A STUDY OF GENETIC POLYMORPHISM IN ATP BINDING CASSETTE PROTEIN GENE (ABCA1) AMONG TYPE 2 DIABETES MELLITUS PATIENTS

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Partial fulfillment of the regulations required for the award of

M.D. DEGREE

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THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH

COIMBATORE

MAY – 2018

CERTIFICATE

CERTIFICATE

This is to certify that the dissertation titled **"A STUDY OF GENETIC POLYMORPHISM IN ATP BINDING CASSETTE PROTEIN GENE (ABCA1) AMONG TYPE 2 DIABETES MELLITUS PATIENTS"** submitted by **Dr.B.Dhanalakshmi** is an original work done by her at PSG Institute of Medical Sciences and Research, Coimbatore. This work was done under the guidance of Dr.B.Gayathri, Professor , Department of Biochemistry, PSG Institute of Medical Sciences and Research, Coimbatore.

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DECLARATION

I solemnly declare that this dissertation titled **"A STUDY OF GENETIC POLYMORPHISM IN ATP BINDING CASSETTE PROTEIN GENE (ABCA1) AMONG TYPE 2 DIABETES MELLITUS PATIENTS"** was written by me in the Department of Biochemistry, PSG Institute of Medical Sciences and Research, Coimbatore under the guidance of **Dr.B.Gayathri**, Professor, Department of Biochemistry, PSG Institute of Medical Sciences and Research.

This dissertation is submitted to the Tamil Nadu Dr.M.G.R. Medical University, Chennai in partial fulfillment of the university regulations for the degree of M.D. Biochemistry – Branch XIII examinations to be held in May 2018.

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I am extremely thankful to all my patients who consented to be a part of my study without whom the whole study would have been impossible. I wish them all good health and long life!

ABBREVIATIONS

ABBREVIATIONS:

DM – Diabetes mellitus

NCD – Non communicable disease

WHO – World health organization

HDL-c – High density lipoprotein cholesterol

ABCA1 – ATP binding cassette transporte A1

apo -A1 – Apolipoprotein A1

HbA1c – Glycosylated hemoglobin

FPG – Fasting plasma glucose

BMI – Body mass index

IFG – Impaired fasting glucose

IGT – Impaired glucose tolerance

NEFA – Non esterified fatty acids

FFA – Free fatty acids

PI3K – Phosphoinositide 3- kinase

Akt – Protein kinase B

mTORC1 – Mammalian target of rapamycin complex 1

HSL – Hormone sensitive lipase

VLDL-c – Very low density lipoprotein cholesterol

LPL – Lipoprotein lipase

LDL-c – Low density lipoprotein cholesterol

TGL – Triglycerides

CETP - Cholesterol ester transfer protein

ATP – Adenosine triphosphate

ADP – Adenosine diphosphate

NAD - Nicotinamide adenine dinucleotide

DCCT – Diabetes control and complications trial

NGSP - National Glycohemoglobin Standardization Program

EDTA – Ethylene diamine tetraacetic acid

SOD – Superoxide dismutase

NEB – New England Biolabs

NCEP ATP – National Cholesterol Education Programme Adult Treatment Panel

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To
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Department of Biochemistry
Guides: Dr B Gayathri / Dr S Thiagarajan
PSG IMS & R
Coimbatore

Ref: Project No.15/376

Date: December 30, 2015,

Dear Dr Dhanalakshmi,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 14.12.2015 to conduct the research study entitled *"A study of genetic polymorphism in ATP binding cassette protein (ABCA1) among type 2 diabetes mellitus patients"* during the IHEC review meeting held on 28.12.2015.

The following documents were reviewed and approved:

1. Project Submission form
2. Study protocol (Version 1 dated 14.12.2015)
3. Informed consent forms (Version 1.1 dated 30.12.2015)
4. Data collection tool (Version 1 dated 14.12.2015)
5. Current CVs of Principal investigator, Co-investigator
6. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 28.12.2015 at Research Conference Room, PSG IMS & R between 10.00 am and 12.30 pm:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Mrs Y Ashraf	MPT	Physiotherapy	Female	Yes	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Mr Gowpathy Velappan	BA., BL	Legal Advisor	Male	No	No
4	Dr A Jayavardhana	MD	Clinician (Paediatrics)	Male	Yes	Yes
5	Mr P Karuppuchamy	M Phil in PSW	Social Scientist	Male	Yes	Yes
6	Mrs G Malarvizhi	M Sc	Nursing	Female	Yes	Yes
7	Mr. R. Nandakumar (Chairperson, IHEC)	BA., BL	Legal Expert	Male	No	Yes
8	Dr. Parag K Shah	DNB	Clinician (Ophthalmology)	Male	No	No

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9	Dr. G. Rajendiran	DM	Clinician (Cardiology)	Male	Yes	Yes
10	Mrs P Rama	M Pharm	Non-Medical (Pharmacy)	Female	Yes	Yes
11	Dr. Seetha Panicker (Vice-chairperson, IHEC)	MD	Clinician (Obstetrics & Gynaecology)	Female	Yes	Yes
12	Dr R Senthil Kumar	MD	Clinician (Endocrinology)	Male	Yes	Yes
13	Dr. S. Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
14	Dr. Sudha Ramalingam (Alternate Member-Secretary, IHEC)	MD	Public Health, Epidemiology, Genetics, Ethicist	Female	Yes	Yes
15	Mrs. Swasthika Soundararaj	MBA	Lay person	Female	No	Yes
16	Dr. D. Vijaya	M Sc, Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
    a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
    b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
    c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
    d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
    e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented

f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review

7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

Thanking You,

Yours Sincerely,

SECRETARY
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COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Dr Sudha Ramalingam
Alternate Member-Secretary
Institutional Human Ethics Committee

URKUND

Urkund Analysis Result

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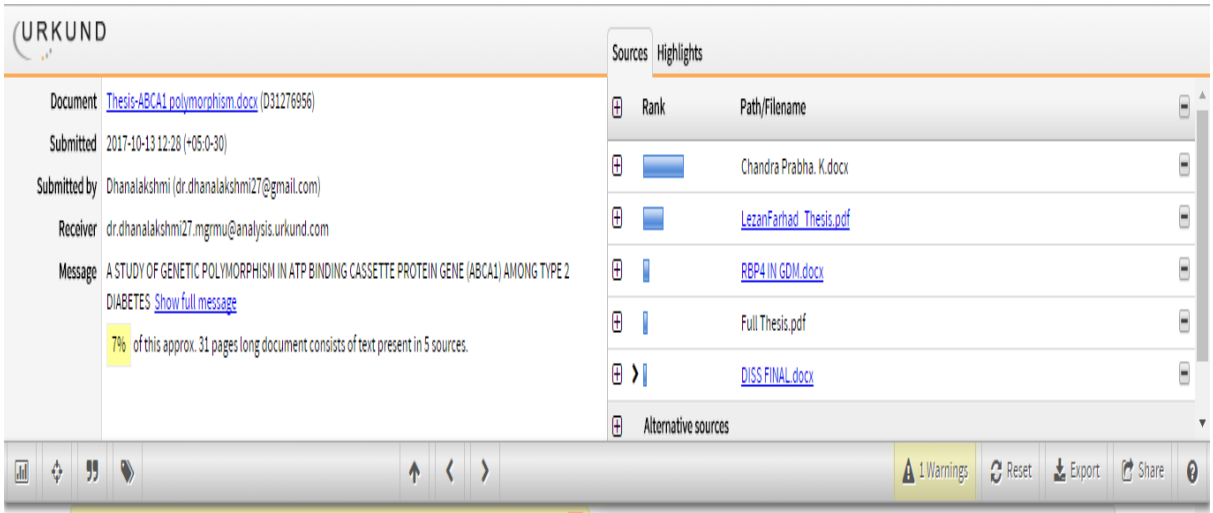

