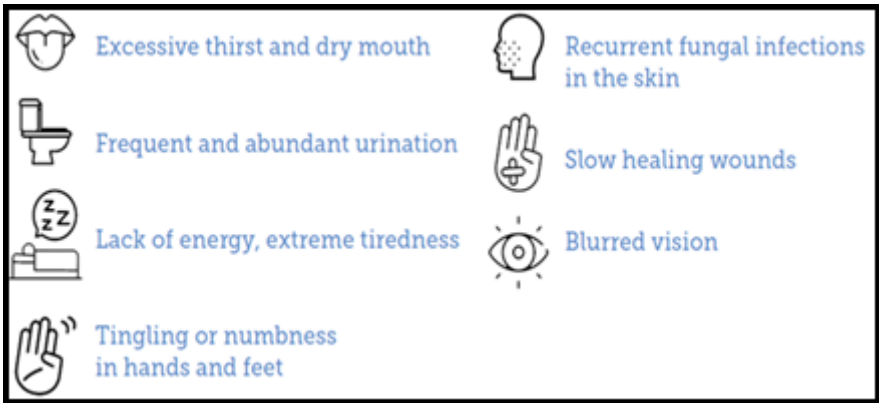

Figure (1.3); the symptom of T2DM. (IDF, 2017b).

Insulin resistance (IR) is a state of insufficient number and inability of the body to respond fully to insulin, result from inhibition of the insulin signaling pathway. Therefore, initially prompts the β- islets to increases the insulin production to reduce the hyperglycemia but over time a state of relative inadequate production of insulin can develop and lead to IR (Holman *et al.*, 2015).

The characteristic of the IR were hyperinsulinemia and hyperglycemia in the fasting state, increased HbA1c, hyperlipidemia, impaired glucose tolerance, impaired insulin tolerance, and increased inflammatory markers in plasma. The primary function of the insulin is reducing blood glucose. Other function is; stimulates the synthesis of fatty acids and glycogen, promotes mitochondrial function, improves microcirculation and induces cell proliferation (He Q *et al*, 2011).

In the general population the obesity and aging is consider a major risk factor for the IR (where it is the earlier metabolic abnormality that detected in those who susceptible to develop T2DM (Reaven, 2011). But the gene background is a key factor where it was supported by many lines of evidence, first the IR patients are often associated with the family history of T2DM and second the high risk of IR in certain ethnic population as shown in epidemiology study (Jianping, 2013).

The primary target of insulin action is the liver (where it plays a major role in the substrate metabolism). After elevated of the glucose the insulin is released from the β cell and transported via the portal vein to the liver directly and bind to the insulin receptor and lead to glycogenesis inhibition loss of inhibition of gluconeogenesis in addition to availability of substrates alanine and lactate from muscle, glycerol from adipose tissue and ATP from β-oxidation leads to exacerbation of gluconeogenesis causes more hyperglycemia (Varman *et al*, 2004).

1.4. Risk Factor of Diabetes Mellitus

There are many environmental and genetic factor such as;

Family history (first degree relative with T2DM), obesity, body mass index (BMI) ≥ 25 kg/m^2, age > 45 years, ethnicity (race), previous gestational diabetes or delive a baby with > 9 pounds, pre-diabetic, hypertension, and abnormal cholesterol level (HDL-c < 35mg/dL, TG > 250 mg/dL) (Valliyto *et al*., 2014).

1.5. Pathogenesis of type 2 Diabetes mellitus

The pathogenesis of T2DM is a complex of metabolic and endocrine disorder resulting from the interaction between genetic and environmental factors, shown in figure (1. 4) which cause different degrees of alteration in insulin functionality on peripheral tissues, as well as in the pancreatic β cell. The main factor that develop T2DM are excess body weight and obesity (Taylor 2013), particularly of the android type [abdominal obesity, describes the distribution of human adipose tissue mainly around the trunk and upper body, in areas such as the abdomen, chest, shoulder and nape of the neck and its more common in males than females (Thornhill, 2008), and its diagnosed by the waist circumference (≥ 80 cm)] (Alberti *et al.*, 2005).

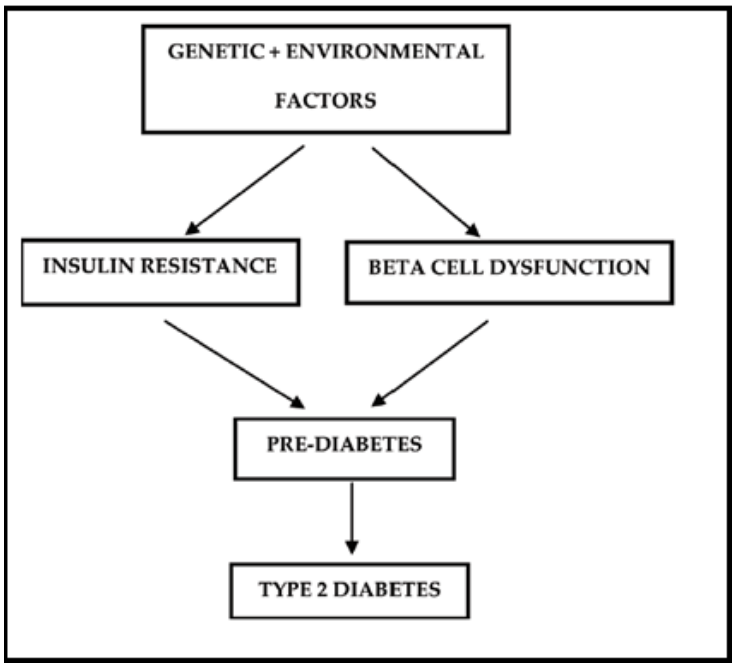

Figure (1. 4); Stages in pathogenesis of type 2 diabetes (Durruty, Pérez-Bravo (2014).

1.6. Diabetes complications

The persistently high blood glucose levels causes generalized vascular damage affecting the heart (causes cardiovascular disease (CVD)), (causes blindness), kidneys (causes kidney failure), neuropathy, nephropathy (leading to retinopathy) and nerves. Generally diabetes complications can be divided into two group as shown in table (1.1); (Papatheodorou *et al*, 2018)

Table (1.1) diabetic complications (Papatheodorou *et al*, 2018)

Acute complications	Chronic complications	
Include hypoglycaemia, diabetic ketoacidosis (DKA), hyperglycaemic hyperosmolar state (HHS), hyrglycaemic diabetic coma, seizures or loss of consciousness and infections.	**Chronic microvascular** include nephropathy, neuropathy and retinopathy	**Chronic macrovascular** Include CAD leading to angina or myocardial infarction (MI), peripheral artery disease (PAD) contributing to stroke, diabetic encephalopathy and diabetic foot.

1.7. Fetuin -A- Protein ;

The human homologue of fetuin A has been called α2- Heremans-Schmid glycoprotein (AHSG). It was first identified in foetal bovine serum, it is a negative acute-phase glycoprotein (Ombrellino *et al*., 2001), and its synthesized and secreted from the liver (Li, *et al.,* 2010). Thus it's found to be related to; T2DM (Li, *et al.*, 2010), the accumulation of fat in the liver (Stefan *et al.*, 2013) and IR (Jung *et al.*, 2013).

As Fetuin -A- is a negative acute phase protein but it may enhances the vascular calcification and may lead to plaque formation (Zwaka *et al*, 2001) and accumulated in it and in the pathologically mineralized tissues, this is due to its high affinity for calcium phosphates (Mori *et al*, 2006). Fetuin-A has been recognized as a potent calcification inhibitor (Shroff *et al*, 2013).

1.7.1 Structure of the Fetuin -A- Protein ;

Chemical structure**;** It is a 367 amino acids (18 amino acid signal peptide), 51–67 kDa glycoprotein (Stefan *et al*, 2008). It is built up from an A-chain (282 amino acids) and B chain (27 amino acids) with a linker sequence (40 amino acids). Originating from the liver, the protein is found at plasma levels of 0.3–0.6 mg/mL (Haglund, 2001).

Fetuin-A belongs to the cystatin super family, a cluster of cysteine protease inhibitors (Ishibashi *et al*. 2010). It possesses two amino-terminal cystatin-like domains designated as D1 and D2 (A chain), and a carboxyl-terminal domain designated D3 (B chain), which lacks sequence similarity to cystatins Fig. (1.5) (Elzanowski *et al*, 1988). Because most of the cystatin-like domains act as inhibitors of cysteine proteases, D1 and D2 were expected to inhibit papain like peptidases or cysteine cathepsin.

However, purified fetuin-A failed to inhibit these enzymes, suggesting that during evolution, the D1 and D2 domains of fetuin-A lost their ability to inhibit cysteine proteases (Brown *et al*, 1992a). This conclusion is supported by the lack of a protease-active site sequence (Gln-Val-Val-Ala-Gly) in fetuin-A (Gln-Gln-Pro-Ser-Gly) (Dziegielewska and Brown, 1995)

A prominent biochemical feature of fetuin-A is its high affinity for apatite (the most common phosphate mineral with typical natural occurrence as $Ca_5(PO_4)_3[F,Cl,OH]$) (Spikings *et al*., 2015). On the basis of sequence analysis, the EF-hand [its a Ca_2^+-binding proteins or "buffers" may play a significant role in Ca_2 homeostasis and signaling. These proteins are characterized by a common sequence of ≈ 30 residues forming a helix–loop–helix motif, which, when bound to Ca_2^+ ,causes a conformational change, allowing for interaction with downstream proteins (Lewit-Bentley and Rety 2000)] motifs within D1 have been proposed (Brown *et al*, 1992b) to serve as calcium-binding sites. Recent structure-based analysis suggests that a dense array of acidic residues (Asp or Glu) on an extended β -sheet in D1 seems to be responsible for binding of fetuin-A to hydroxyapatite Fig. (1.5) (Heiss *et al*, 2003). The binding site of fetuin-A for transforming growth factor- β (TGF-β) superfamily members is also located within D1 Fig. (1.5) (Katsuhito *et al*, 2011).

Biosynthesis of fetuin-A involves posttranslational modifications. Thus, proteolytic cleavage yields an N-terminal heavy chain (321 amino acid residues) and a C-terminal light chain (27 amino acid residues) covalently bound to each other by interchain disulfide bonds Fig. (1.5) (Jahnen *et al*, 1994). Proteolytic processing rarely produces an A chain lacking 40 amino acid residues from the C-terminus of the heavy chain and a B chain that is identical to the light chain Fig. (1.5) (Lee *et al*, 1987).

The missing C-terminal stretch of the heavy chain, the socalled "connecting peptide," seems to occur in pathological conditions such as sepsis (Nawratil *et al*, 1996). Among the six disulfide bridges within fetuin-A, the first and last half-cysteine residues are engaged in connecting the heavy (A) and light (B) chains Fig. (1.5) (Kellermann *et al*, 1989).

Fetuin-A expressed by a human hepatoma cell line (HepG2) was demonstrated to be modified by phosphorylation on multiple serine residues (Jahnen *et al*, 1994). In addition, Asn residues 156 and 176 have been identified as sites of N-linked glycosylation, and Thr256 (766 (C/G), Thr270, and Ser346 have been identified as three O-linked glycosylation sites Fig. (1.5) (Yoshioka *et al*, 1986).

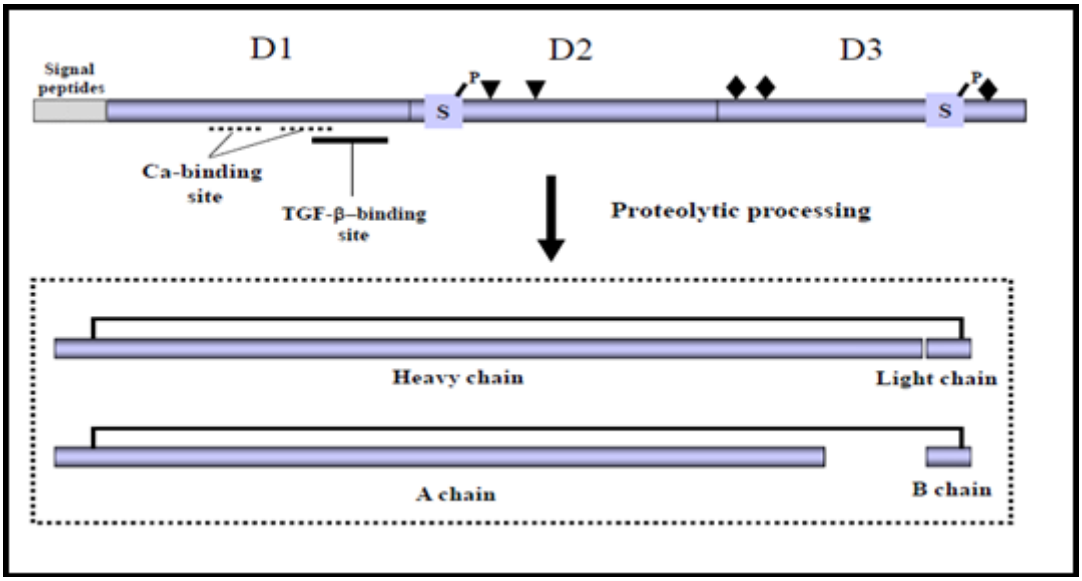

Fig. (1.5); Protein structure and posttranslational modification of fetuin-A. S: Phosphorylated Ser sites,▼: N-Linked glycosylation sites, ◊; O-Linked glycosylation sites (Katsuhito *et al*, 2011).

1.7.2 Fetuin A Function;

Fetuin -A- can be served as an important biomarker for numerous physiological and pathological processes (Marechal, 2011),

Role of Fetuin- A- in inhibition of insulin receptors tyrosine kinase;

Fetuin- A reversibly binds to insulin receptors tyrosine kinase in peripheral tissues (liver and muscles) thereby inhibiting the insulin induced intracellular signal cascade, which is shown in figure (1.6) (Fatima *et al*., 2013).

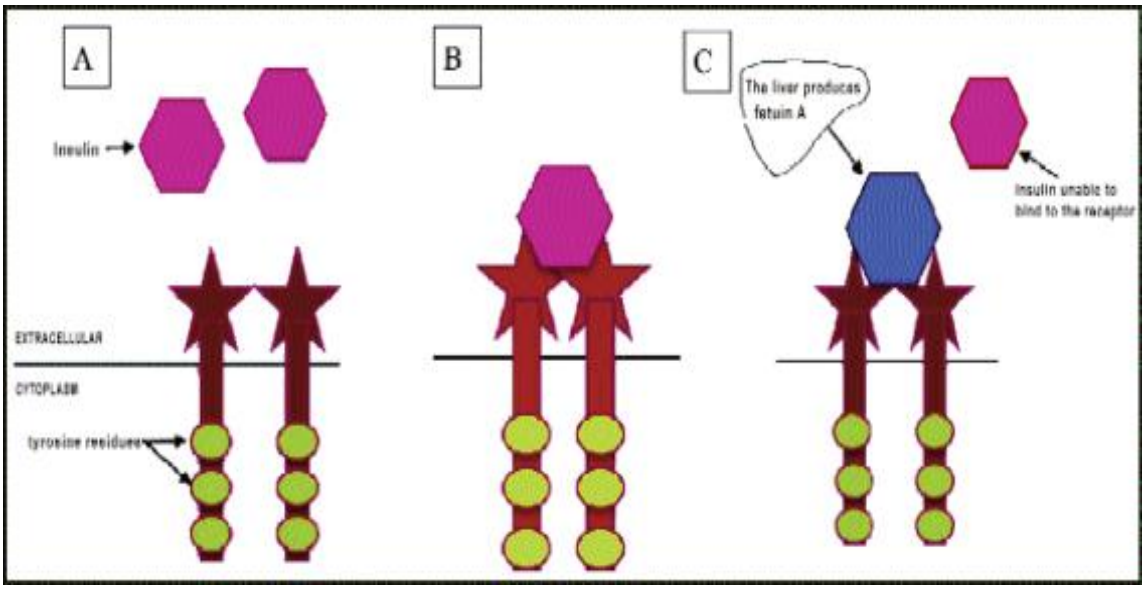

Figure (1.6): Schematic diagram showing insulin and it's inactivated receptor**, (A)** insulin binding to its receptor, which works through tyrosine kinase to activate it **(B)** and Fetuin-A binding to insulin's receptor, which blocks binding of insulin to its receptors**, (C)** contributing to insulin resistance (Fatima *et al.,* 2013).