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INTRODUCTION Diabetes mellitus (DM) is a metabolic disorder characterized by chronic inflammation and derangement in the metabolism of

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INTRODUCTION Diabetes Mellitus (DM) is a metabolic disorder characterized by the presence of chronic hyperglycemia associated with impairment in the metabolism of

carbohydrate, protein and lipid and is considered to be contributed by several etiologies . It results from defective secretion of insulin, action of insulin or both1.Based on this, two major forms of diabetes, type 1 and type 2were recognized. Lack of or severe reduction in insulin secretion due to autoimmune or viral destruction of β cells is responsible for type 1 diabetes, which accounts for about 10% of diabetic population.Type 2 DM is the most common form which constitutes for more than 90% of diabetic patients1. Type 2 DM was earlier known as non-insulin dependent DM2. The multifactorial etiology of type 2 DM is contributed by genetic, environmental and behavioral risk factorsand found to be one of the four major non-communicable diseases (NCDs) recognized by the World Health Organization (WHO). Over the past two decades type 2 DM has affected more than 150 million people worldwide3. Asia has become anoteworthy site of early onset type 2 DM epidemic and a major increase is expected in India in the next 20 years. Currently,more than 62 million individuals are diagnosed with type 2 DM in India. It is projected that by 2030, up to 79.4 million individuals in India may be affected by type 2 DM4. The complications of type 2 DM have become the leading cause of mortality and morbidity worldwide. There is escalation in the incidence of cardiovascular disease in patients with type 2 DM6. Even though epidemiological studies have shown that levels of HDL-cholesterol(HDL-c) is inversely associated to the risk of coronary artery disease and its thrombotic complications,it is becoming increasingly evident that HDL-c functionality is as important as HDL-c levels. This is because, HDL-c not only promote reverse cholesterol transport but also exert anti-oxidative, anti-inflammatory,anti-thrombotic and vasodilatory activities7. ATP- binding cassette transporter A1 (ABCA1) is a transmembrane protein also known as cholesterol efflux regulator protein, mediates the efflux of cholesterol and phospholipids to lipid-poor apolipoproteins (apo-A1 and apoE), which then form nascent high-density lipoproteins. Down regulation of

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INTRODUCTION

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic inflammation and derangement in the metabolism of carbohydrate, protein and lipid and is considered to be contributed by several etiologies . It results from defective secretion of insulin, action of insulin or both[1]. Based on this, two major forms of diabetes, type 1 and type 2 were recognized. Lack of or severe reduction in insulin secretion due to autoimmune or viral destruction of β cells is responsible for type 1 DM, which accounts for about 10% of diabetic population. Type 2 DM is the most common form which constitutes for more than 90% of diabetic patients[1].

Type 2 DM was earlier known as non-insulin dependent DM[2]. The multifactorial etiology of type 2 DM is contributed by genetic, environmental and behavioral risk factors and found to be one of the four major non-communicable diseases (NCDs) recognized by the World Health Organization (WHO). Over the past two decades type 2 DM has affected more than 150 million people worldwide[1]. Asia has become a noteworthy site of early onset type 2 DM epidemic and a major increase is expected in India in the next 20 years. Currently, more than 62 million individuals are diagnosed with type 2 DM in India. It is projected that by 2030, up to 79.4 million individuals in India may be affected by type 2 DM[3].

The complications of type 2 DM have become the leading cause of mortality and morbidity worldwide. There is escalation in the incidence of cardiovascular disease in patients with type 2 DM[4,5]. Even though epidemiological studies have shown that levels of HDL-cholesterol (HDL-c) is inversely associated to the risk of

coronary artery disease and its thrombotic complications, it is becoming increasingly evident that HDL-c functionality is as important as HDL-c levels. This is because, HDL-c not only promote reverse cholesterol transport but also exert anti-oxidative, anti-inflammatory, anti-thrombotic and vasodilatory activities[6].

ATP- binding cassette transporter A1 (ABCA1) is a transmembrane protein also known as cholesterol efflux regulator protein, mediates the efflux of cholesterol and phospholipids to lipid-poor apolipoproteins (apo-A1 and apo E), which then form nascent HDL. Down regulation of ABCA1, disrupts the cell's ability to remove cholesterol from its cytoplasm, leading to pathologic atherogenesis[7].

ABCA1 gene polymorphisms have been associated with abnormalities of both serum levels of HDL-c and the quality of HDL-c. Understanding the pathological mechanisms underlying HDL-c dysfunction may allow the allocation of unique therapies for specific population. Furthermore, characterization of the genetic pathways regulating HDL-c function, composition, and particle sizes will bring us closer to the breakthrough in preventing cardiovascular disease, the most dreaded complication of type 2 DM.

This study is done to find the association between ABCA1 gene polymorphism and type 2 DM, which might serve as a breakthrough for the treatment of dyslipidemia in type 2 DM.

AIM AND OBJECTIVES

AIM & OBJECTIVES

Aim:

To determine the genetic polymorphisms of R219K(rs2230806) and C69T (rs1800977) of ABCA1 gene in type 2 DM

Objectives:

1. To study the genotype and allele frequencies of ABCA1 gene polymorphism (R219K and C69T**)** in type 2 DM patients and non-diabetic healthy individuals

2. To find out the association between ABCA1 gene polymorphism and type 2 DM

REVIEW OF LITERATURE

REVIEW OF LITERATURE

DM is the foremost non-communicable disease which is prevalent worldwide. It is a complex metabolic disorder characterized by chronic hyperglycemia, related with alterations in metabolism of carbohydrate, protein and fat, primarily due to defective insulin secretion or action or both[8].

Epidemiology of type 2 DM:

The disease burden associated to type 2 DM is rising globally, fuelled by the comprehensive rise in the prevalence of obesity and detrimental lifestyles. The latest estimates show a worldwide prevalence of 382 million people with diabetes in 2013, anticipated to rise to 592 million by 2035[9]. Caribbean region and North America has got the maximum prevalence of disease (11%), subsequently the Middle East and North Africa have a prevalence of 9.2%[10] .It was estimated that almost 1 million Indians expire due to type 2 DM annually with the common age of onset being 42.5 years [11,12]. When compared to urban population, prevalence of type 2 DM is just one-fourth in the rural areas[13,14]. Type 2 DM has a male preponderance in early middle age when compared to women[15].In developing countries, people with diabetes are usually in 45 to 64 years of age while in developed countries majority of them are greater than 64 years of age[16].

Criteria for diagnosing type 2 DM[17]:

- Glycosylated hemoglobin (HbA1c) ≥ 6.5% or
- Fasting Plasma Glucose (FPG) ≥ 126 mg/dL or

- 2-hour plasma Post prandial glucose ≥ 200mg/dL during an Oral Glucose Tolerance Test or
- Random plasma Glucose ≥ 200 mg/dL with signs and symptoms of hyperglycemia.

Type 2 DM:

The foremost type of DM is type 2. It affects more than 90% of the inhabitants suffering from DM worldwide. There is a swift increase in the number of type 2 DM patients and this sizzling growth is noted in both rural and urban areas. It is characterized by disproportionate hepatic glucose production, erratic level of resistance to insulin action, decreased insulin secretion, and abnormalities in fat metabolism.

Risk factors for type 2 DM[17]:

Sedentary lifestyle, diet and associated epidemiological conversion has been recognized as risk factors for type 2 DM. Other major risk factors are listed below.

- Family history of DM
- Overweight with body mass index(BMI) ≥ 25 kg/m^2
- Lack of physical activity
- Ethnicity
- History of impaired fasting glucose(IFG)/impaired glucose tolerance(IGT)
- Blood pressure ≥ 140/90 mm of Hg

- Triglyceride(TGL) levels ≥ 250 mg/dL
- HDL-c ≤ 35 mg/dL
- Gestational DM
- Polycystic ovary syndrome (PCOS)

Pathogenesis of type2 DM:

Type2 DM is found to be as a result of combined effect of both genetic and acquired etiologies. This establishes a negative influence on the beta cell function and also on the sensitivity of the tissues to insulin [18]. For more than a few years, it was speculated whether the derangements in beta cell function or resistance to the action of insulin was the principal element underlying the pathogenesis. Insulin resistance being the prime defect has been tracked back to the classic studies of Kerr and Himsworth[19]. A combination of genetic as well as environmental factors contributed to the development of insulin resistance.

Insulin resistance:

Insulin resistance may be defined as an incompetence of insulin to yield its standard biological effects at usual circulating concentrations, which is habitually effective in normal subjects. There is loss of suppression of hepatic glucose production and also stimulation of glucose uptake by peripheral tissues is lost[20].Suppression of lipolysis in adipocytes by insulin is lost resulting in increased non-esterified fatty acids (NEFA) levels in plasma .This in turn results in stimulation of gluconeogenesis and triglyceride (TGL) synthesis in the liver. Inhibition of hepatic

glucose production in the face of hyperglycemia is absent leading to inappropriately high levels of glucose output from the liver[21].

Obesity:

Obesity is strongly associated with type 2 DM. Individuals with BMI >35 kg/m^2 have forty times greater risk of developing the disease than individuals with BMI <25 kg/m^2. Truncal obesity, inherited or a result of sedentary lifestyle is associated with insulin resistance, the key factor in the pathogenesis of the disease[21]. Type 2 DM is known to occur as a late phenomenon in obese subjects and is heralded by normal glucose tolerance or IGT for many years. This is described by the overworked beta cell hypothesis, according to which during initial years of obesity the patients are normoglycemic due to high rate of insulin secretion by the beta cells[22]. Later on, the beta cells are not able to compensate for the increased plasma glucose levels thereby resulting in the progression to type 2 DM from IGT.

Metabolic derangements in Type 2 DM:

Type 2 DM which is known for its complex polygenic nature is associated with dysregulation of carbohydrate, lipid and protein metabolism**.** Exposure to prolonged hyperglycemia causes irreversible changes to tissue metabolism.

Carbohydrate metabolism:

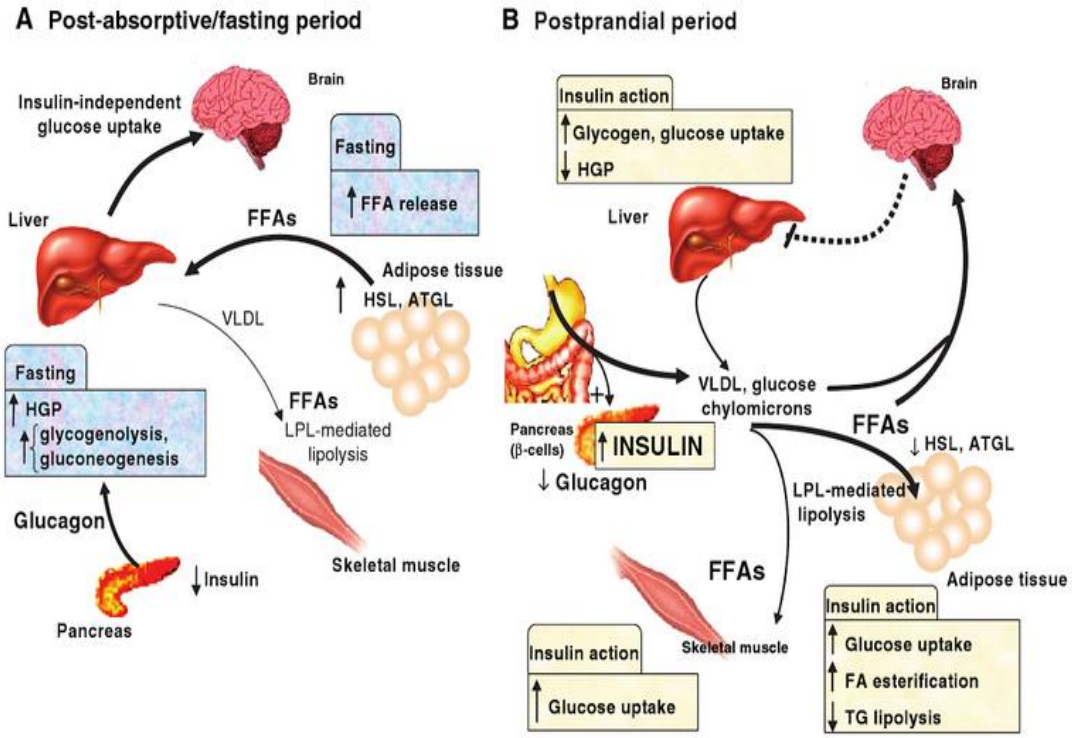
People with type 2 DM have high FPG levels and have a rapid change in glycemic levels following the ingestion of carbohydrate. This is possibly due to the decreased and delayed insulin secretion following ingestion of food[23].Chronic hyperglycemia alone or in combination with elevated free fatty acids (FFA) impairs insulin secretion. Glucose is also an important regulator of its own metabolism. In the presence of basal insulin concentrations, an increase in plasma glucose stimulates glucose uptake and suppresses glucose production. The ability of glucose to regulate its own metabolism is impaired in type 2 DM. This is commonly referred to as a defect in " glucose effectiveness[24]."

Following glucose ingestion, there is reciprocal change in the concentration of insulin and glucagon. Marked hyperglycemia results from failure of glucagon secretion when secretion of insulin is decreased and delayed in type 2 DM. For non-diabetic individuals, due to the low insulin secretion in fasting state its role in suppression of gluconeogenesis and glycogenolysis is negligible. Thus the source of glucose in healthy individuals during fasting is from glucagon stimulated glycogenolysis.

During the fasting state in type 2 DM, signaling from glucagon activates glycogenolysis and gluconeogenesis resulting in the production of plasma glucose.Insulin supplemented exogenously, brings about the utilization of peripheral glucose. But there is insulin deficiency in the portal circulation, which ensues in lack of appropriate regulation of gluconeogenesis and glycogenolysis in liver. Exogenous

insulin for individuals with type 2 DM in the fed state, is futile in quashing glucagon secretion through the physiological paracrine route. This causes a rise in the hepatic glucose production. The end result is postprandial hyperglycemia because the disappearance of glucose from circulation is exceeded by its appearance[25].