Fetuin -A- and it association with lipid profile;

Kotronen and Yki-Jarvinen (2008) showed that fetuin-A levels were negatively correlated with high density lipoprotein (HDL-C). Khalil and Kuobaili (2013) reported that elevated serum fetuin-A levels found in T2DM patients were significantly associated with atherogenic dyslipidemia, thus indicating that fetuin-A- may be one of the contributing factors to the increased incidence of coronary heart disease (CHD) in T2DM patients. Ix *et al,* (2012) reported that higher level of fetuin-A was associated with higher triglycerides, and LDL-C.

The Role of Fetuin-A in Insulin Resistance, and inflammation;

Fetuin -A- is known as a major carrier protein of free fatty acids (FFAs) in the circulation via the nuclear factor kB (NFkB) (Dasgupta *et al.*, 2010). The α 2-Heremans - Schmid glycoprotein (AHSG) molecule binds to FFA By binding to the Toll-like receptor 4 (TLR4), the FFA-AHSG complex generates inflammatory signals and IR (Pal *et al.*, 2012), the serum AHSG and FFA interact with each other in predicting insulin sensitivity (Stefan *et al*, 2013) as shown in figure (1.7).

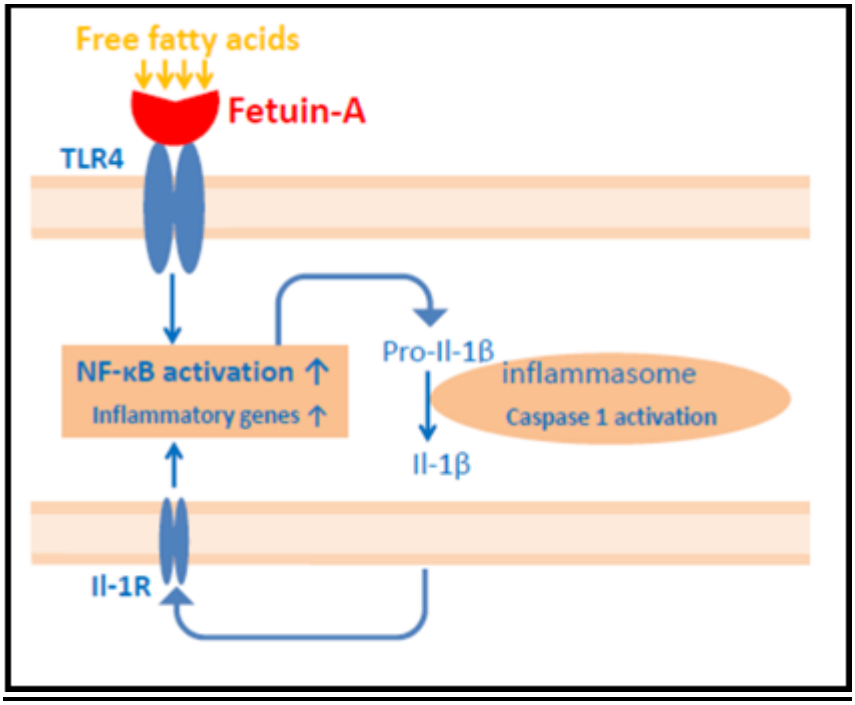

Figure 1.7: Model for Fetuin-A linking FFAs to TLR4 signaling**.** (Spanien, *et al*, 2017).

Role of fetuin -A- in non-alcoholic fatty liver disease (NAFLD), and Obesity;

Obesity, particularly that involving visceral fat, based on high caloric intake and lack of physical activity, leads to increased fatty acid and pro-inflammatory cytokine levels and decreased adiponectin in the bloodstream. Previously was reported that fatty acids directly enhance fetuin-A secretion by the liver (Dasgupta *et al.*, 2010). In addition, decreased adiponectin can induce fat accumulation in the liver (Roden, 2019), resulting in increased fetuin-A secretion.

Stefan *et al* (2013), has reported the role of fetuin -A- in fatty liver. Increased fetuin-A causes insulin resistance in skeletal muscle and the liver. At the same time, Dasgupta *et al.*, (2010), has reported that an increased in fetuin-A promotes the adipocyte dysfunction by decreasing the levels of adiponectin while increasing those of fatty acids and inflammatory cytokines. Ix *et al.*, (2012) has reported that higher level of fetuin-A was associated with higher triglycerides, LDL-C, BMI, and IR. Reinehr and Roth (2008) has reported the role of fetuin -A- in developing obesity.

Basar *et al*. (2011) stated that Fetuin-A is an anti-inflammatory mediator that participates in macrophage deactivation. Fetuin -A enhances the cellular uptake of cationic inhibitors of proinflammatory cytokine synthesis by macrophages and hence it prevents the morbid sequelae of infection and inflammation that would result from overproduction of pro-inflammatory cytokines. This event mainly result from an interleukin-1β (IL-1β) induced down-regulation of its hepatic mRNA level (Christophe *et al*, 2003). The role of fetuin -A- in insulin resistance, nonalcoholic liver disease, and obesity are shown in figure (1. 8) (Katsuhito *et al.*, 2011).

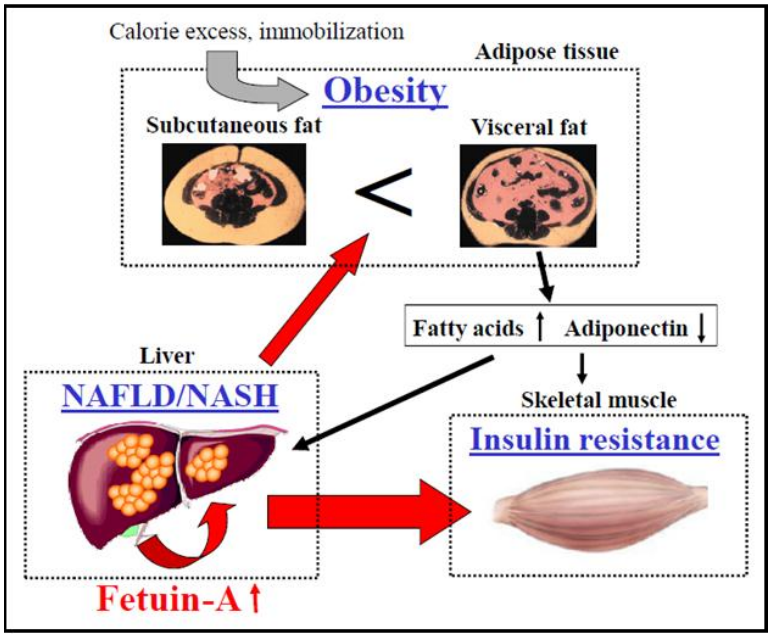

Fig. (1. 8). The role of fetuin-A in insulin resistance, nonalcoholic liver disease, and obesity (Katsuhito *et al.*, 2011) (NASH nan-alcoholic steatohepatitis).

Fetuin -A- role in protection against ectopic calcifation;

Fetuin -A- protect against ectopic calcification (Zhou *et al*, 2016) through formation of a fetuin-mineral complex (FMC) (Hamano *et al*, 2010) due to the physiological phosphorylation of fetuin -A- at Ser138 and Ser330 Fig. (1.5) (Haglund, 2001, Katsuhito *et al*, 2011).

Fetuin-A can form complexes with calcium and phosphorous in the circulation and prevents the sedimentation of these minerals in serum (Shidfar *et al*, 2014). Therefore, Fetuin-A by binding to calcium ion inhibits ectopic calcium deposition and protects vascular calcification (Yin *et al*, 2014).

Effect of changing fetuin -A- level on disease:

As the source of fetuin A is the liver also for it mainly secreted from liver, thus it's effected by fat accumulation in the liver and CVD and other diseases. Fisher *et al* (2009) and Weikert *et al*. (2008) has showed that high plasma fetuin-A levels were associated with myocardial infarction and ischemic stroke, suggesting that fetuin-A may influence the pathophysiology of cardiovascular disease. Tuttolomondo *et al*. (2010), whose findings supported the conclusions of Weikert *et al.* (2008), which proposed fetuin-A as a candidate proinflammatory marker of ischemic stroke. Voros *et al*. (2011) reported elevated serum fetuin-A levels among 171 Hungarian patients with previous myocardial infarction and normal renal function.

Also its level influence the body's defense mechanisms such as inflammatory regulation for macrophage deactivation, and a decreased serum levels predict mortality of patients with liver cirrhosis (Kalabay *et al*, 2007) and end-stage renal disease (Ketteler *et al*, 2003), causes a several negative growth effects (Brylka and Jahnen, 2013).

On the other hand, the elevation level of Fetuin -A- has linked to many disease such as obesity (Reinehr and Roth, 2008) , T2DM (Stefan *et al*, 2014), metabolic syndrome (Ix *et al*, 2006), adipocyte dysfunction (Dasgupta *et al*, 2010) and fatty liver (Stefan and Haring, 2013). The variation of fetuin -A- level effect are shown in figure (1. 9).

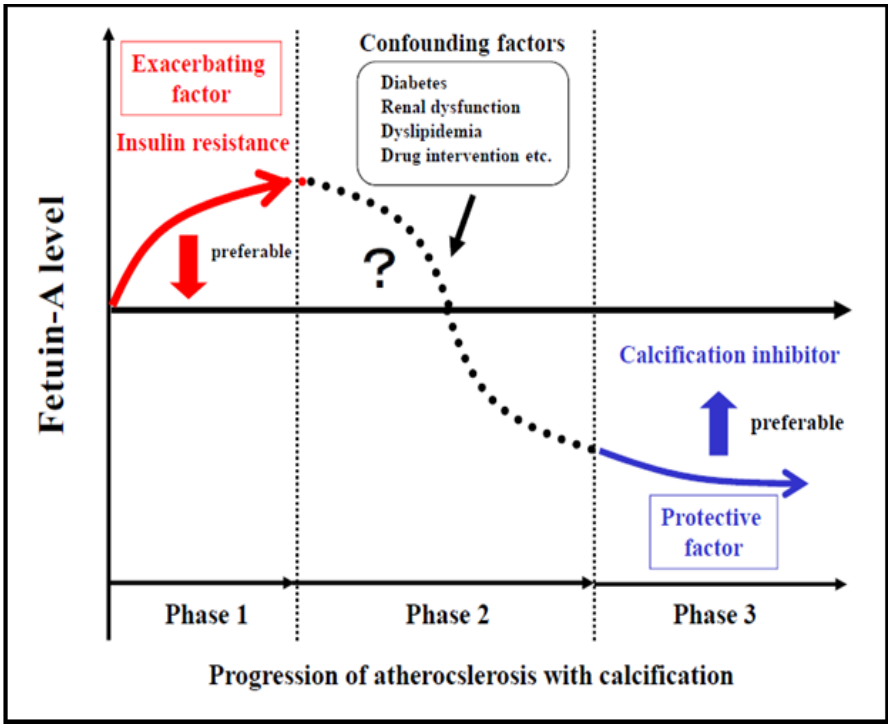

Figure (1. 9); The variation of fetuin -A- effect on some disease. (Rose *et al.*, 2009)

1.8. The alpha 2- Heremans-Schmid glycoprotein (AHSG) gene

1.8.1. The genomic Structure of AHSG gene;

Genomic structure; It is resides on chromosome 3 (3q27) (Rizzu and Baldini, 1995) and contains seven exons and six introns, shown in figure (1.10) (Maréchal *et al.*, 2011), spanning an approximately 8.2- Kilo base (Kb) region (Osawa *et al*, 1997). A single mRNA transcript encodes the single-chain of fetuin-A precursor (Lee *et al.*,, 1987). The transcriptional activity of the fetuin-A gene is mediated by several CCAAT enhancer-binding proteins (C/EBP)-β and NF-1-binding sites in its promoter region (Banine *et al*, 2000).

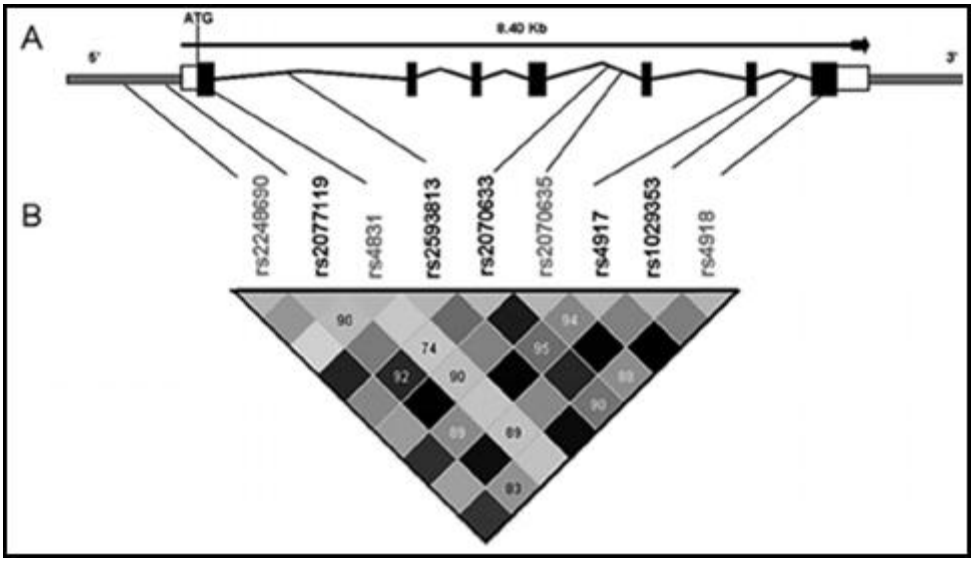

Figure (1.10); The *AHSG* gene Structure, polymorphisms, and haplotype analysis. **(A)** Structure of *AHSG* gene, with black boxes representing exons 1 to 7 and shaded regions representing the 5'- and 3'-untranslated regions, and its indicated the genomic location of the -9- single nucleotide polymorphism (SNPs) covering the gene. **(B)** Linkage disequilibrium between the nine SNPs covering *AHSG* and selection of the four SNPs capturing the common genetic variation (rs2248690, rs4831, rs2070635, and rs4918) (Maréchal, *et al.*, 2011).

1.8.2 Genomic Location of AHSG Gene;

The AHSG gene (rs4918) is located at chromosome 3q27 and exon 7, which is a susceptibility locus for T2DM and the metabolic syndrome (Shanshan, *et al* 2013) shown in figure (1.11) and encodes a protein that is present both in tissue and circulation, and mainly secreted by the parenchymal cell (Li M, *et al.*, 2010).

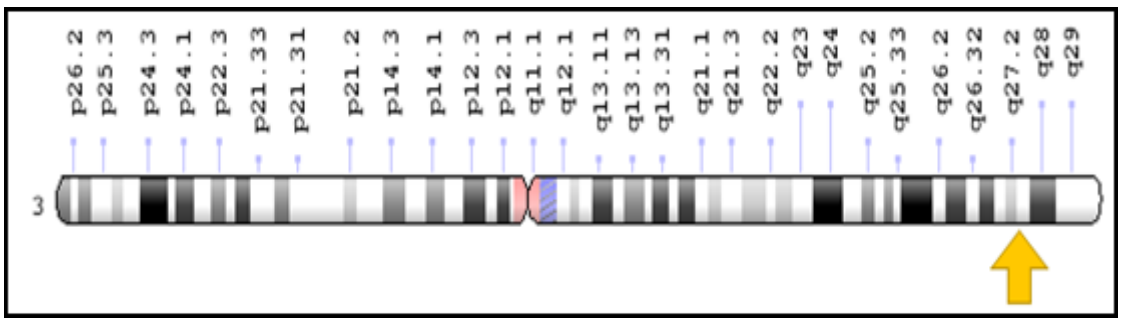

Figure (1. 11); genomic location of AHSG. (https://ghr.nlm.nih.gov)

1.8.3. Fetuin A Gene Polymorphism (rs4918);

The rs4918 is found at position 766 of Exon 7. The most common polymorphism recorded for this area is the G (Ser) : C (Thr) substitution. This SNP may be associated with maintaining good BMI, body fat and controlling insulin sensitivity. Furthermore, it shows a protective effect against diet induced obesity (Catharina *et al*, 2005), diabetes and metabolic syndrome (Siddiq *et al.*, 2005), and is therefore called the 'lean gene'.