Glucose and Free Fatty Acid homeostasis



Source: Mark N. Feinglos, Mary Angelyn Bethel: Type 2 Diabetes Mellitus:: An Evidence-Based Approach to Practical Management.

Protein metabolism:

Insulin is a vital hormone involved in the regulation of protein synthesis and breakdown. Deficit of insulin causes an increase in the levels of amino acids in the circulation due to escalated breakdown of protein along with decrease in the

synthesis[26]. Signaling of insulin through the PI3K pathway is diminished in type 2 DM resulting in the block of insulin transport to destined tissues.Through the Akt pathway,PI3K activates mTORC1 and thereby plays a part in conveyance of amino acids to the muscles. But in type 2 DM due to the flaw in this pathway,synthesis of proteins in muscle cell is affected[27]. The knowledge about the changes caused by type 2 DM on protein metabolism is vague when compared to that of type 1 DM. Catabolism of protein is limited by the residual secretion of insulin in type 2 DM. This helps in the conservation of the lean body mass. Nevertheless, in individuals with poor control of type 2 DM, there is a rise in the whole body nitrogen flux along with synthesis and breakdown of proteins[26].

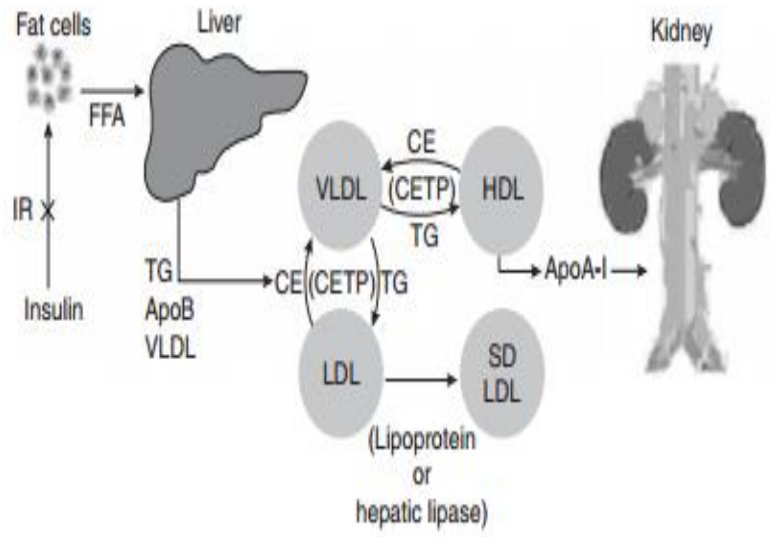
Lipid metabolism:

The foremost regulator of the release of FFA from the adipose tissue is hormone - sensitive lipase (HSL) and this is intricately sensitive to insulin. Insulin is the main hormonal regulator of lipolysis. The rise in the concentration of plasma glucose after a meal usually results in enhanced secretion of insulin, which thereby prevents lipolysis[28].In individuals with type 2 DM, there is a surge in FFA concentration during the post-prandial and post-absorptive period. It is probable that the insensitivity of HSL to insulin in type 2 DM causes increased lipolysis. This results in increased levels of FFA which thereby kindles gluconeogenesis and TGL synthesis in the liver. Prolonged increase in FFA levels inhibit the secretion of insulin contradictory to its acute increase. Therefore, increased levels of FFA have been concerned with several of the metabolic abnormalities associated with type 2 DM[29].

Lipoprotein abnormalities in DM:

DM is one of the most common source of derangement in lipid metabolism. Hypertriglyceridemia which is considered to be the paramount abnormality of lipid metabolism in type 2 DM is because of the increase of TGL-carrying lipoproteins namely chylomicrons and very-low-density lipoprotein cholesterol (VLDL-c). Amplified FFA flux into the liver with added decrease in the activity of lipoprotein lipase (LPL) is responsible for increased TGL synthesis. The abnormalities observed are surge in LDL-cholesterol(LDL-c) levels and decline in HDL-c concentration[30].

Insulin resistance considered as the fundamental metabolic defect in type 2 DM causes release of FFA from the adipocytes. The FFA inturn enters the liver and kindles the assembly and secretion of VLDL-c which leads to hypertriglyceridemia. This VLDL-c stimulates the exchange of its triglycerides for cholesteryl esters from both HDL-c and LDL-c mediated by Cholesterol Ester Transfer Protein (CETP).Free apoA-I dissociated from HDL-c is promptly cleared from the circulation by excretion through the kidney.These changes decreases the availability of HDL-c for reverse cholesterol transport. Triglyceride enhanced LDL-c undergoes lipolysis and becomes smaller and denser. Both the decline in levels of HDL-c and the presence of small dense LDL-c are individual risk factors for cardiovascular disease[31].



Source: Krentz AJ: Lipoprotein abnormalities and their consequences for patients with type 2 diabetes. Diabetes Obes Metab. 2003 ;5 (1):S19-27.

The distinctive feature of dyslipidemia in type 2 DM namely fasting and post-prandial hypertriglyceridemia results from above said increased synthesis of VLDL-c in the liver along with decline in LPL activity[31]. The capacity of triglyceride-rich remnant lipoproteins is more than LDL-c when concerned with the delivery of cholesterol to macrophages. Lipolysis results in the removal of the core of LDL-c and the resultant surface materials are transferred to HDL-c. This paves the way for LDL-c to be derived from VLDL-c. An important physiological role of LDL-c is delivery of cholesterol for the repair of cellular membrane and also to specified endocrine organs, for vitamin D and steroid hormones synthesis. Small and dense LDL-c particles are found to have more atherogenicity than their larger equivalents because of their liability to oxidation and their readiness to adhere and invade the arterial wall[31].

HDL-c and reverse cholesterol transport:

Reverse cholesterol transport is considered to be the movement of cholesterol to the hepatocytes from various tissues and organs. The fate of cholesterol on entering the liver cell maybe its catabolism to bile acids, its excretion as free cholesterol into the bile, secreted as a component of lipoproteins into the plasma or esterification and storage in the hepatocytes. A portion of the bile acid and cholesterol eliminated into bile is lost through feces and is considered as the foremost loss of cholesterol and its metabolites from the body[32].Cholesterol efflux from the cells is mainly facilitated by HDL-c which acts in juxtaposition with lecithin: cholesterol acyl transferase(LCAT)[33].