For many years, association studies in a population group showed that this polymorphism is related to various physical statures such as bone mineral density (Sritara *et al*., 2014) and other major diseases such as lipid levels (Temesszentandrasi *et al*., 2015), and ischemic heart disease (Ma *et al*., 2013).

Shanshan, *et al*., (2013) was the first study which provided the evidence for an independent association between AHSG gene rs4918 and ischemic stroke in China, their finding showed that the GG genotype and G allele frequencies of AHSG gene (rs4918) in ischemic stroke patients were significantly higher than those in unrelated healthy controls.

Marion Verduijn *et al*, (2010) has reported that the AHSG gene polymorphism at 766 (C/G) was weakly associated with the mortality of dialysis patients, and no causative effect of fetuin- A levels on this outcome was observed. The fetuin -A- might be lowered by inflammation and diabetes.

Healthy individuals with the G allele have been found to have lower inflammatory cytokines such as the tumor necrosis factor alpha (TNFa) and adiponectin levels while higher levels of leptin are found (Temesszentandrasi *et al*, 2016). Even in patients with a history of myocardial infarction (MI), the presence of rs4918 G allele renders better anthropometric parameters such as BMI and waist circumference (Temesszentandrasi *et al*, 2016). On the contrary, the allele also predisposes carriers like those with a single G allele to certain conditions including IR. The nucleotides sequence shown in the figure (1.12)

Figure (1.12); the nucleotides sequence of the rs4918.

(https://www.ncbi.nlm.nih.gov)

1.9. Biochemical Marker;

1.9.1. Lipid profile

Lipoproteins Cholesterol and triglycerides are insoluble in water and therefore these lipids must be transported in association with proteins (Feingold and Grunfeld, 2018), which is the lipoproteins, which are a complex particles where the central core consist from cholesterol esters and triglycerides and where surrounded by free cholesterol, phospholipids and apolipoproteins, they facilitate lipoprotein formation and function. The lipoproteins can be divided into five classes based on size lipid composition and apolipoproteins (chylomicrons, very low density lipoprotein (VLDL-C), intermediate density lipoprotein (IDL-C), LDL-C {they all consider pro- atherogenic} and HDL {which is consider anti-atherogenic}) (Feingold and Grunfeld, 2018). The Apolipoproteins have four major functions including (Feingold and Grunfeld, 2018);

- Serving a structural role.
- Acting as ligands for lipoprotein receptors.
- Guiding the formation of lipoproteins.
- Serving as activators or inhibitors of enzymes involved in the metabolism of lipoproteins.

The lipoprotein have two pathway, the exogenous pathway which is start from the dietary lipid intake and convert it into chylomicrons on the intestine, which carried the triglycerides into the adipose tissue and by the lipoprotein lipase release the free fatty acid, which are subsequently metabolized by muscle and adipose tissue and chylomicron remnants are formed, then they are taken up by the liver (Miller *et al*., 2011).

To begins the endogenous lipoprotein pathway, which start in the liver by formation of the VLDL-C, which carry the triglycerides into the muscle and adipose tissue then release its free fatty by the lipoprotein lipase and then the IDL-C are formed. Then its further metabolized into LDL-C, which are bind to the LDL-C receptor in many tissue such as; liver.

Then a revers cholesterol transporting begins with formation of nascent HDL-C by the liver and intestine (Conroy *et al*., 2018). These lipoproteins play an important role in the absorption and transport of dietary lipids intake by the small intestine in the transport the lipid from the liver to the peripheral tissues and/or transport it back (reverse cholesterol transport). A secondary function is to transport toxic foreign hydrophobic and amphipathic compounds, such as bacterial endotoxin, from areas of invasion and infection (Yassine *et al*., 2015). The whole metabolic pathway of the lipids (exogenous and endogenous pathway are shown in figure (1. 13) (Karam *et al*, 2017).

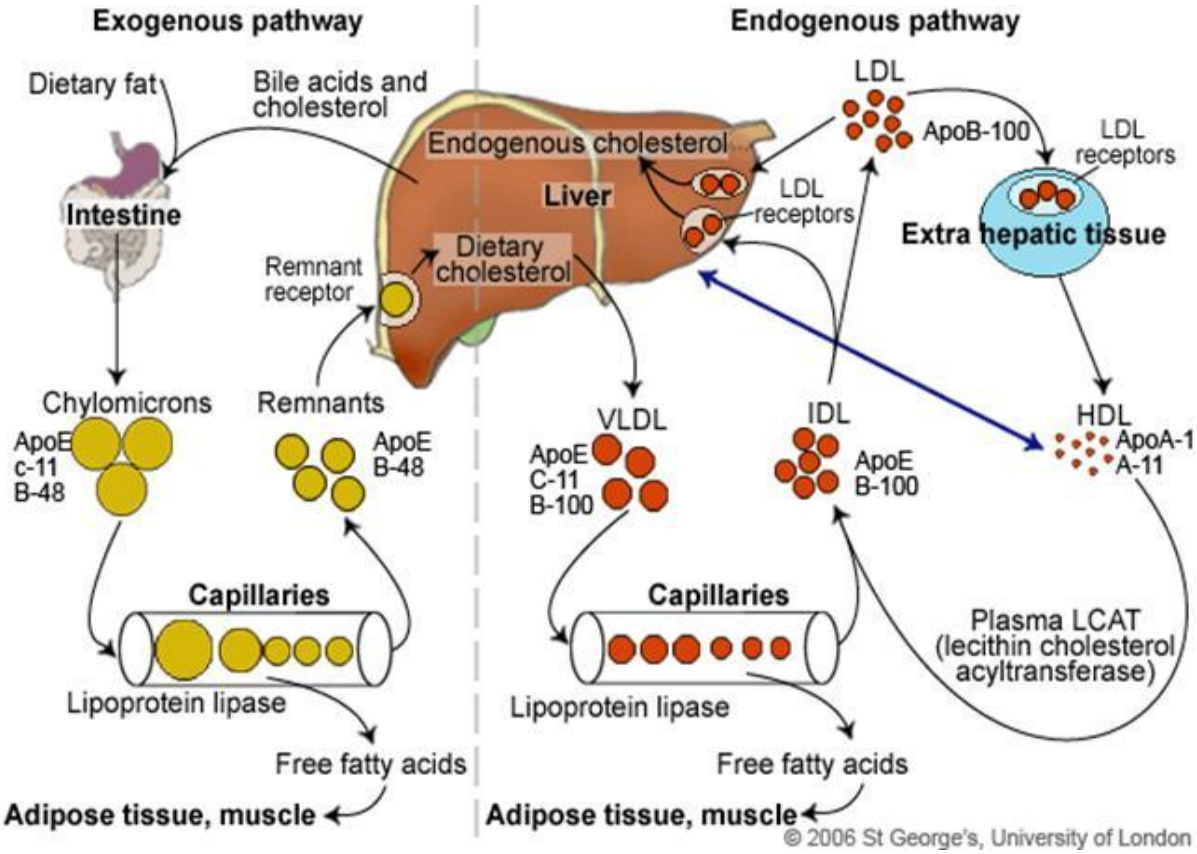

Figure (1. 13); The whole metabolic pathway of the lipids (exogenous and endogenous pathway) (Karam *et al*, 2017).

1.9.2. Classification of Lipoproteins

1.9.2.1. Chylomicrons (CM)

They are large TG rich particles made by the intestine, which involved in the transporting of dietary TG and cholesterol between the peripheral tissues and liver. The size of CM are directly proportional by the fat ingested, increased in case of large fat meal, and decreased in case of fasting (Julve *et al*, 2016).

1.9.2.2. Chylomicron remnants

They are result from the removal of TG from CM by the peripheral tissues, when compared to chylomicrons these particles are enriched in cholesterol (Julve *et al*., 2016)

1.9.2.3. Very low density lipoproteins (VLDL-C)

These particles are produced by the liver and are rich by TG. They contain apolipoprotein B-100, C-I, CI1. C-III, and E. Apo B-100 is the core structural protein. Similar to chylomicrons, where the size of the VLDL-C particles also directly proportional by the quantity of TG carried, but it is smaller than CM (Dallinga-Thie *et al*., 2016).

1.9.2.4. Intermediate density lipoproteins (IDL-C; VLDL-c remnants)

They are formed after the removal of TG from VLDL-c by muscle and adipose tissue, they are enriched in cholesterol. These particles contain apolipoprotein B-100 and E. These IDL particles are pro-atherogenic (Chapman *et al*., 2011)

1.9.2.5. Low density lipoproteins (LDL-C)

These particles are derived from VLDL-C and IDL-C particles and they are even further enriched in cholesterol. They carries the majority of the cholesterol that is in the circulation. The predominant apolipoprotein is B-100. LDL-C consists of a spectrum of particles varying in size and density. An abundance of small dense LDL particles are seen in association with hypertriglyceridemia, low HDL-C levels, obesity, T2DM, infectious and inflammatory states (Sato *et al*., 2016)

1.9.2.6. High density lipoproteins (HDL-C)

The HDL-C are the only anti-atherogenic particles due to it reverse cholesterol transporting from the peripheral tissues to the liver, also due to the HDL-C particles contains anti-oxidant, anti-inflammatory, anti-thrombotic and anti-apoptotic properties, which may also contribute to their ability to inhibit atherosclerosis. HDL particles are enriched in cholesterol and phospholipids (Annema and von Eckardstein, 2013). Table (1. 2) shown the classes of the lipoproteins.

Table (1. 2). Lipoprotein Classes

lipoprotein	Source	Lipid	Apolipoprotein
CM	Gut	TG	Apo B-48, Apo C, Apo E, Apo A-I, A-II, A-IV
VLDL-C	Liver	TG	Apo B-100, Apo E, Apo C
IDL-C	HDL-C	TG, cholesterol	Apo B-100, Apo E, Apo C
LDL-C	VLDL-C via IDL-C	Cholesterol	Apo B-100
HDL-C	Gut/ liver	Cholesterol phospholipid	Apo A-I, Apo A-II, Apo C, Apo E

Whre CK is chylomicron.

1.9.3 The atherogenic index (the nonHDL-C)

Some authors believe to determine the non-HDL-C concentration instead of using the real LDL-C level in initiating the management of dyslipidemia because the precipitation methods that used in determination of HDL-C and LDL-C may be not accurate (Masana *et al.*, 2013).

It is measured as total cholesterol – HDL-C.

1.9.4 Body Mass Index (BMI)

Body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify overweight and obesity in adults. It is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2). (WHO, 2016).

1.9.5 Glycosylated hemoglobin (HbA1c)

The origin of the naming derives from Hemoglobin type A being separated on cation exchange chromatography. The first fraction to separate, probably considered to be pure Hemoglobin A, was designated HbAo, the following fractions were designated HbAia, HbA1b, and HbAis respective of their order of elution. There have subsequently been many more sub fractions as separation techniques have improved (Meshram, 2017).

The HbA1c is determine the normal lifespan of red blood cell (120-day) of blood glucose molecules joined hemoglobin. Thus, it is a routinely marker for long term diabetic control preceding 8-12 weeks of time. According to American Diabetes Association (ADA), Glycated Haemoglobin (HbA1c) $\geq$ 6.5% is considered diabetes (ADA, 2018).

Measuring glycosylated haemoglobin assesses the effectiveness of therapy by monitoring long- term serum glucose regulation. The HbA1c is formed in a non-enzymatic glycation pathway by hemoglobin's exposure to plasma glucose. HbAlc is a measure of the beta-N-1-deoxy fructosyl component of hemoglobin (AbdAlrhman, 2016).

The elevation level of HbA1c in the DM patients indicates a poor control of blood glucose level, also recently HbA1c have been associated with CVD, nephropathy, neuropathy and retinopathy (Areosa *et al*., 2017). It is estimated that there is an 18% increased risk of CVD for each 1% rise in absolute HbA1c levels in the diabetic population. This positive correlation between HbA1c and CVD has been demonstrated in nondiabetic cases, even within the normal range of HbA1c (Syed and Khan, 2011).

1.10 Polymerase Chain Reaction (PCR)

It was performed by using thermocycler which amplified a desired region of the genome. The concentration of the desired target sequence theoretically increases from one molecule to several million copies. There are three steps on any PCR that are cycled about 25-35 times, included the following steps based on (Champe *et al*, 2004);

1- Denaturation: it is the unwind step of the double strand DNA into two single strands by heating (94^{o}C).

2- Annealing: it is done at (55 - 65^{0}C) to allow the oligonucleotide primers to hybridize to the template. The single strands of the template are too long and complex to be able to reanneal during this rapid cooling phase. During this annealing step the thermostable DNA polymerase will be active to some extent and will begin to extend the primers as soon as they anneal to the template.

3- Extension (DNA synthesis): the reaction is heated to between (72 - 74^{0}C) for efficient DNA synthesis by the thermostable DNA polymerase and entails the extension of the primers to form a new strand that is complementary to the template strand. This occurs in the presence of the Taq DNA polymerase, a DNA polymerase (isolated from the organism Thermus aquaticus, a bacterium that can survive at high temperatures without denaturation).

1.10.1. Real time PCR (RT-PCR)

It is highly sensitively and reproducibly quantifies the initial amount of starting template (transcript) by monitoring PCR amplification product (amplicon) accumulation during each PCR cycle, in contrast to conventional methods which detect the final end product. Furthermore, real-time RT-PCR is rapid, it is possible to analyze several transcripts (genes) simultaneously the dynamic range of real-time RT-PCR is higher (up to 1010-fold) than conventional quantitative RT-PCR (1000-fold), which means that a wide range of amplification products can be accurately and reproducibly quantified (Michael and Simon, 2006).

There are two fundamental differences between conventional and RT-PCR: (i) amplicon accumulation is detected and quantified using a fluorescent reporter and not by conventional gel electrophoresis, and (ii) amplicon accumulation is measured during each PCR cycle in contrast to standard end-point detection.

A real-time RT-PCR reaction contains all the components used for conventional RT-PCR but in addition contains a fluorescent reporter either in the form of a fluorescent DNA-binding dye (Ex. Cybergreen) or as a fluorescent oligonucleotide primer (Ex. FAM, VIC) (Michael and Simon, 2006).

1.10.2. The TaqMan PCR;

The TaqMan probe contains a reporter dye (FAM and VIC) at the 5´ end of the probe and a quencher dye minor groove binder (MGB) at the 3´ end of the probe. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence.

During PCR, if the target of interest is present, the probe specifically anneals to the target. The 5´ to 3´ nucleolytic activity of the enzyme, polymerase enzyme cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target (Michael and Simon, 2006).

The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3´ end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle, and it does not interfere with the exponential accumulation of product (Michael and Simon, 2006).