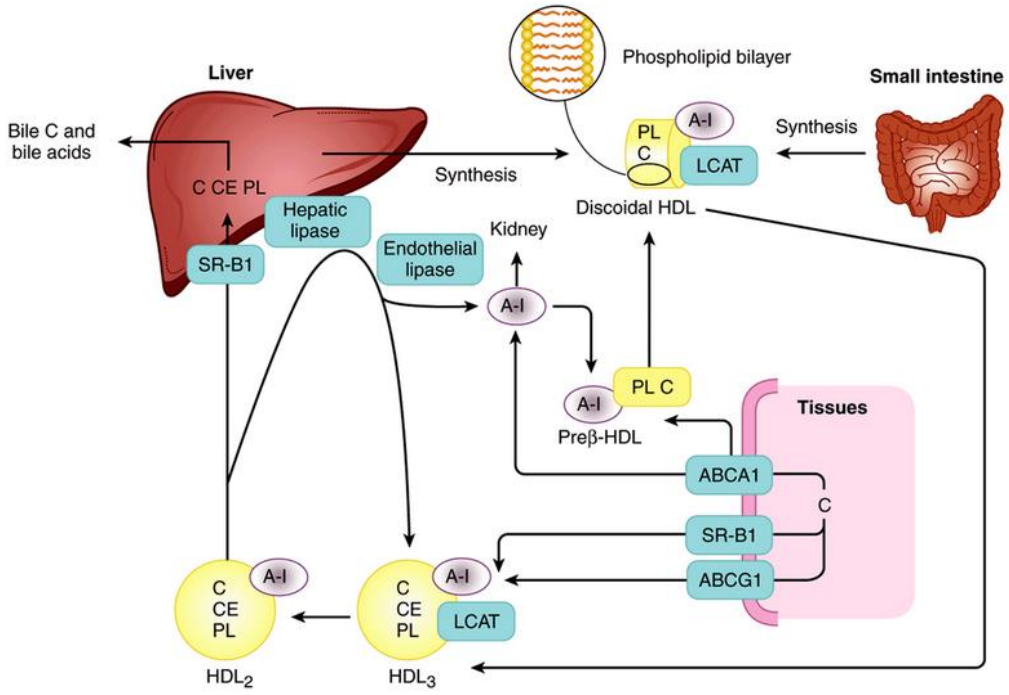
The important steps involved in reverse cholesterol transport pathway is as follows: 1) HDL-c mediated uptake of cellular cholesterol 2) esterification of cholesterol present in HDL-c mediated by LCAT and 3) confiscation of HDL-c esters by the liver which will later on be metabolized for excretion into the bile[34].

The nascent HDL-c considered as discoidal, pre-β-migrating complexes of phospholipid and apoA-I accepts cholesterol. These nascent HDL-c particles are synthesized by liver and small intestine apart from the formation during the metabolism of TGL-rich lipoproteins from excess surface material (phospholipid and soluble apoproteins). This in turn triggers cholesterol efflux in the macrophages and from the fibroblasts of sub-endothelial space. Externalized cholesterol is absorbed by nascent HDL-c and further undergoes esterification by LCAT to cholesterol esters. These cholesterol esters move into the core of the HDL-c particles due to their hydrophobic nature and the resultant particle is HDL3[35].

The class B scavenger receptor B1 (SR-B1) which has a dual role in metabolism of HDL-c is acknowledged as a HDL-c receptor .The major role of SR-B1in the tissues is, the acceptance of the effluxed cholesterol from the cells due to the action of HDL-c.This cholesterol is further transported to the hepatocytes for excretion through bile. In the liver and in steroidogenic tissues, SR-B1binds HDL-c via apo A-I, and cholesteryl ester is selectively delivered to the cells. HDL3, which is synthesized from discoidal HDL-c due to the feat of LCAT, accepts cholesterol from the tissues through SR-B1 and the cholesterol undergoes esterification by LCAT. This contributes to the formation of less dense HDL2 due to its increase in size. HDL3 is then renewed, after selective delivery of cholesteryl ester to the liver mediated by SR-B1 or by hydrolytic action of hepatic lipase and endothelial lipase on HDL2 phospholipid and triacylglycerol. This interconversion of HDL2 and HDL3 is known as the HDL cycle. The free apo A-I released by these routes forms pre-β HDL after coupling with a least amount of phospholipid and cholesterol. Excess apo A-I undergoes destruction in the kidney[36].

A second significant mechanism for reverse cholesterol transport involves proteins, namely the ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1). These proteins are members of a family of transporter proteins that pair the hydrolysis of ATP to the binding of a substrate, thereby enabling its transport through the membrane. ABCG1 mediates transportation of cholesterol to HDL from the cells, whereas ABCA1 favours the efflux of cholesterol to particles poor in lipids like pre-β HDL or apo A-1, which are then converted to HDL3 by means of discoidal HDL[36].

Cholesterol ester transfer protein (CETP) facilitates the equimolar exchange of cholesteryl esters from HDL for TGL in apoB100-containing lipoproteins namely VLDL-c and LDL-c. The delivery of the cholesteryl esters to the liver is mediated by low density-lipoprotein receptor (LDL-R). Then the cholesterol esters are transformed to bile salts to be eliminated via the gastrointestinal tract[37].



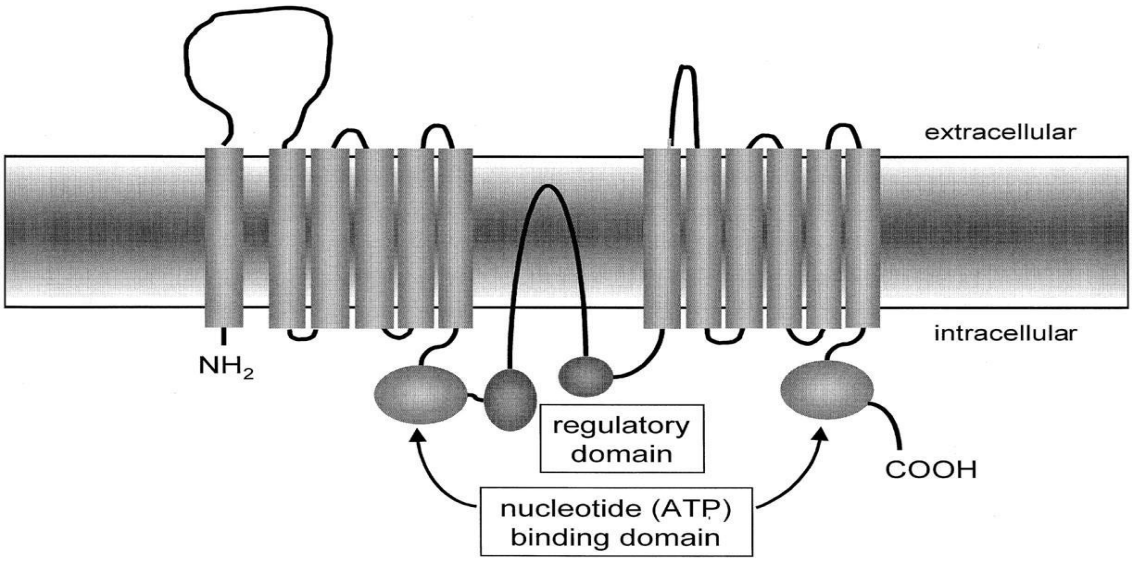
Source: Figure 25-5 Metabolism of HDL in reverse cholesterol transport. Harper's Illustrated Biochemistry, 29th Edition (2012).