The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and if it is amplified during PCR. Because of these requirements, nonspecific amplification is not detected (Michael and Simon, 2006). As shown in figure (1. 14)

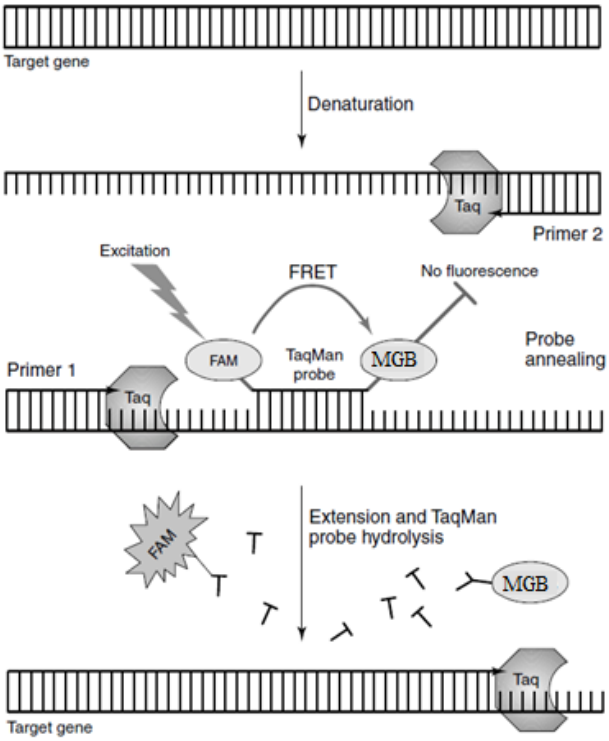

Figure (1. 14); the principle of real-time polymerase chain reaction (RT-PCR) using the TaqMan technique (Michael and Simon, 2006).

1.10.3. Primers and probes

1.10.3.1. Primers properties;

The primer should be 16 - 30 nucleotides long, which provides good specificity for a unique target sequence, even with a starting template as complex as human genomic DNA**;** should contain approximately equal numbers of each nucleotide**;** should avoid repetitive sequences because this lead to 'slipping' of the primer on the template**;** should avoid runs of three or more G or Cs at the 3'-end as this can lead to mispriming at GC-rich regions**;** should not be able to form secondary structures due to internal complementarity**;** should not contain sequences at the 3'-ends that will allow base pairing with itself or any other primer to not form primer-dimers (Michael and Simon, 2006).

1.10.3.2 TaqMan probes properties

It should be longer than the amplification primers (20 and 30 nucleotides)**;** the melting temperature (Tm) approximately 10°C higher than for the amplification primers to allows hybridization to the target gene during the extension step to ensure that the emitted fluorescence after TaqMan probe displacement is directly proportional to the amount of target DNA present in the reaction**;** avoid G at the 5'end because this results in quenching of the fluorescent signal even after probe cleavage**;** it must contain more C than G and this can be achieved by designing either a sense or an antisense probe**;** the possibility of co-amplification of genomic DNA together with the target cDNA**.** Thus, it should be designed so that it spans two exons, so that only correctly spliced versions of the cDNA are amplified (Michael and Simon, 2006).

Hypothesis

Insulin resistance is one of the key pathophysiological mechanism of T2DM, which may contribute to the development of T2DM and associated with rs4918 SNP in AHSG gen polymorphism as well as the metabolic syndrome.

Aim of the study

To study the association of AHSG rs4918 at 766 (C/G) gene polymorphism with DM in Kerbala population.

2. Subjects, Materials and Methods

2.1. Subjects

This study was carried out in the college of medicine in Kerbala University and the Institute of Genetic Engineering and Biotechnology University of Baghdad for postgraduate studies through the period from April 2018 till the end of November 2018. Two study groups were investigated:

2.1.1. Patient groups

This study was a randomize case - control study which included: 100 DM patients, (50 males and 50 females). Patients groups was selected from Al-Imam Al-Hassan Centre for endocrine and diabetes in Al-Imam Al-Hussein medical teaching hospital in holy Kerbala province and were examine by a physician. Taking from the individuals some information's (questionnaire) as shown in the appendix list.

The exclusion criteria of DM patients;

1- Individual those diagnosed with T1DM.

2- Patients suffering from kidney (Roos *et al.,* 2009) or liver diseases (Katsuhito *et al.,* 2011), IR (Ix JH *et al.,* 2012), osteoporosis (Sritara *et al.*, 2014), ischemic stroke (Shanshan, *et al*., 2013), myocardial infraction, pregnant woman, as this diseases might be affected the fetuin A and it gene.

3- Patients with tumor diseases due to it will give a false results.

4- Patients who taken drug therapies known to interfere with lipid metabolism (to avoid any interfering with measured parameters).

The inclusion criteria;

1- Individual those diagnosed by specialized physician as having T2DM according to WHO guidelines.

2- Age of patients was ≥ 45 years old.

3- The following parameter FBG > 126 mg\dl, HbA1c > 6.5%, and BMI ≥ 23 Kg\m2.

2.1.2. Control group

The control group included 100 apparently healthy persons (48 female and 52 male) were randomly selected from the general population.

The exclusion criteria included; Patients with diabetic, heart, kidney or liver disease and intake statin drug.

The inclusion criteria included;

1- Individual with age at examination ≥ *4*5 years.

2- The FBG < 120mg/dL.

3- The HbA1c values <6.5%.

4- No past medical history of T2DM.

5- No family history of T2DM.

6- The BMI ≤ 23 kg/m^2.

2.2. Collection of blood Samples

Blood samples were collected in the morning after 12 hours of fasting from the venous by using 5 ml disposable syringes. This blood was divided into;

1- The first part (3 ml) was put in gel tube and then centrifuged at 4000 r.p.m. for 10 minutes, then collected the serum and used for the lipid profile , FBS and troponin I (positive or negative only).

2- Second part of venous blood (2ml) was put into Ethylenediaminetetraacetic acid (EDTA) tubes and used (1ml) for HbA1c measurement, and (1ml) preserved in -4˚C for DNA extraction.

Approval of the ethical committee

The protocol of the study was approved by Ethical Committee of Kerbala medical college, and Kerbala diabetic diseases (Imam Al-hassan Centre)

2.3. Materials:

2.3.1. Instruments and Equipment's

The general instruments and equipment's used in this study are listed in table (2. 1).

Table (2. 1): Instruments and equipment used in the study

No.	Instruments and equipment	Manufacture/Origin
1	Disposable syringe 5ml	China
2	EDTA tubes	
3	Eppindrof tube different size (0.5and 1.5 ml)	
4	Gel tube	
5	Microcentrifuge tube	
6	Microceterfuge	Hettich /Germany
7	Minispin	Bioneer / EU
8	Nano-drop (UV-Vis spectrophotometer Q5000)	Quawell / USA
9	Oven	Binder / Germany
10	Real time PCR/ Roto-Gene/ 5 plex	QIAGEN Q/ Germany
11	Small Desk Centrifuge H-19α	Kokusan/Japan
12	Vortex- Mixture	Cyan / Belgium
13	Water bath	Memmert / Germany

2.3.2. Reagents and Chemicals

General reagents and chemicals used throughout the study are listed in table (2. 2).

Table (2. 2): The reagents and chemicals used in the study.

No.	Chemicals and Kits	Manufactured/origin
1	DNA extraction kit (Catalog No. A5082)	Promega/ USA
2	Glucose assay kit	Linear / Spain
3	GoTaq® qPCR Master Mix	Promega/ USA
4	HbA1c	Biosystem / Spain
5	HDL-C kit	Linear / Spain
6	LDL-C	Linear / Spain
7	Nuclease Free Water	Rongsheng Biotech/Shanghai
8	Primers	Alpha DNA/Canada
9	Total cholesterol kit	Linear / Spain
10	Triglyceride kit	Linear / Spain

2.3.3 The kits used in the study for DNA extraction (A5082);

The ReliaPre™ Blood gDNA miniprep system (USA/ Promega) are presented in Table (2. 3).

Table (2. 3): components of DNA Extraction Kit (ReliaPre™);

Components	Quantity
Binding Buffer (BBA)	1 x 27.5ml
Cell Lysis Buffer (CLD)	1 x 55ml
Collection Tubes	5 x 200/pk
Column Wash Solution (CWD)	1 x 412.5 ml
Nuclease-Free Water	1 x 50 ml
Proteinase K (PK) Solution	1 x 5.5ml
ReliaPrep™ Binding Columns	5 x 50/pk

2.3.4. qPCRMaster (PROBE)

The components of GoTaq® qPCR Master (PROBE) include:

One eppendorf tube (1 ml) 2X qPCRMaster (PROBE) contains:-

- GoTaq® probe qPCR master mix, dTTP
- Nuclease Free Water.
- Hot start (HS) - Taq DNA Polymerase.
- PCR buffer buffer, $MgCl_2$ and dNTPs, except DNA template and primers,

2.4. Methods:

2.4.1. Clinical data:

Every participant has been interviewed and asked to answer information shown in the questionnaire in the appendix. They have been also subjected to medical checkup for signs of DM by specialized doctor.

2.4.2. Body Mass Index (BMI)

Body mass index (BMI) was estimated by measuring an individual's weight and height to lean body mass. The BMI is thus an index of weight adjusted for stature. Body mass index was figured by dividing weight in kilograms by height in meters squared. The diagnostic criteria for BMI shown in table (2. 4) (WHO, 2016).

BMI = weight in (Kg) / height (m^2).

Table (2. 4); Distribution of weight status according to BMI.

BMI Kg / m^2	**Weight Status**
< 18.5	Under weight
18.5 – 24.9	Healthy weight
25 – 29.9	Over weight
≥ 30	Obese

2.4.3. Genomic DNA isolation

Total genomic DNA isolated from the whole fresh blood have been collected in EDTA containing tubes for molecular studies, was applied using genomic DNA purification kits (ReliaPre^{TM} Blood gDNA miniprep system USA, Promega DNA extraction kit (Catalog No. A5082).

2.4.3.1. Genomic DNA Isolation protocol

The protocol supplied by Promega Company was used for DNA isolation as follow:

1- The blood sample was thoroughly mixed for 10 minutes at room temperature.

2. A 20µl of Proteinase K (PK) was dispensed into a 1.5ml microcentrifuge tube.

3. A 200µl of blood was added to the tube contained the Proteinase K (PK) Solution, and briefly mix.

4. A 200µl of Cell Lysis Buffer was added to the tube, caped and mixed by vortex for at least 10 seconds. This step was essential for obtaining good yields.

5. Incubated at 56°C for 10 minutes.

6. As the blood sample was incubated, the ReliaPrep™ Binding Column was placed into an empty collection tube.

7. The tube was removed from the heating block. A 250µl of binding buffer was added, then the tube was caped, and mixed by vortexed for 10 seconds with a vortex mixer.

8. The contents of the tube were added to the ReliaPrep™ Binding Column, caped and placed it in a microcentrifuge.

9. All compound was centrifuged for 1 minute at maximum speed. Then the binding column was checked to make sure the lysate has completely passed through the membrane. If lysate was still visible on top of the membrane, the tube was centrifuged the column for another minute.

10. The collection tube containing flowthrough was removed, and the liquid discarded as hazardous waste.

11. The binding column placed into a fresh collection tube. A 500µl of Column Wash Solution was added to the column, and centrifuged for 3 minutes at maximum speed. Discarded the flowthrough.

12. Step 11 was repeated twice for a total of three washes.

13. The column placed in a clean 1.5ml microcentrifuge tube.

14. A 50 - 200µl (100 µl) of Nuclease-Free Water added to the column. Centrifuged for 1 minute at maximum speed.

15. The ReliaPrep™ Binding Column discarded, and saved the eluate at 4^0C - -20^0C.

2.4.4. Estimation of DNA Concentration

A Nanodrop was used to estimate the purity and the concentration for DNA samples in this study by putting 2µl of the extracted DNA in the Nanodrop apparatus to detect concentration in ng/µl and the purity was detected by noticing the ratio of optical density (OD) 260/280 nm to detect the contamination of samples with protein. The purity of DNA was ranged 1.7 - 1.9 and that accepted 260/280 ratio with previous literatures (Sambrook *et al*., 1989).

2.4.5. Primer design

The SNP rs4918 at 766 (C/G) on the 3q27 chromosome of the AHSG was chosen due to it is location at a susceptible locus for T2DM and metabolic syndrome. By using the National Center for Biotechnology Information (NCBI) website found the position of this SNP on the chromosome (g.12533 G>C,), and taken from the SNP position ± 200 nucleotides and chosen a melting temperature (55-65 ^{o}C) and the GC% (55-65%) then pick the primer and probe and chosen the best sequences which shown in figure (2. 1) and send to the ALPHA company for the production.

2.4.6. Primers and probes preparation;

The primers units are given as a mass (in picomoles). The primers reconstitute in nuclease free water to obtain master stock of primers which can be used again to obtain working stock. It's done as;

1. The tube was spin down before opening the cap.
2. The nuclease free water (NFW) was added according to the manufacturer to obtain a 100 pmoles/ µl (Master Stock). Samples was stored at -20^{0}C.
3. The tube was shacked properly to re-suspend the primers equally.
4. A 10 µl of the master stock was taken for farther dilution with 90 µl of NFW and this used for PCR product preparation (working solution). It was stored at -20^{0}C.

Once the primers were reconstituted and distributed into single use aliquots. The use of single-use aliquots limited the freeze-thawing of primers and, therefore, extended their life. The sequence of primers and probe used for PCR amplification of Fetuin -A- gene rs4918 is demonstrated in Table (2. 5).

2.4.7. Primer and Probe sequence matching:

TaqMan fluorescent oligonucleotide probes and primers sequences were prepared according to William *et al*., (2004), synthesized by Alpha DNA Ltd (Canada) and stored lyophilized at (-20°C). The sequences of each of the probes and primers used in the allelic discrimination experiments are shown in table (2-5) it included the *AHSG* gene SNP exon 7 (G to C; rs4918 at the position 766).

Table (2. 5): The sequence of primers and probe used for PCR amplification of Fetuin A gene;

Primers	Sequences	Concentration in Picomoles	Water volume in diluting	GC %	Melting Temp. ^{o}C
Forward	CTGGGAGGAGGAAGCAAACT	92300	923 µl	55%	57.27
Revers	TAACACATGGGAGTCTGGGG	125790	1258 µl	55%	56.67
FAM	TGAGCTCACAGCCCCAAC	83919	839 µl	61.11%	56.92
VIC	ACCTCACAGCCCCAACCAG	87548	875 µl	63.16%	59.49

Primer and probe sequence were designed and matched by www.ncbi.nlm.nih.gov as shown in Figure (2. 1), the wild type detecting probe was labeled with FAM in the ′5 end and MGB in the ′3 end. While the probe prepared for the mutant allele (SNP) was labeled with VIC in the ′5 end and MGB in the ′3 end (Fig 2.1).

Exon 7 (C to G; AGSH gene, rs4918)

rs4918, Band size: 186 bp, g. 766 C>G.

Homo sapiens AHSG RefSeqGene on chromosome 3

NCBI Reference Sequence: NG_011436.1

Probe 1 FAM

CTGGGAGGAGGAAGCAAACTAACTGAAGGAAATGGTCCTTTTTCC
AGCCCGTGAGCTCACAGCCCCAACCAGAAGGTGCCAATGAAGCAG
TCCCCACACCCGTGGTGGACCCAGATGCACCTCCGTCCCCTCCACT
TGGCGCACCTGGACTCCCTCCAGCTGGCTCACCCCCAGACTCCCAT
GTGTTA

Probe 2 VIC

CTGGGAGGAGGAAGCAAACTAACTGAAGGAAATGGTCCTTTTTCC
AGCCCGTGACCTCACAGCCCCAACCAGAAGGTGCCAATGAAGCAG
TCCCCACACCCGTGGTGGACCCAGATGCACCTCCGTCCCCTCCACT
TGGCGCACCTGGACTCCCTCCAGCTGGCTCACCCCCAGACTCCCAT
GTGTTA

Figure (2. 1): Matching of the primers and probes sequences on the bioinformatics programs/ NCBI (the blue color represented as the probe nucleotides, while the yellow color represented as forward and reverse primer, and the red nucleotide color represented the SNP C>G).

2.5. Real-time PCR run

DNA samples from DM patients (n=100), and apparently healthy subjects (n=100) were genotyped for the *AHSG* gene SNP (rs4918) with a Taqman SNP genotyping assay using real time thermocycler according to the protocol recommended by the manufacturer, the kit content as show in table (2. 6):

Table (2. 6): Components of Real-time PCR/ allelic discrimination reaction.