ABCA1 gene:

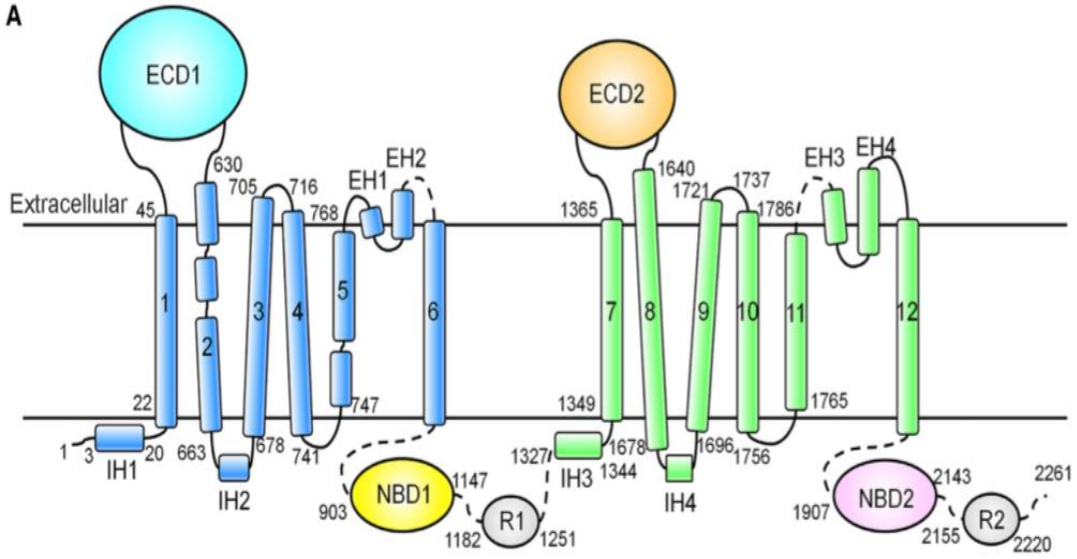
Reverse cholesterol transport as aforementioned, is a pathway in HDL-c metabolism which aids in the removal of excess cholesterol from the peripheral tissues to be transported to the hepatocytes to be dismissed from the body[38].A protein known as ABCA1 is considered as the gatekeeper in reverse cholesterol transport[39].

ATP Binding Cassette(ABC) transporters belong to a superfamily of proteins which utilize ATP as an energy source for transporting substrates across the cellular compartments[40].The ABC transporters are categorized into 8 subfamilies from A to H, based on their amino acid sequences.

The ABCA1 gene in humans maps to chromosome 9q31 with its 50 exons spanning over 150 kbp[41]. ABCA1 is an integral protein with 2,261-amino acids. It has two transmembrane domains(TMD) each with six helices and also two nucleotide binding domains (NBD) in the cytoplasm. The nucleotide binding domains have 2 peptide motifs called Walker A and Walker B. An unique amino acid signature is present between the two Walker motifs, which is characteristic of ABC transporters family. In contrast to the other ABC transporters, in ABCA1 there are 2 regulatory domains with a hydrophobic segment located in the cytoplasmic region, one for each TMD and this favours the cooperative functioning of the two units of ABCA1[42].



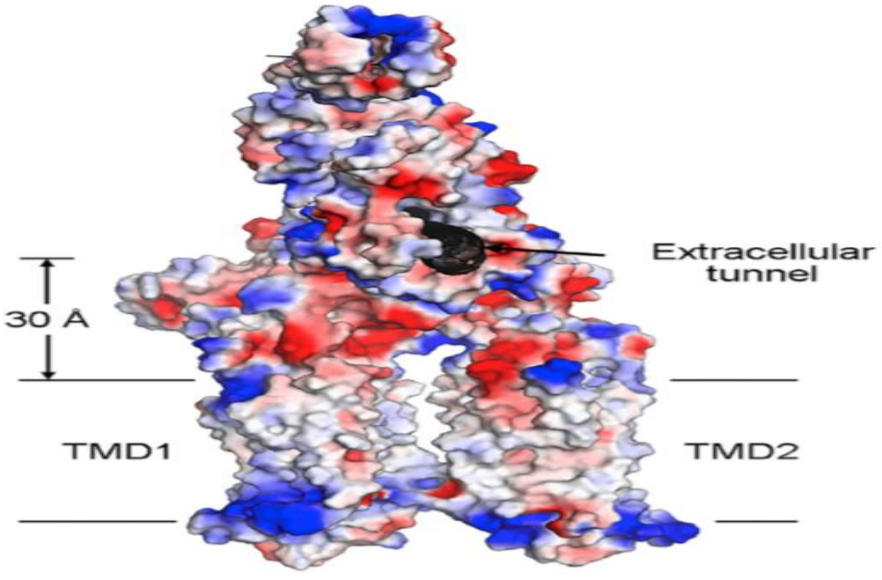
Source: Fitzgerald M. L.,et al. ABCA1 contains an N-terminal signal-anchor sequence that translocates the proteins first hydrophilic domain to the exoplasmic space. J. Biol. Chem.2001; 276: 15137–15145.



Source: Qian et al. Structure of the Human Lipid Exporter ABCA1. Cell 2017;169: 1228–1239.

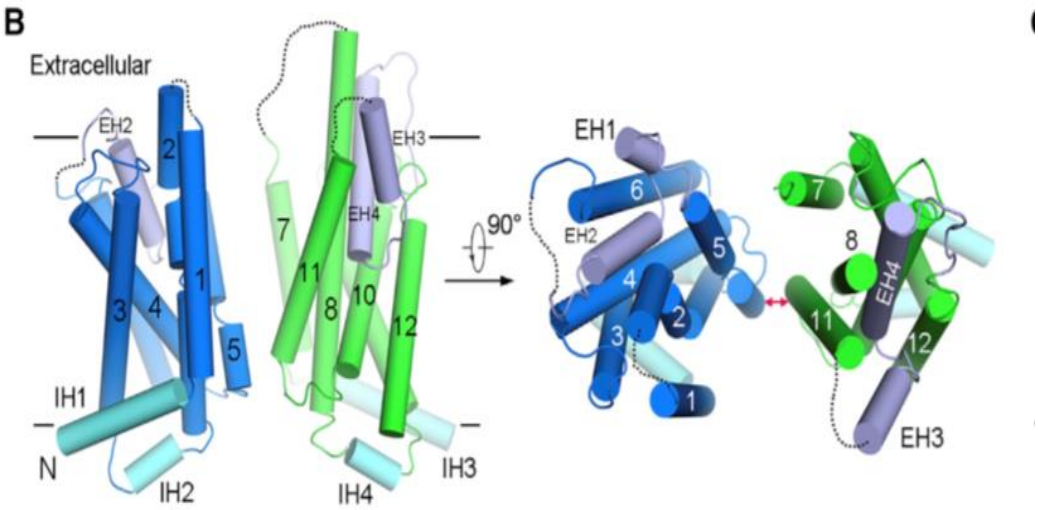
ABCA1 has 2 extracellular domains, ECD1 and ECD2 which are liable for binding to apoA-I. ECD1 has 583 amino acids whereas ECD2 has 270 amino acids.The three dimensional structure reveals that ECD1 is placed above TMD2 and ECD2 is placed above TMD1. The extracellular domains form a flamed shaped structure which is stabilized by disulphide bonds. Regardless of this stability, the ECD has a hydrophobic hollow interior. This tunnel serves as a potential passage for the transportation of lipids to apoA-I from the interior of the membrane[43].

The tunnel has both proximal and distal ends, with the opening at the distal end narrow than that of the proximal end .The proximal end opening is situated at a distance of 30Å from the transmembrane cavity.This structural arrangement shows that conformational change has to take place to facilitate the delivery of the lipids to the ECD from the transmembrane cavity[43].