No.	Components	Volume	Final conc.	Volume (µl)	
1	qPCR Master (PROBE)	10 µl	1X	10	
2	Forward primer	0.2-2.0 µl	0.1-1.0 µM	10 µl	0.5
3	Reverse primer	0.2-2.0 µl	0.1-1.0 µM	10 µl	
4	Fluorescence Probe	Variable	≤500ng/reaction	20 µl	
5	Template DNA	Variable	-	4	
6	Water , RNase free	Up0 to 20	-	5.5	
	Final Volume			20	

The thermal profile of allelic discrimination Real-time PCR program is shown in table (2. 7).

Table (2. 7); Real Time PCR program for *AHSG* gene SNP (rs4918).

Cycle step		Temp. (°C)	Time	Cycle
Hold1		50	15 min.	1
Hold2		95	15min.	
Cycling 1	**Denaturation**	95	5 sec.	5
	Annealing	60	20 sec.	
	Extension	72	15 sec.	
Cycling 2	**Denaturation**	95	5 sec.	40
	***Annealing**	60	20 sec.	
	Extension	72	15 sec.	

*** In this step we added the acquiring Green and Yellow (FAM and VIC) respectively.**

2.6. Hardy - Weinberg equilibrium (HWE);

It is a mathematical relationship that related genotypes to allele frequencies. States that in a large randomly breeding population, allelic frequencies will remain the same from generation to generation assuming that there is no mutation, gene migration, selection or genetic drift, illustrated mathematically with the following equation (Dorak., 2014): $p2+2pq+q2 = 1$

Where (p) and (q) represent the major and minor allele frequencies respectively (National Biological Information Infrastructure. 2005). To analyze HWE, the allele frequencies was calculated from the above equation .Then expected genotype frequencies can be calculated to determine how far a population deviates from HWE which can be measured using the chi-squared test by comparing the observed genotypes with expected values. If P value is less than 0.05 mean deviation from HWE, small population size is one of the causes of deviation from HWE (National Biological Information Infrastructure, 2005). HWE was calculated using the online software Encyclopedia for Genetic Epidemiology studies (OEGE).

2.7. Multinomial logistic regression

By SPSS (V 22.0) windows software was used to assess the association of genotype and allele frequencies with T2DM by different inheritance models of Fetuin -A- gene, the major allele was G and the minor allele was C, and the wild type GG was the reference, the models were shown in (table 2.8):

Table (2. 8): Genetic models of inheritance

Genetic model	Fetuin A gene
Allelic model	G vs. C
Dominant model	CC + GC vs. CC
Recessive model	GC + GG vs. GG
Major allele	G
Minor allele	C

2.8. Statistical analysis

Data are presented descriptively as means and standard deviation (Mean ±SD). Significant differences in continuous variables among two group were confirmed by the t-test to compare mean levels of continuous characteristics across genotypes using (SPSS v. 22.0) software (SPSS Inc., Chicago, IL). Categorical data (genotypes and alleles) were expressed as frequency. P values less than 0.05 were considered statistically significant for all of the statistical analysis. Genotyping and allele frequencies of rs4918 in *AHSG* gene were compared in groups using a chi-squire ($\chi 2$) test. Odds ratio (OR) and confidence intervals (CI) were calculated to assess the relative risk conferred by a particular allele and genotype.

3. Result and discussion

The identification of genetic polymorphism which effect DM is a key entreaty of research to detect the causal mechanisms of the pathogenesis of this disorders as well as associated with pathological progress. This effort may improve the plans of protection, diagnosis and treatment of Iraqi population.

The genetic involvement of several genes related to DM considered to be a field of continuous researches. Basically, the main objectives of these studies were to understanding the disease, and identifying individuals that are at risk to develop DM.

In this study, the genotype of the AHSG gene polymorphisms was determined in the Kerbala population. This SNP located at the chromosome 3q27 and of exon 7 and spanning approximately 8.2 kb of deoxyribonucleic acid (DNA) (Ma *et al*, 2013). The most common polymorphism recorded for this area is 766 (C/G) which is a missense mutation (a change in one DNA base pair) (Zeidan, *et al* 2012). Because fetuin-A is an AHSG product, researchers have hypothesized that may be related between the gene and DM, which is approved by many studies {Dahlman *et al* (2004), Siddiq *et al* (2005), Andersen *et al* (2008)}. According to our knowledge this is the first study that deals with the AHSG polymorphisms in the kerbala population.

Genome Wide Association Studies (GWAS) showed that T2DM has strong genetic link and many genes are responsible to develop T2DM and also overt its complications (Phani *et al*, 2016). There are 136 SNPs at the *AHSG* locus achieved by genome-wide significance among European Americans and one of the common genetic variant is (rs4918) were strongly proposed to be associated with DM (Joachim *et al* 2017).

In the present study, the promoter of AHSG gene was noted as the serine changing into threonine at 766 (rs4918) and analyzed for the association with DM, the selection of this SNP based on the fact that the variation in the promoter may direct the transcriptional status of the phenotype, ie; fetuin -A-. Thus, it logically can be expected that such changes may increase or decrease the concentration of the fetuin -A- as well as the extent of the function, also due to the location of AHSG on 3q27 which is the susceptible locus for DM, and metabolic syndrome (Shanshan, *et al* 2013), also for the location of the fetuin A which is specially in the liver organ (Celebi, *et al* 2015).

Thus, make it important to studied the polymorphism of AHSG (rs4918) with T2DM, where generally the T2DM is affects over 415 million people worldwide, with the global prevalence expected to double in the next 15 years (International Diabetes Federation, 2015). The principal complication of DM is CVD, almost 65% of patients with DM dying from CVD complications despite numerous advances in treatment (Grundy, *et al* 1999). So it is important to lower the most widely used in clinical measure of glycemic control as HbA1c, and circulating glucose levels.

The exact pathogenesis of T2DM is unclear, it is generally accepted that T2DM is a multifactorial disorder resulting from several environmental factors, or genetic disorder (Ben-Salem *et al*, 2014). One of the most linked factor is the obesity which is a serious health problem that associated with many chronic diseases, it was reported over the past three decades that the prevalence of obesity was increased in alarming rate worldwide. Almost, one out of three people in the global population is overweight or obese (Goutzelas *et al*, 2017), thus this study was supported and completed for both DM and control group were overweight (26.04 ± 3.08, 26.78 ± 2.03) respectively.

3.1. Clinical and biochemical characteristics of study subjects.

Subject data

A total of 200 blood samples were collected from two groups of Kerbala population which include (100 DM) patient and (100) apparently healthy control, they were matched in age, gender and BMI. The clinical and biochemical characteristics of the recruited individuals were presented in Table (3.1). The result showed a significant differences ($p<0.05$) in all parameters comparing DM patients with control group.

Table (3.1): Clinical and biochemical characteristics of study subjects**.**

Parameter	DM (n=100) (mean ± SD)	Control Group (n=100) (mean ± SD)	P value
Number (M/F)	50 / 50	52 / 48	----
BMI	26.04 ± 3.08	26.78 ± 2.03	0.215
Age (y)	56.57 ± 5.98	55.84 ± 5.58	0.053
FBS (mg/dl)	241.09 ± 71.81	95.88 ± 17.11	**< 0.001***
TC (mg/dl)	167.17 ± 39.02	171.26 ± 31.39	**< 0.001***
TG (mg/dl)	239.53 ± 92.48	170.25 ± 85.07	**0.045 ***
VLDL (mg/dl)	47.91 ± 18.49	34.05 ± 17.01	**0.454 ***
LDL (mg/dl)	112.65 ± 27.41	93.82 ± 34.14	**0.021 ***
HDL-C (mg/dl)	37.29 ± 10.74	47.59 ± 5.99	**< 0.001***
HbA1c%	9.4 ± 1.8	4.48 ± 0.87	**< 0.001***
Non HDL-C	130.6 ± 36.85	123.43 ± 33.33	**0.001 ***
LDL/HDL	3.22 ± 0.926	2.02 ± 0.882	**0.001***

T-test P value of (*) was significant <0.05.

The result of this study was observed an elevation in total cholesterol, and HbA1c with a significant differences (P<0.05) and there mean ± SD was 167.17 ± 39.02, 171.26 ± 31.39 for patients and control group respectively. These results show the abnormal total cholesterol, and HbA1c metabolism remains as a strong risk factors for DM, and also observed an elevation in FBS (P<0.01), and decrease HDL-C (P<0.01) in patient with T2DM, these results was shown in table (3.1).

It has been reported that fetuin-A directly or indirectly leads to these changes through its inhibitory effects on the insulin receptor tyrosine kinase, which lead to increases lipolysis and efflux of free fatty acids from adipose tissue (Stefan, *et al*, 2013). This might lead to increased production of apolipoprotein B - containing very low density lipoprotein (VLDL-c). Furthermore, the hypertriglyceridemia may lead to a decrease in the cholesterol content of HDL-C enhancing HDL-C clearance from the circulation, thereby potentially leading to the atherogenic lipid profile (Khalil, 2013).

3.2: Results of the Genotype Analysis

3.2.1: Measurement of DNA Concentration and Purity

DNA concentration and purity were estimated by the measurement of A260/A280 ratio. Results are clarified in Table (3.2) and Figures (3.1) and (3.2). DNA samples were seemed to be pure and the concentrations ranged from 0.01-0.24 Ng-µL (10 - 240 ng / µl) (Desjardins, P., & Conklin, D. 2010).

Table (3.2): DNA concentration and purity**.**

	Mean ± SD
DNA Concentration (µg/ml)	82.91 ± 59.1
DNA Purity	1.96 ± 0.15

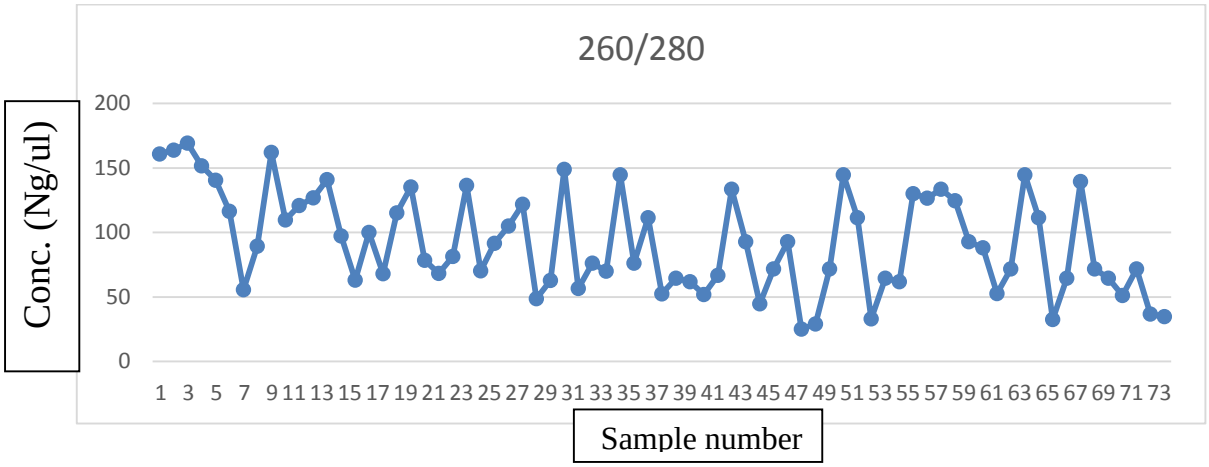

Figure (3. 1): Concentration of DNA extracted from studied individuals**.**

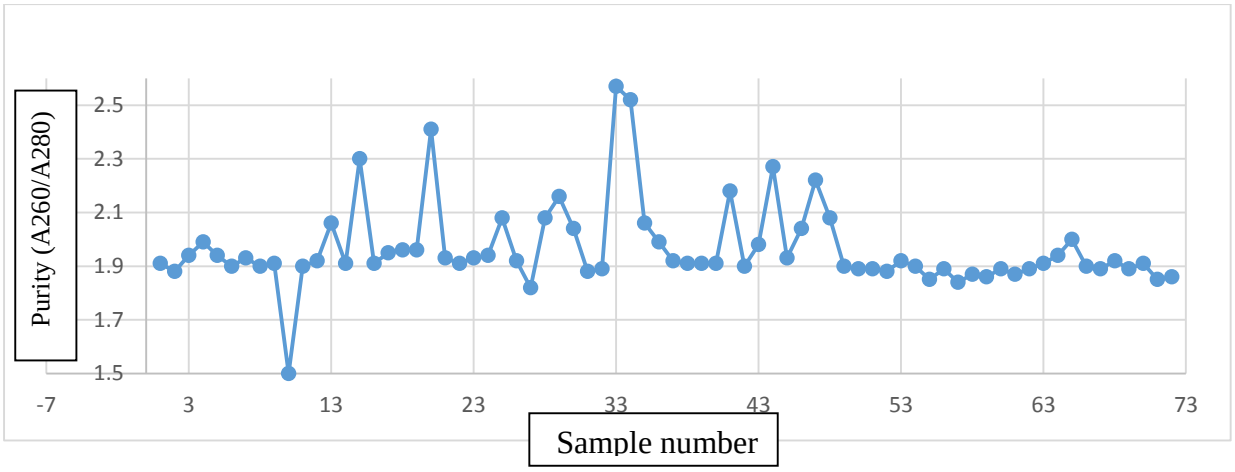

Figure (3. 2): Purity of DNA extracted from studied subjects**.**

3.2.3. The result of the Roto- Gene Q Real-time PCR;

The result were taken from the Roto-Gene Q series software 2.1.0 (Build 9) for the real time PCR as shown in the figure (3. 3), the green color is the wild genotype (GG), the light blue color is the heterozygous genotype (GC), and the red color is the mutant genotype (CC). The straight line is the FAM probe (major allele G) and the line with circles is the VIC probe (minor allele C). When there is a reaction and emitted florescences in the samples there line well increases above the threshold.

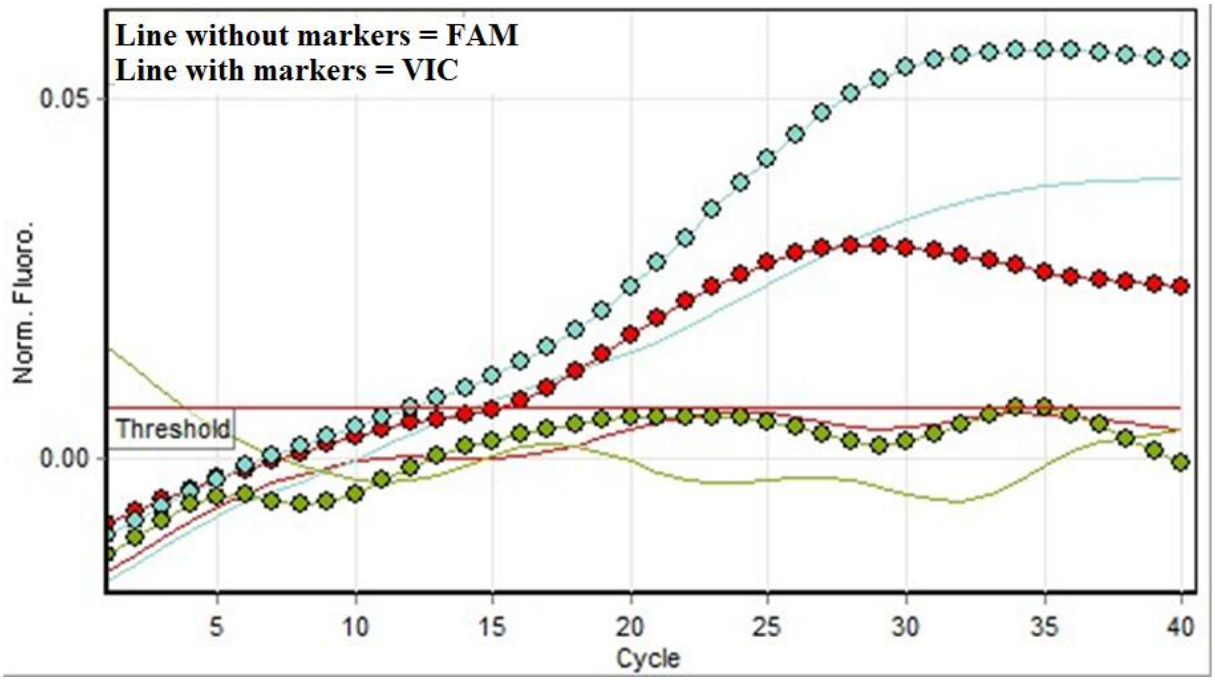

Figure (3-3); The qPCR curves, The photograph was taken directly from Roto-Gene Q Series Software 2.1.0 (Build 9).

3.2.4. Estimation of the genetic power and examination of Hardy-Weinberg equilibrium (HWE) for AHSG gene polymorphism;

Genotyping frequencies of AHSG gene were consistent with Hardy Weinberg Equilibrium (HWE) ($p>0.05$) in control group have ($\chi2 = 1.35$, G allele frequencies = 0.74 and the C allele frequency is C=0.26, P= 0.245), on the other hand the DM group have ($\chi2 =2.13$, G allele frequencies = 0.62 and for allele C = 0.38, P= 0.144).

The analysis of results indicated that the rs4918 AHSG gene (G→C) genotype frequencies of wild genotype (GG), heterozygous genotype (GC) and homozygous genotype (CC) were 35%, 54% and 11 % in cases of DM, and in the control group 57 %, 34% and 9%. The heterozygous genotype GC was found to increase the risk of DM two folds with respect to those of the wild genotype CC (OR= 2.27, C.I; 95% =1.288 - 4.033, $P<0.001$).

On the other hand, the homozygous genotype CC was increase the risk of DM but not significantly (OR=1.249, 95% C.I= 0.494 - 3.161, P=0.639) as indicated in table (3.3). Jensen *et al*., 2013 found that probability of his results was significantly consistent with HWE it was recorded as (p= 0.04),

Table (3.3): Distribution and association of genotypes, allele frequencies of AHSG (rs4918) gene polymorphism and their association with the risk of DM in cases and control groups.

AHSG	Group				X^2	P value	OR	CI 95%
	Control n=100		DM n=100					
Genotype	H W O.F.	H W E.F.	H W O.F.	H W E.F.				
GG	57	55	35	38	9.74	0.001*	-	-
GC	34	38	54	47	9.78	0.001 *	2.58	1.41-4.71
CC	9	7	11	15	1.95	0.162	1.99	0.74-5.28
Total	100	100	100	100				
X^2	1.35		2.13					
P-value HWE	0.245 NS		0.144 NS					
Allele frequency								
G	0.74		0.62					
C	0.26		0.38					

Where the X^2 = chi-sequear, p= probability, OD= Odd ratio, H W O.F. = Hardy Weinberg observed frequency, H W E.F. Hardy Weinberg expected frequency

Table (3.3) shown the percentage of patients group with wild GG genotype carriers was significantly (p=.0001) and lower as 35% compared with healthy control group have 57%. Also, the present study demonstrated that AHSG gene polymorphism may be linked with the danger of DM two time then in healthy control, these results were disagreement with some studies like (György *et al*,. 2016) who was observed that CC genotype in DM patient have 50%, CG genotype have 42.85%, and GG genotype have 7.14%.

Otherwise, Maréchal, *et al*., (2011) found the genotype of CC, CG, GG were recorded as 47.65%, 42.23%, 10.10 % respectively, but it is in agreement with the HWE which have (0.78) and p value (0.17).

The genetic variation of fetuin A was revealed an impact of AHSG polymorphism on patient with T2DM. The importance of the AHSG polymorphism was located in exon 7, which changes the sequence at 766 from the serine (AGC) changing into threonine (ACC) (Catharina *et al*, 2005). Theoretically, this SNP which affects the exon 7 of the molecule (D3 domain region), may also result in altered function of the molecule. But there was no functional differences of this missense mutation for rs4918 have been reported (Catharina *et al*, 2005).

Our result was studied one hundred of DM patient and one hundred apparently healthy as control group who admitted to Imam Al-Hussein teaching hospital. At this moment a very reasonable question may be asked; why did we not choose an appropriate sample size regarding the *AHSG* gene polymorphism SNP rs4918? The answer is that the selection of sample size is depending on the findings of past investigations. Unluckily there was a little published research concerning this SNP, therefore the current study results may be used as a base for improvement of the genetic power for future studies on SNP rs4918.

3.2.5. The assessment of genotype AHSG rs4918 at 766 (C/G) gene polymorphism in the patients and control groups;

The genotype of fetuin -A- gene polymorphism (rs4918) at 766 (C/G) in DM and control groups was examined under the co-dominant, dominant, and recessive models with the use of multinomial logistic regression analysis.

Under the co-dominant model, the DM patients are significant in heterozygous genotype (GC) (OR= 0.38, 95% CI= 0.21- 0.7, p value = 0.001) and the homozygous genotype (CC) are not significant (OR= 0.5, 95% CI= 0.18-1.33, p value 0.167).

Under the dominant model, the DM patients with (CC + GC) genotype were significant (OR= 0.4, 95% CI= 0.22 – 0.71, P=0.001) higher than those of control group, with the minor allele C, was exhibited to be significantly (p=0.001) higher than those of control group table (3-4).

Table (3. 4); Result of the assessment of genotype AHSG at 766 (C/G) gene polymorphism in DM patients and control group.

Genotypes	**DM** n=100	**Control** N=100	**Crude R (95% CI)**	**P vaule**	**Risk ratio(95% CI)**	**P value**
Co-dominant						
GG (Reference)	35	57				
GC	54	34	0.38 (0.21- 0.7)	0.001 *	0.61 (0.45-0.84)	0.002 *
CC	11	9	0.5 (0.18-1.33)	0.167	0.69 (0.43-1.11)	0.127
Dominant						
CC+GC	65	43	0.4 (0.22 – 0.71)	0.001 *	0.63 (0.46 - 0.85)	0.002 *
Recessive						
GG+GC(Ref)	89	91				
CC	11	9	0.8 (0.31 - 2.02)	0.637	0.89 (0.58 - 1.37)	0.621
Additive						
2(CC)+GC	76	52	0.66 (0.42-1.05)	0.085	0.83 (0.67-1.02)	0.08
Frequency of C allele	76	52				

Results of the assessment of genotype distribution of the rs4918 SNP under various inheritance models exhibited significant increase of the C allele in DM patients when compared with those of the control group. However, the minor G allele frequency in the DM was also elucidated to be non-significantly higher than those of the control group.

The mutation of AHSG [rs4918, 766 (C/G)] on chromosome 3q27 is a missense mutation (Zeidan *et al* 2012) which is a change in one DNA base pair that results in the substitution of one amino acid for another in the protein made by the gene, which is the A**G**C serine changing into A**C**C threonine at 766 (C/G), and this change have been linked to low level of fetuin -A- (Verduijn *et al*, 2011, El-Batch *et al*, 2015), thus, it well loss its function in inhibition of the insulin receptor tyrosine kinase (Fatima *et al*, 2013) and have low effect on the prevalence of DM, but it has been reported the low level of fetuin A is associated with increased risk of CVD (Stenvinkel *et al.,* 2005) On the other hand, other scientific have been reported that the increase level of fetuin A is associated with DM, thus why we did not have increase the prevalence of G allele.

The current results are consistent with those of (Siddiq *et al* 2005) who showed that the C allele of rs4918 SNP is associated with DM. Also they are in agreement with AHSG Tag Single Nucleotide Polymorphisms Associate with T2DM and dyslipidemia Studies of Metabolic Traits in 7,683 White Danish Subjects (Andersen *et al,*. 2008). On the other hand, the present study is inconsistent with those of (El-Batch *et al*, 2015) which report that the G allele is not significant between DM and control group (p=0.9, χ^2= 0.27). It is a big differences are principally attributed to the ethnic diversity as reported in Chinese population were studied the genotype distribution and allele frequency of AHSG (rs4918) was found no significantly different (P> 0.05) (Wang *et al*; 2012).

Also there are increasing number of the observations suggesting that race (ethnic) differences may explain the contradictory effects of different AHSG genotypes and their putative function, it was reported by Dvornyk *et al*. (2003) that the 766 (G) allele frequency is similar to that among American Caucasians, but Osawa *et al*. (2001) founds slightly higher than that in German Caucasians. Furthermore, Hispanic and Caucasian populations have different frequencies of minor alleles at *AHSG*, making this a gene of interest in the study of health disparities Katie *et al.*, (2017). Also Jensen *et al.*, (2013) has been reported that the G allele are present more in Caucasians 13 % than in African Americans (11%).

The relationship between Fetuin -A- and obesity appears to be mediated at least in part by genetics. A variant of the *AHSG* gene that is associated with lower Fetuin -A- level is more common among normal weight men compared to overweight and obese men. Also Osawa *et al*. (2005) reported that individuals carrying the rare alleles for 766 (C/G) amino acid substitutions had significantly reduced serum fetuin A levels. Which in turn suggests a protective role of AHSG gene variation in body fat accumulation in man.

3.2.6. Biochemical characteristics of DM in relevance to the distribution of the genotypes of (rs4918) of AHSG gene polymorphism

To verify the involvement of the investigated SNP in directing the changes of the pathophysiology in DM patients, data were analyzed with respect to the distribution of the genotypes. Genotypes of the (rs4918) of AHSG gene were considered only due to the significant changes of this SNP with respect to the occurrence of T2DM that were obtained.

The FBS, HbA1c, and lipid profile of the DM subjects relative to codominant model (GG, GC, CC) of AHSG gene polymorphism (rs4918) were analyzed by ANOVA (table 3. 5). The FBS, HDL/LDH, and HbA1c were highly significant (p value < 0.001) with the GG, GC and CC genotypes, while the HDL-C with GG and GC genotype, the LDL-C with GC genotype, the VLDL-C and TG with GG genotype. As the HDL-C and LDL-C was significant (P value 0.001) with the CC genotype, while the LDL-C was significant (P value = 0.037) with the GG genotype, the VLDL-C and TG was (p value = 0.004) with the GC genotype, the non-HDL (p value = 0.028) with the CC genotype all parameter and genotype was comparing between the DM patients and the control group (table 3. 5).

The current study found no significant differences in BMI in both group and these result were consistent with György *et al,*. (2016), and Aisha *et al*, 2018, but not consistent with Catharina *et al,*. (2005).

Table (3. 5): Biochemical characteristics of DM individuals according to AHSG gene SNP (rs4918) genotype (codominant model)

AHSG SNP	**rs4918**			
Genotypes		**GG (35)** (Mean ±SD)	**GC (54)** (Mean ±SD)	**CC (11)** (Mean ±SD)
BMI (Kg/m^2)	DM	25.35 ±2.2	26.26±3.21	27.37±4.36
	Control	25.37±2.2	26.98±2.18	22.74±7.56
	P-value	0.5	0.107	0.066
FBS (mg/dL)	DM	248.83±77.25	245.18±68.27	194.83±57.83
	Control	96.15±15.96	96.14±20.19	91.28±10.11
	P –value	**< 0.001**	**< 0.001**	**< 0.001**
HDL (mg/dL)	DM	37.77±13.01	36.97±9.23	35.09±10.88
	Control	48.02±6.35	46.64±5.53	48.97±6.26
	P –value	**< 0.001**	**< 0.001**	**0.001**
TC (mg/dL)	DM	167.71±38.92	166.8±37.85	171.59±43.54
	Control	169.89±39.99	175.44±14.01	160.48±10.96
	P –value	0.398	0.066	0.215
LDL (mg/dL)	DM	111.71±28.08	110.81±24.84	126.96±34.4
	Control	98.36±42.79	88.42±17.15	84.6±6.73
	P –value	**0.037**	**< 0.001**	**0.001**
VLDL (mg/dL)	DM	45.57±18.97	50.28±18.52	41.96±15.7
	Control	32.27±9.91	36.18±26.07	34.9±8.41
	P –value	**< 0.001**	**0.004**	0.108
TG (mg/dL)	DM	227.68±94.91	251.43±92.61	209.81±78.52
	Control	161.39±49.58	180.91±130.36	174.51±42.05
	P –value	**< 0.001**	**0.004**	0.108
Non-HDL (mg/dL)	DM	129.93±38.78	129.83±35.91	136.5±38.15
	Control	121.87±42.2	128.8±16.41	111.51±6.93
	P –value	0.176	0.427	**0.028**
HDL/LDL (mg/dL)	DM	3.19±1	3.11±0.77	3.83±1.17
	Control	2.11±1.09	1.92±0.48	1.76±0.32
	P –value	**< 0.001**	**< 0.001**	**< 0.001**
HbA1c (%)	DM	9.39±1.93	9.36±1.72	9.71±1.87
	Control	4.44±0.93	4.44±0.86	4.84±0.37
	P –value	**< 0.001**	**< 0.001**	**< 0.001**

The association between the FBS and HbA1c with the genotype may be due to the inhibitory function of the tyrosinase kinase of the fetuin A protein as reported in (Fatima *et al*., 2013). While the association between the lipids profile with the genotype as reported a previously in Kotronen and Yki-Jarvinen (2008), Khalil and Kuobaili (2013), and Ix *et al,* (2012)

Our result were in agreement with Shanshan *et al.*, 2013 by the association between the BMI with the genotype where the is no significant association between them in both reports.

Our result were disagreement with György *et al*., 2016 that reported there is no significant association between the SPN rs4918 with the BMI, total cholesterol, LDL-C, TG, and gloucose (p value > 0.05). But we disagree with György *et al,*. (2016) by the G allele frequent which we have more frequent in overweight, but in his report, they found that the G allele was more frequent among lean than obese patients (RR=1.067, 95%CI=1.053–2.651, p=0.015).

Also out result were disagreement with Jensen, *et al,* 2013 result were have been reported that rs4918 is do not appear to be related to metabolic markers including insulin levels, lipids, BMI, and fasting glucose, as it in agreement with our result.

Siddiq *et al.,* 2005 also disagreed with us by the association between the SNP genotypes with the BMI, plasma level of glucose, triglycerides or cholesterol. But in agreement by the association between the SNP and the diabetic.

Chapter Four: Conclusion, Recommendation and Future Work

4. Conclusion, Recommendation, and Future Work:

4.1. Conclusion

1- The AHSG (rs4918) gene polymorphism is associated with DM.

2- The C allele frequency of rs4918 at 766 (C/G) was more frequent in diabetic patients than the control group.

3- The significant association between the FBS, HbA1c and lipid profile may be due to the effect of the SNP rs4918 at 766 (C/G) on their level.

4.2. Recommendation, and Future Work

1- Analysis of another SNPs of the AHSG gene and observe their effect on causing DM.

2- Studying the effect on AHSG on subject with diabetic, and check for the effect of this gene on causes the DM.

3- Increasing the population number and measure the level of fetuin -A- in serum and observe there effect on DM.

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Appendices

Appendix (A)

Questionnaire

University of Karbala – College of Medicine

Department of Biochemistry

((Experimental Data))

Association between Fetuin –A- Gene Polymorphism With its level in Iraqi type -2- diabetic patient.		
Sample No.:	**Type of study:** Case-Control	**Gender:**
BMI :	**Diet Habit:**	**Smoking:** Slightly ; Moderately ; Severe
Hypertension: **Diastolic** **Systolic**	**Age:**	**Exercise :** Heavy ; Moderate ; Slightly
Case Severity and Description:		
Exclusion criteria:		
Type of treatment; **Dietary** restriction ; Hypoglycemic tablets ; Insulin Therapy		

Biomarkers		
Biomarker	Concentration	Unit
Blood Sugar:		
Duration of diabetes		
Troponin I		
Lipid profile: TC HDL-C VLDL-C TG LDL-C		
Notes:		

Appendices

Appendix (B)

- **Determination of lipid profile**
- **Determination of Total Cholesterol (TC) concentration**

* Principle;

This method was involved the enzymatic reaction as shown;

Cholesterol ester + H_2O -- $^{Cholesterol\ Esterase\ (CE)}$ → Cholesterol +Fatty acids

Cholesterol + ½ O_2 + H_2O -- $^{Cholesterol\ Oxidase\ (CO)}$ → Cholestenone +H_2O_2

$2H_2O_2$ + Phenol + 4-aminoantipyrine -- $^{Peroxidase\ (POD)}$→ Quinoneimine + $4H_2O$

Where; Cholesterol Esterase (CE), Cholesterol Oxidase (CO) and Peroxidase (POD).