Source: Qian et al. Structure of the Human Lipid Exporter ABCA1. Cell 2017;169: 1228–1239.

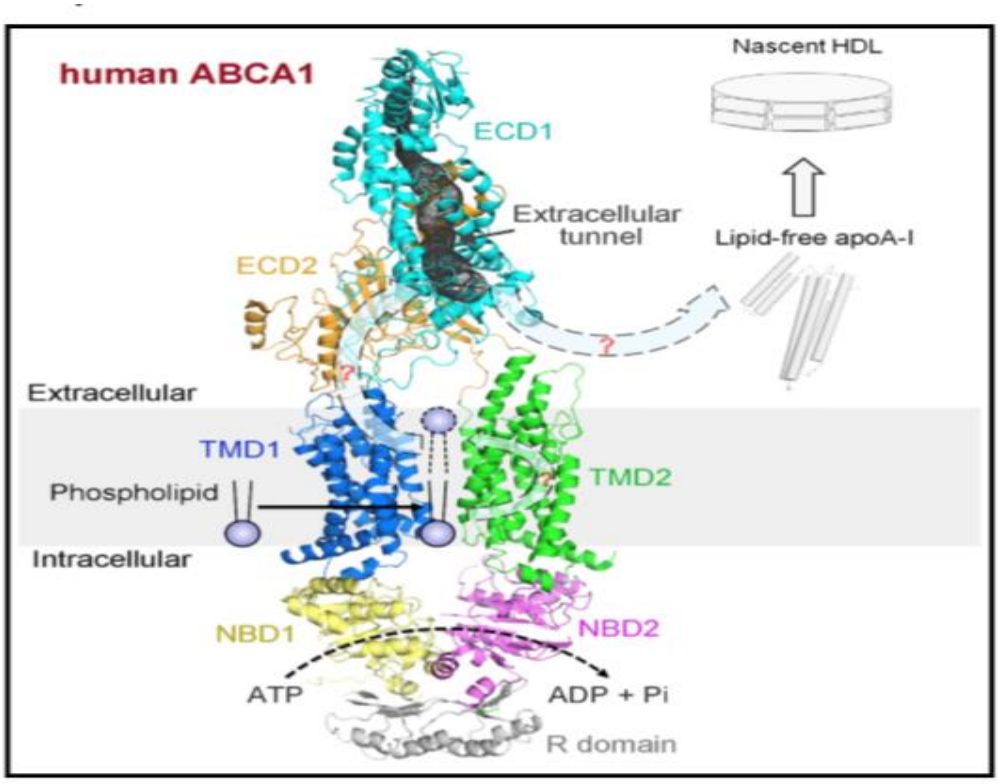
V-shaped α helical hairpins called extracellular helices, EH are present between transmembranes TM 5 & 6 of TMD1 and TM 11 & 12 of TMD2.These EH penetrate halfway through the membrane. Apart from a narrow interface between TM5 and TM11 which is responsible for the contact of TMD1 and TMD2, all the other areas are exposed to the lipid bilayer. Thus this "outward facing conformation" favours the access of lipid substrates from inner side of membrane to the substrate binding site[43].



Source: Qian et al. Structure of the Human Lipid Exporter ABCA1. Cell 2017;169: 1228–1239.

The proposed mechanism is lateral access lipid- linked oligosaccharide (LLO) flopping by a protein called PgIK. The focal step in lipid flopping mechanism is the transport of the polar head across the hydrophobic barrier of membrane.A polar cluster present in TMD1 close to the inner membrane leaflet is responsible for binding the polar moiety of lipid. The conformational change caused in NBD due to ATP

binding/hydrolysis and dissociation of ADP, leads to changes in TMD1 and TMD2 leading to delivery of the polar head to a potential binding site in TMD located close to the outer leaflet. It is speculated that ABCA1 and lipid bilayer bind cooperatively with apoA-I and deliver the substrate for nascent HDL-c formation[43].



Source: Qian et al. Structure of the Human Lipid Exporter ABCA1. Cell 2017;169: 1228–1239.

Thus, ABCA1 plays an important role in the transportation of cholesterol and phosopholipids from the cell, failure of which might lead to intracellular accumulation of lipids. This in turn leads to atherogenic changes, the most dreaded complication of type 2 DM.

In view of type 2 DM, there are few studies which were done to find out the association between ABCA1 gene polymorphism and the pathological mechanism underlying HDL-c dysfunction which is a significant derangement in lipid metabolism.

Polin et al[44] have shown that there is a significant difference between cases and controls for R219K(rs2230806) polymorphism in Malayasian population.

Udita Singh et al[45] have studied that ABCA1 gene(C69T) polymorphism has strong association with type 2 DM among central Indians.

Alharbi K K et al[46] have conducted a study on the association of ABCA1 C69T gene polymorphism and the risk of type 2 DM in Saudi population.

MATERIALS AND METHODS

MATERIALS AND METHODS:

The study was conducted at PSG Institute of Medical Sciences and Research, Coimbatore. Ethical clearance for conducting the study was acquired from the Institutional Human Ethics Committee. An informed consent was acquired from the patients before collecting blood samples from them.

The study design is case control study in which type 2 DM patients (n =50) were selected as cases. The diagnosis of type 2 DM was based on the description in medical records using the American Diabetic association (ADA) criteria. Cases were selected from type 2 DM patients attending endocrinology and medicine outpatient department. Patients satisfying the diagnostic criteria, inclusion criteria and exclusion criteria were explained about the study. After obtaining their consent blood samples were collected, processed and stored for further analysis.

Individuals for control group were selected mainly from the patients who came for master health checkup. Persons satisfying the criteria for control group were requested to participate in the study. After getting the consent forms duly filled from them, samples were collected and stored for analysis after processing. Patient data namely height, weight and other details were retrieved from their case sheet.

INCLUSION CRITERIA:

- Males and females
- Age > 30yrs

- Type 2 DM(ADA criteria)- maximum duration of 2 years on treatment(oral drugs / insulin)

EXCLUSION CRITERIA:

Subjects with:

- Malignancy
- Type 1 DM
- Type 2 DM with microvascular (Diabetic nephropathy, neuropathy, retinopathy) and macrovascular (Coronary artery disease, peripheral arterial disease,stroke) complications
- Genetic malformation
- Acute illness
- Pregnancy
- Other endocrine abnormalities

Collection of blood samples:

For both cases and control individuals, blood sample was collected in sodium fluoride vacutainer for fasting and post-prandial plasma glucose estimation, in EDTA vacutainer for estimation of HbA1c and DNA extraction. Whereas for lipid profile and estimation of urea and creatinine blood was collected in red topped serum tubes.

The sample for DNA extraction was transferred to labelled sterile cryovial and stored at -80 degree celsius.

The methods used for estimation of biochemical parameters are given below.

PLASMA GLUCOSE

Method:

Enzymatic reference method with Hexokinase.

Principle:

The phosphorylation of glucose by ATP to form glucose-6 phosphate and ADP is catalysed by Hexokinase (HK). This is followed by the action of another enzyme, glucose-6 phosphate dehydrogenase (G6PDH). This enzyme catalyzes oxidation of glucose-6 phosphate by NAD^+ to form NADH.