Procedure:

1- Reagents and samples was brought at room temperature, and was added to the labelled tubes;

Tubes	Blank	Sample	Cal Standard
R1. Monoreagent	1.0 mL	1.0 mL	1.0 mL
Sample	-	10 µL	-
CAL. Standard	-	-	10 µL

2- The tubes were mixed and incubated for 10 min at room temperature.

4- The absorbance (A) of the samples and the standard was read at 500 nm against the reagent blank.

The color is stable for at least 30 min protected from light.

Calculation:

TC (mg/dL) = A_{sample} / $A_{standard}$ * $C_{standard}$ (200 mg/dL)

Reference Value; (200 - 239 mg/dL)

Appendices

- **Determination of serum Triglyceride concentration:**

Principle:

The method was based on the enzymatic hydrolysis as shown;

Triglyceride + $3H_2O$ -- LPL → Glycerol + 3 fatty acid

Glycerol + adenosin triphosphate -- GK → G3P + adenosine diphosphate

G3P+ O_2 -- GPO → Dihydroxyacetone-phosphate + H_2O_2

H_2O_2 + 4-Chlorophenol + PAP -- POD → Quinoneimine (pink) + H_2O

Where; LPL (lipoprotein lipase), GK (glycerol kinase), G3P (glycerol-3-phosphate), and GPO (glycerophosphate oxidase).

Procedure:

1- Reagents and samples was brought at room temperature, and was added to the labelled tubes;

Tubes	Blank	Sample	Cal Standard
R1. Monoreagent	1.0 mL	1.0 mL	1.0 mL
Sample	-	10 µL	-
CAL. Standard	-	-	10 µL

2- The tubes were mixed and incubated for 15 min at room temperature.

3- The absorbance (A) of the samples and the standard was read at 500 nm against the reagent blank.

The color is stable for at least 30 min protected from light.

Calculation:

TG (mg/dL) = $A_{sample} / A_{standard} * C_{standard}$

Reference range: (60-160 mg/dL).

Appendices

- **Determination of high density lipoporten concentration:**

Principle:

This method was based on the selective precipitation of apoliprotein B-containing lipoproteins (VLDL-C, LDL-C and (a) Lpa) by phosphotungstic acid/$MgCl_2$ and centrifugation, then subsequent enzymatic analysis of HDL-C as residual cholesterol remaining in the clear supernatant

Procedure:

I- Precipitation:

1- Reagents and samples was brought at room temperature, and was added to the labelled tubes;

Sample or Standard	0.2 mL
Precipitating reagent	0.4 mL

Ratio sample / reagent = 1/2
Dil. Factor = 3

2- The tubes was shaken by vortex and allowed to stand for 10 min at room temperature, then was centrifuged for 10 min at 4000 rpm.

3- The clear supernatant was separated within 2 hours.

In case of turbid supernatants caused by elevated TG (>350 g/dl) the sample was diluted 1:2 with saline and steps 2,3,4, and 5 was repeated.

II. *Colorimetry:*

1. the cholesterol MR monoreagent and cholesterol standard (50 mg/dL) of the kit was brought to room temperature, and was added as;

Tubes	Blank	Sample	Standard
Monoreagent	1.0 mL	1.0 mL	1.0 mL
Supernate	–	50 µL	–
Standard	–	–	50 µL

2- The tubes were mixed and let to stand for 10 min at room temperature.

3- The absorbance (A) of the samples and the standard were read at 500 nm against the reagent blank.

The color is stable for at least 30 min protected from light.

Calculation: HDL-C (mg/dL) = $A_{sample} / A_{standard} * C_{standard}$

Reference range: < 40 mg/d.

Appendices

- **Determination of serum VLDL – C concentration:**

VLDL-C = TG/5 mg/dL.

Reference Value; 2-30 mg/dL

- **Determination of serum LDL - C concentration:**

LDL-C = TC - (HDL-C +VLDL-C) mg/dL.

Reference Value; 100-160 mg/dL

- **Measurement of Fasting Serum Glucose level:**

Principle;

This method was based on the oxidation reaction as fallowing;

Glucose +H_2O + O_2 -- GOD → Gluconate + O2 + H_2O_2

H_2O_2 + phenol + 4-aminoantipyrin --POD→ 4H2O + Quinoneimine

Where glucose oxidase (GOD), glycerophosphate oxidase (POD).

PROCEDURE

1- Reagents and samples was brought at room temperature, and was added to the labelled tubes;

Tube	Blank	Sample	Standard
R1 Monoreagent	1.0 mL	1.0 mL	1.0 mL
Supernate	-	10 µL	-
CAL Standard	-	-	10 µL

2- The tubes were mixed and let to stand for 10 min at room temperature.

3- The absorbance (A) of the samples and the standard was read at 500 nm against the reagent blank.

The color is stable for about 2 hours protected from light.

Calculation:

Glucose (mg/dl) = $A_{sample} / A_{standard} * C_{standard}$

Reference range: (70 -110) mg/dl.

Appendices

- **Measurement of HbA1c level;**

Principle;

This procedure was utilized a weak binding cation-exchange resin for the rapid separation of glycated hemoglobin A1c from all the other hemoglobins. A hemolyzed preparation of the whole blood was mixed for 5 minutes with a weak binding cation-exchange resin, where all the hemoglobins except A1c which remains in the solution was bounded to the resin. Then the A1c was separated from the mixture, and determined by measuring the absorbance at 415 nm of the A1c fraction and the total hemoglobin fraction. The ratio of the two absorbances gives the percent of HbA1c.

Hemolysate Preparation:

A 500 µL of lysing Reagent was dispensed into a labeled tubes as, Standard, Control, Sample. Taken from it a 100 µL of well-mixed, and allowed to stand for 5 min.

Glycohemoglobin preparation:

1. A 70 µL of the hemolysate was added into the resin tube (RA).
2- Filter Separators was puts in the tubes so that the rubber sleeve is approximately 1 cm above the liquid level.
3. The tubes were mixed for 5 min.
4. The filter separator was pushed into the tubes until the resin is firmly packed.
5. The supernatant can be poured into a cuvette for absorbance measurement.
6. The absorbance (A) was read at 415 nm against the reagent blank, where they was represent the glycohemoglobin.

Total Hemoglobin Fraction:

1. A 5.0 mL of nuclease free water, and 20 µL of the hemolysate was placed into a labeled tubes: Standard, Control, Sample, and mixed.
2. The absorbance (A) was read at 415 nm against the reagent blank, where they was represent the total hemoglobin.

Appendices

Calculation:

% HbA1c = $R_{unknown} / R_{standard} * C_{standard}$

$R_{unknown}$ = Ratio $_{unknown}$ = Abs of HbA1c ($_{unknown}$) / Abs of Hb Tot ($_{unknown}$)

$R_{Standard}$ = Ratio $_{Standard}$ = Abs of HbA1c ($_{Standard}$) / Abs of Hb Tot ($_{Standard}$)

Reference range: < 6.5%.

الخلاصة:

الخلفية: يعتبر مرض السكري (DM) المرض الصامت و المشترك في العالم. العديد من عوامل الخطر البيئية والجينية في حدوث مرض السكري. في السنوات الأخيرة ، ازدادت نسبة الاشخاص المصابين بالسكري من بين امراض اخرى في العراق. وقد أظهرت العديد من الدراسات أن تعدد الأشكال النوكليوتيدات الواحده (SNP) ضمن جين fetuin A (rs4918) at 766 (C/G) ترتبط مع DM ولهذا يمكن اعتبار SNP هي احدى مسببات داء السكري النوع الثاني.

الهدف: لدراسة ارتباط تعدد الاشكال الجينيه AHSG (rs4918) بموقع 766 (G/C) في عينة من سكان كربلاء.

طرائق العمل: تتألف الدراسة من مائتين شخصًا ، شملت حوالي 100 مريض من داء السكري من النوع الثاني (T2DM) ، و 100 منهم يبدون بصحة جيدة. وشملت البيانات المظهرية: مؤشر كتلة الجسم ، ومستوى السكر في الدم (FBS) ، السكر التراكمي (HbA1c) ، ومستوى الدهون في الجسم. تم إجراء الكشف عن تعدد الأشكال AHSG (rs4918) بواسطة تفاعل البلمرة المتسلسل (TaqMan PCR). تم استخلاص الحمض النووي من الدم الكامل وتم تحقيق التنميط الجيني باستخدام بوادئ لجين AHSG. طبقت برامج التحليلات الإحصائية المختلفة لتحليل بيانات البحث. و تم فحص النمط الوراثي وتردد الأليل تحت النمط السائد, المشترك, المتنحي مع استخدام تحليل الانحدار اللوجستي متعدد الحدود.

النتائج: عثر على توزيع النمط الوراثي للطفره (rs4918) لتكون داخلة في معادلة هاردي-واينبرغ للتوازن، وكان هنالك اختلاف معنوي في المرضى الذين يعانون من النمط الجيني المتغاير (GC) (X^2= 9.78, P =0.001, OR = 2.58, CI 95%= 1.41 – 4.71), ولم يكن هنالك ختلاف معنوب في المرضى الذين يعانون من النمط الجيني المتغاير (CC) (CI 95%=0.74 – 5.28, OR= 1.99, X^2= 1.95,P value =0.162). والمنط الجيني السائد (G) كان (0.74) في الاشخاص المصابين بالسكري و (0.62) في الاصحاء و النط الجيني المتنحي (C) كان (0.26) في الاشخاص المصابين بالسكري و (0.38) في الاصحاء. بينما المرضى الذين يعانون من النمط الجيني المتغاير (GC) في اطار النمط المشترك كان هنال اختلاف معنوي (OR = 0.38, CI 95%= 0.21 – 0.7, P =0.001) وكانت اعلى من مجموعة الاصحاء, بينما المرضى الذين يعانون من النمط الجيني المتغاير (CC) في اطار النمط المشترك لم يظهروا اختلاف معنوي (OR = 0.5, CI 95%= 0.18 - 1.33, P = 0.167) وكانت أعلى من مجموعة الاصحاء. اما في إطار النموذج السائد ، اظهر المرضى مع الأنماط الجينية

(CC + GC) وجود اختلاف معنوي (OR = 0.4, CI 95% = 0.22 - 0.71, P = 0.001) وكانت أعلى من مجموعة الاصحاء بشكل ملحوظ . و لم يحتوي تواتر الأليل الرئيسي (C) على اختلاف معنوياً (P = 0.085) وكانت أعلى في DM بالمقارنة مع مجموعة الاصحاء. وتم فحص السكر والدهون والسكر التراكمي وnon-HDL و HDL/LDL حسب طريقة co-dominant بين الاشخاص المصابين بالسكري والاشخاص الاصحاء وكان هنالك الاختلاف المعنوي كبير (p <0.001) بين FBS, HDL/LDH, and HbA1c مع GG, GC, CC, وايضاً في HDL-C مع GG, GC, وايضاً في LDL-C مع GC, وايضاً VLDL-Cو TG مع GG. و كان هنالك اختلاف معنوي (P < 0.001) بين HDL-C و LDL-C مع CC, وايضاً LDL-C مع GG (P value = 0.037), وايضاً VLDL-C و TG مع GC (p value = 0.004), وايضاً non-HDL مع CC (p value = 0.028).

<u>الاستنتاجات:</u> ارتبط تعدد الأشكال الجيني AHSG (rs4918) مع داء السكري. بالاضافه الى الارتباط المعنوي بين الطفره و مستوى السكر في الدم والسكر التراكمي والدهون, حيث ان هذا الارتباط بسبب الطفره.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء / كلية الطب

فرع الكيمياء الحياتية

العلاقة بين تعدد الاشكال الجينية جين -a- fetuin مع مستواه في مرضى السكري من النوع الثاني في كربلاء.

رسالة مقدمة الى مجلس كلية الطب / جامعة كربلاء كجزء من متطلبات

نيل شهادة الماجستير في الكيمياء السريرية

من قبل

حسين سعد محمدعلي

(بكلوريوس تقنيات تحليلات مرضية , 2012)

اشراف

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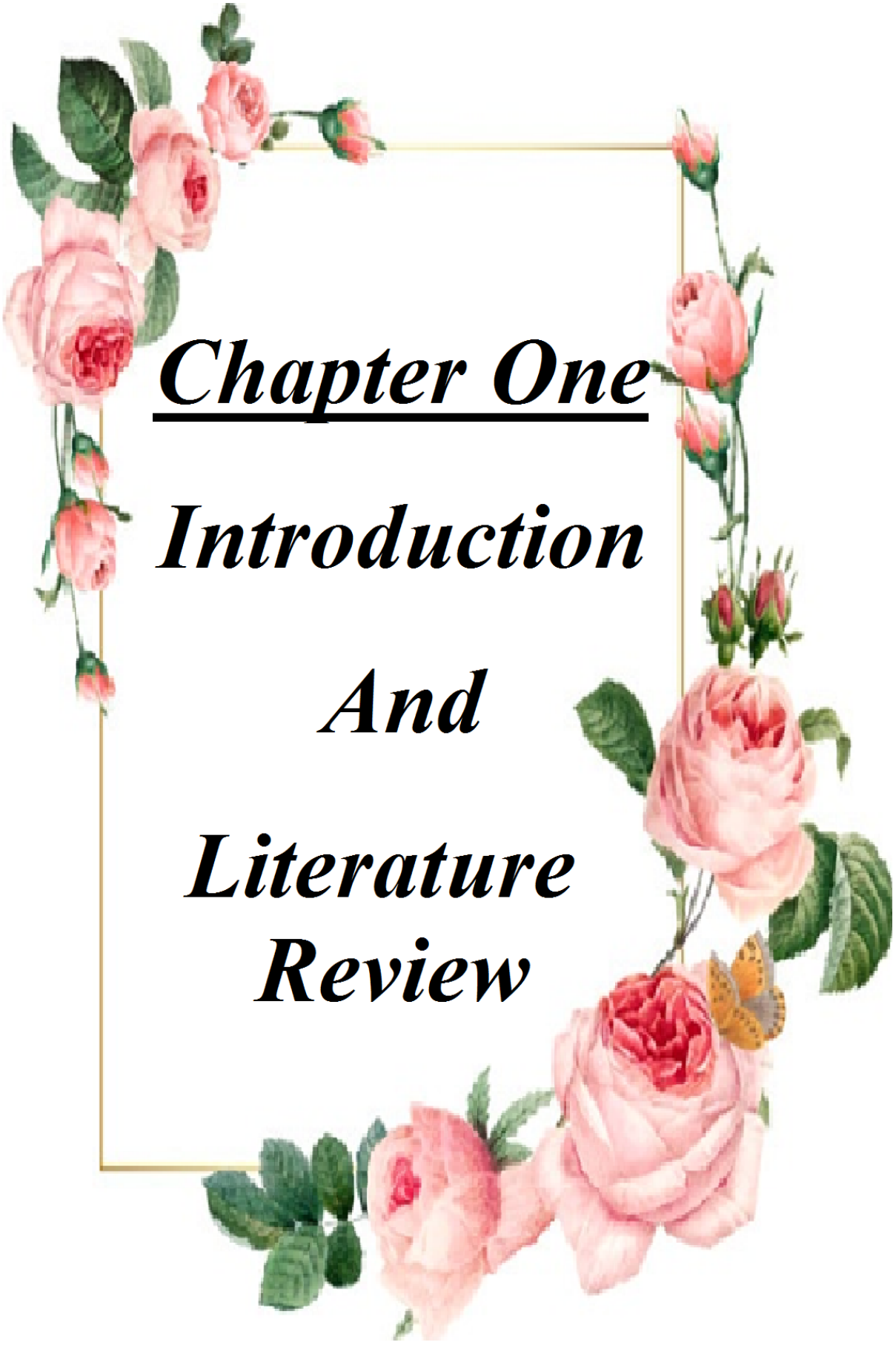
معهد الهندسة الوراثية
جامعة بغداد

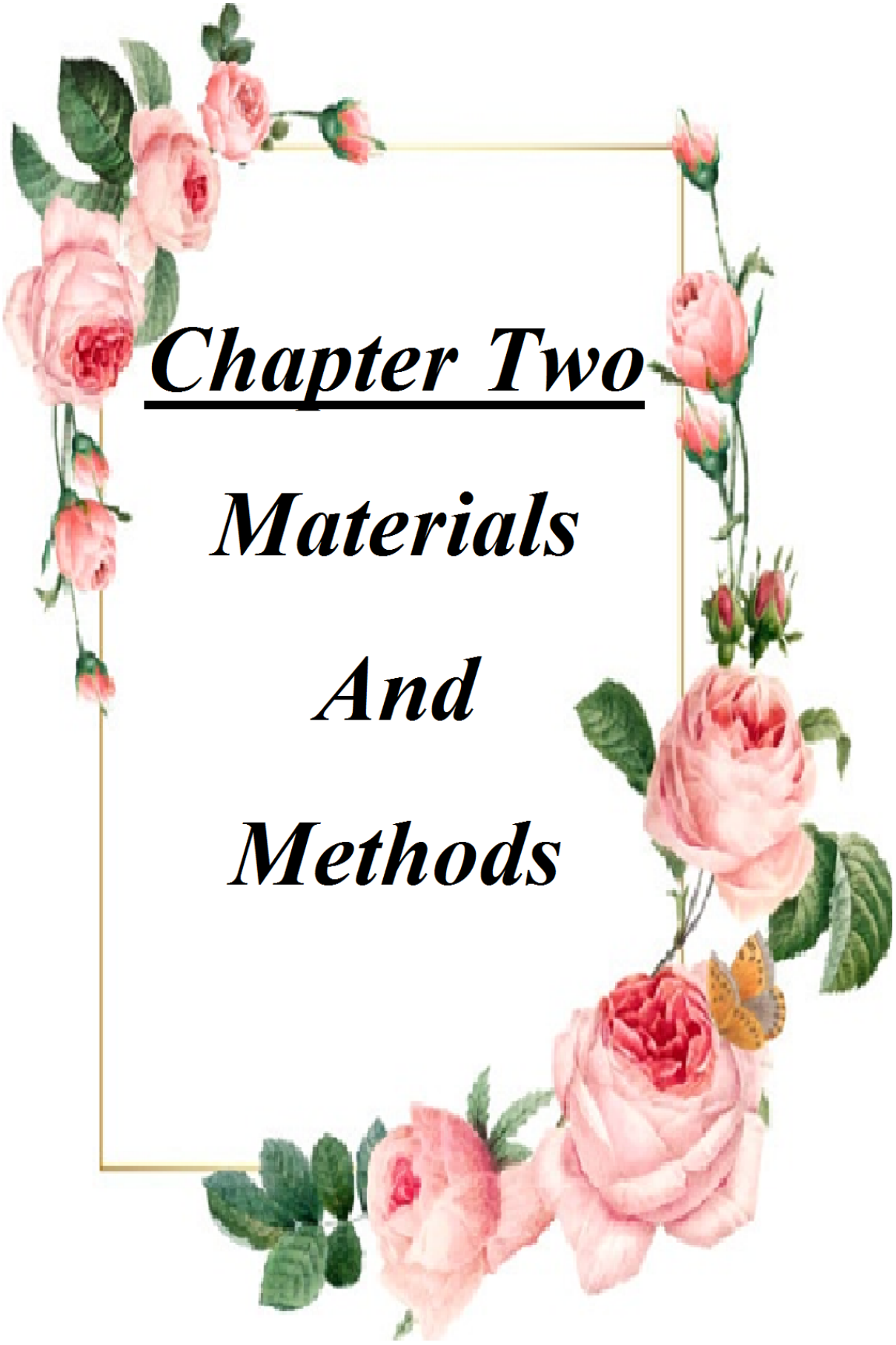
ميلادي (2019)

هجري (1440)



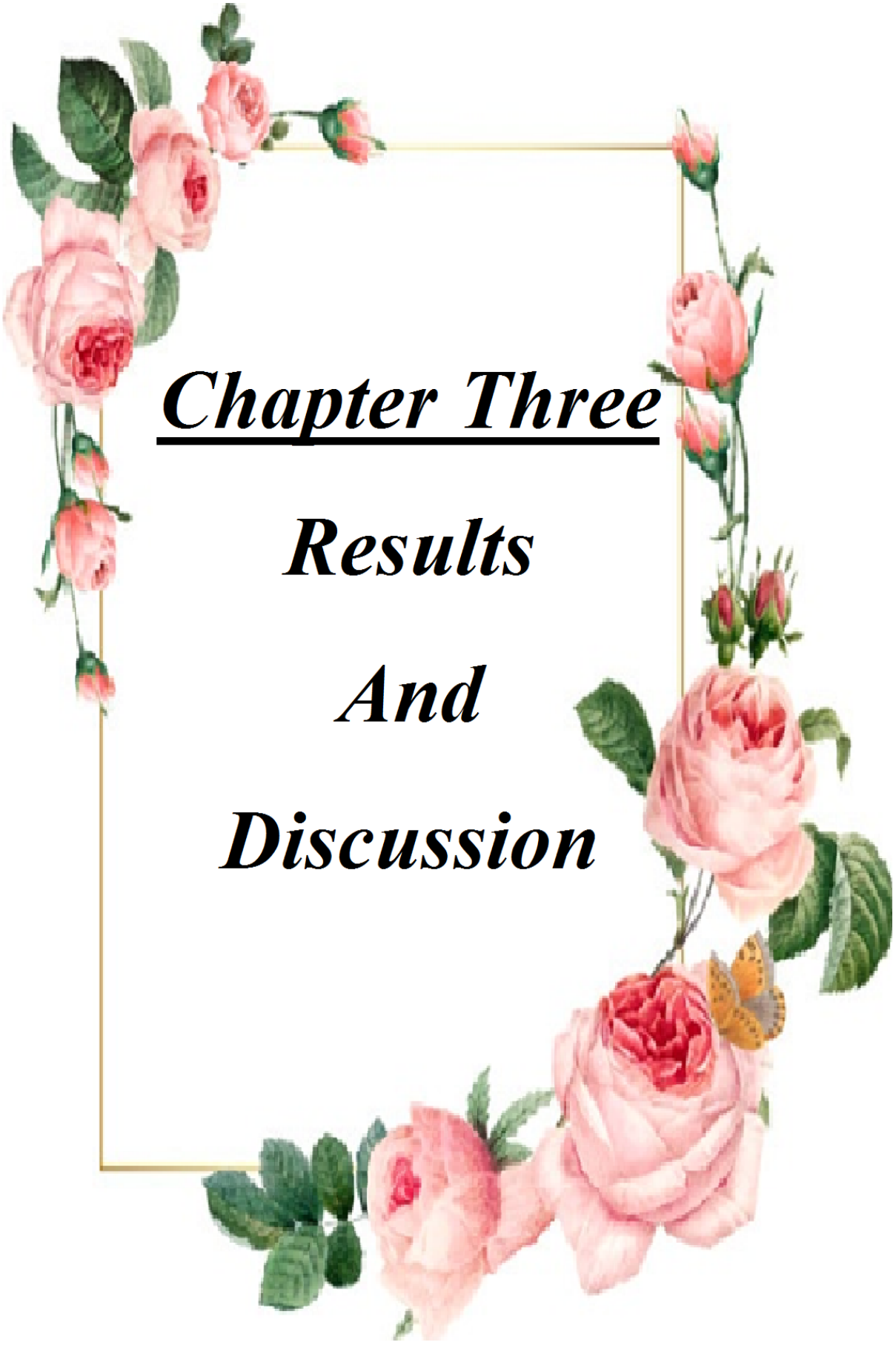
Chapter One

Introduction And Literature Review



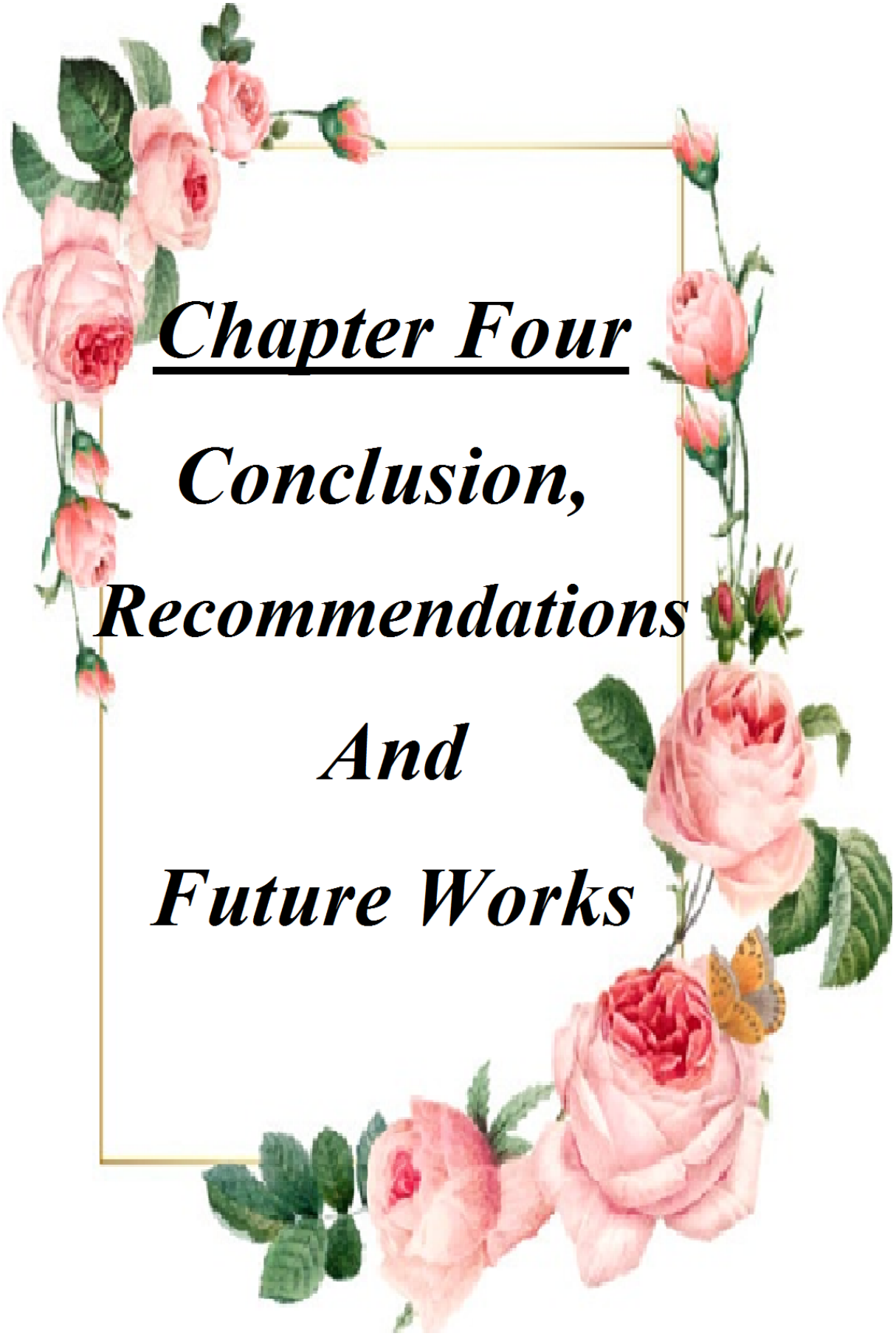
Chapter Two

Materials And Methods



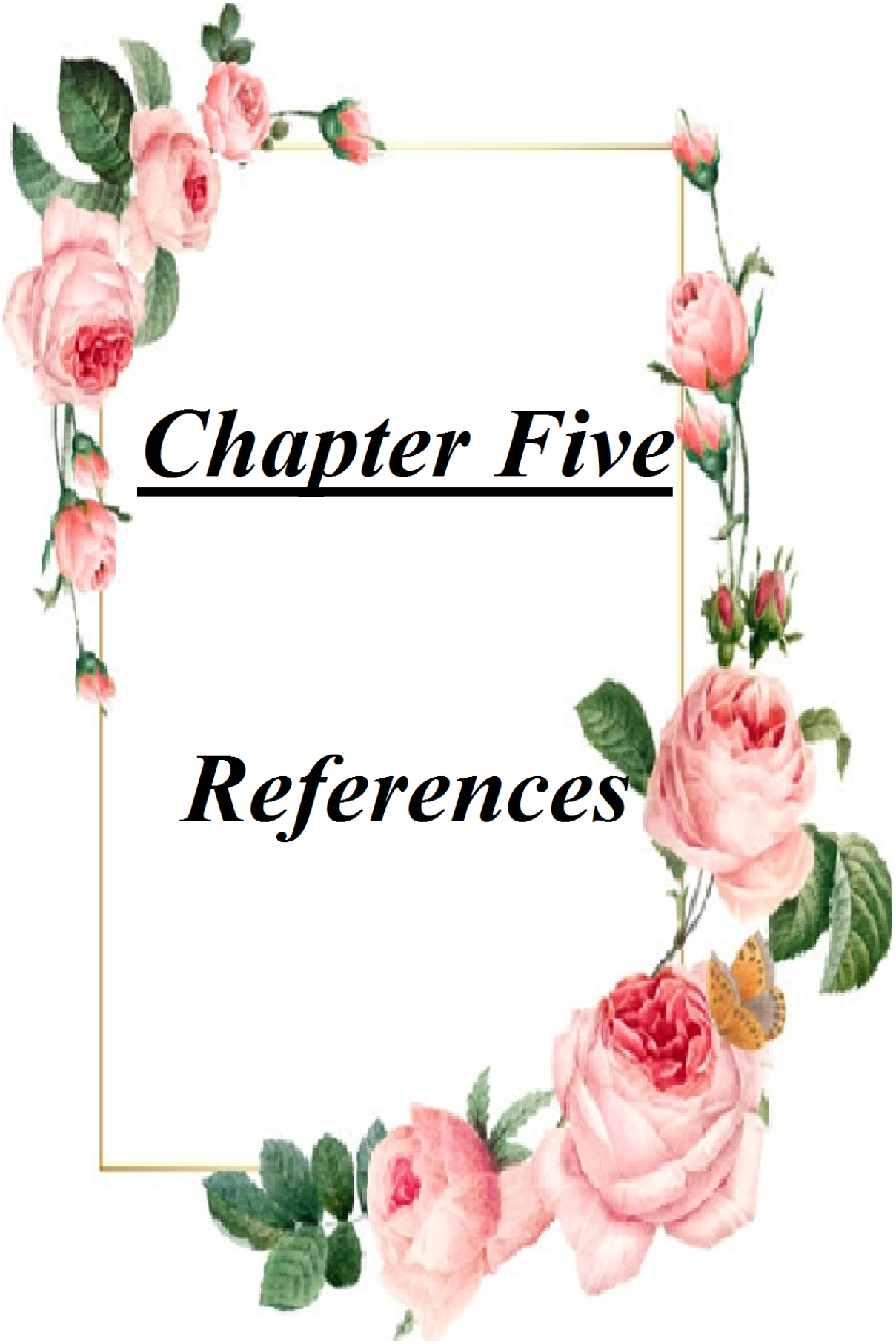
Chapter Three

Results And Discussion



Chapter Four

Conclusion, Recommendations And Future Works



Chapter Five

References

Appendix