Reaction:

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

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

The NADH concentration is directly proportional to the concentration of glucose and the increase in absorbance is measured at 340nm.

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R-S
Direction of Reaction	Increase
Wavelength A/B	340/409 nm
Test value	0-40 mmol/L
Unit	mmol/L

Conversion factor: mmol/L x 18.02 = mg/dL

GLYCOSYLATED HAEMOGLOBIN: (HbA1c)

Method:

Turbidimetric inhibition immunoassay

Principle:

EDTA tube collected whole blood specimen is hemolysed after collection. Total hemoglobin and HbA1c concentrations are determined. Total hemoglobin is measured colorimetrically. The percentage of HbA1c is determined immunoturbidimetrically. The ratio of both these levels gives the final percent HbA1c result.

EDTA collected blood is hemolysed with hemolysis reagent in the predilution cuvette. This leads to reduction in osmotic pressure which lyses the erythrocytes. Lysis of erythrocytes releases the haemoglobin. It is degraded by the proteolytic action of the enzyme pepsin. This renders the beta-N terminal structures more available for the immunoassay. In the hemolysate, a colorimetric method helps in the determination of total haemoglobin. This is done on the basis of production of a brownish-green chromophore. This occurs in alkaline detergent solution using a cyanide free method. The intensity of color relates to the level of total hemoglobin in the sample. The concentration is calculated by sensing the raise in the absorbance at 552nm. A fixed factor that is obtained from the primary calibrator chlorohemin calculates the test results.

Turbidimetric method to measure HbA1c is done using monoclonal antibodies which are present attached to latex particles. The monoclonal antibodies bind the amino terminal fragments of HbA1c. The unbound free antibodies agglutinate with an artificial polymer. The change in turbidity is inversely related to the quantity of bound glycated proteins. This is measured turbidimetrically at 552nm. HbA1c result is given as percentage of HbA1c. It is evaluated from the ratio of HbA1c/Hb as given below:

According to IFCC

HbA1c (%) = (HbA1c / Hb) x 100

According to DCCT/NGSP

HbA1c(%) = (HbA1c/Hb) x 87.6 + 2.27

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	D-R1-S-SR
Direction of Reaction	Increase
Wavelength A/B	552nm
Test range	1.4-4.7µmol/L (2.4-75.8mg/dL)
Unit	µmol/L

SERUM TOTAL CHOLESTEROL

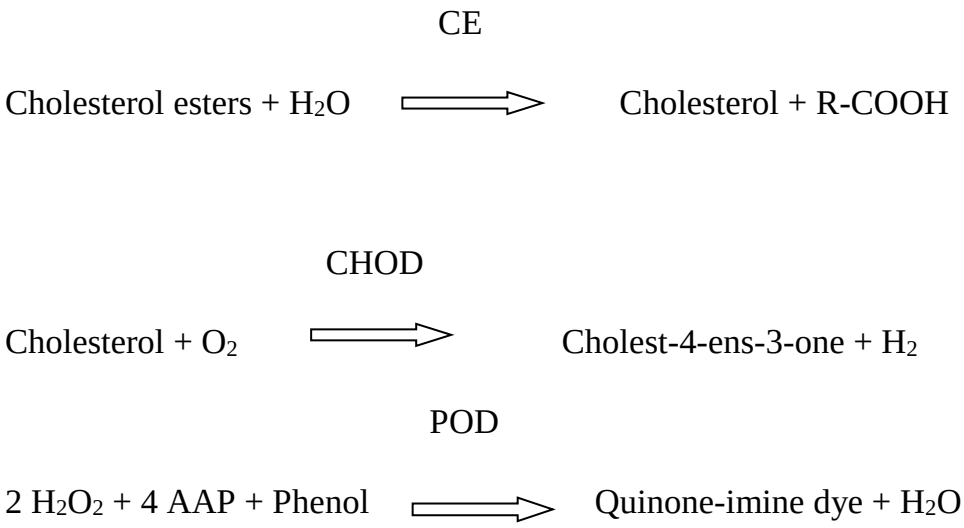
Method:

Enzymatic colorimetric test.

Principle:

Cholesterol esters are cleaved by the action of cholesterol esterase (CE) to yield free cholesterol and fatty acids. Cholesterol oxidase (CHOD) in a second reaction helps in the formation of cholest-4-en-3-one and hydrogen peroxide by the oxidation of cholesterol. Peroxidase (POD) converts the hydrogen peroxide that is formed and helps in the oxidative coupling of phenol and 4-aminoantipyrine (AAP) forming a quinone-imine(red).

Reaction:



The intensity of the red colour produced is directly proportional to the serum cholesterol level and is determined by the increase in absorbance measured at 512 nm.

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R-S
Direction of Reaction	Increase
Wavelength A/B	512/659 nm
Test value	0-20.7 mmol/L
Unit	mmol/L

Conversion factor: mmol/L x 38.66 = mg/dL

SERUM TRIGLYCERIDES:

Method:

Enzymatic colorimetric method

Principle:

Lipoprotein lipase (LPL) hydrolyzes the triglycerides to glycerol and fatty acids. Glycerol kinase (GK) catalyses the conversion of glycerol to glycerol-3-phosphate with the help of ATP phosphorylation. Glycerol phosphate oxidase (GPO) catalyses the oxidation of glycerol-3-phosphate to form dihydroxyacetone phosphate and hydrogen peroxide. Peroxidase (POD) converts hydrogen peroxide, 4-chlorophenol and 4-aminophenazone to form a red-coloured quinoneimine dye. The intensity of color is measured at 512 nm and the concentration of triglyceride is assessed in the sample.

Reaction:

$$\text{Triglycerides} \xrightarrow{\text{LPL}} \text{Glycerol + Fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol-3 phosphate + ADP}$$

$$\text{Glycerol -3 phosphate + } O_2 \xrightarrow{\text{GPO}} \text{Dihydroxy acetone Phosphate+} H_2O_2$$

POD

$2H_2O_2$ + 4 aminophenazone ⟹ Quinoneimine dye+$4H_2O$+4 Chlorophenol

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R-S
Direction of Reaction	Increase
Wavelength A/B	512/659 nm
Test range	0-10 mmol/L (0-885 mg/dL)
Unit	mmol/L

Conversion factor: mmol/L x 38.66 = mg/dL

SERUM HDL CHOLESTEROL:

Method:

Homogenous enzymatic colorimetric assay.

Principle:

With magnesium ions, dextran sulphate complexes with LDL, VLDL and chylomicrons which are water soluble and resistant to polyethylene glycol (PEG)

modified enzymes. Cholesterol esterase and cholesterol oxidase associated with PEG to the amino groups is used to determine the cholesterol level using enzymatic methods. Cholesterol ester is partitioned into free cholesterol and fatty acids with the help of cholesterol esterase. Oxidation of cholesterol by cholesterol oxidase is done in the presence of oxygen to Δ^4-cholestenone and hydrogen peroxide. Hydrogen peroxide which is generated reacts with 4-aminoantipyrine and HSDA in the presence of peroxidase, forms a purple-blue dye. The cholesterol concentration is directly proportional to the intensity of blue colour and it is measured photometrically.

Reaction:

$$\text{HDL Cholesterol ester} + H_2O \xrightarrow{\text{PEG cholesterol esterase}} \text{HDL Cholesterol} + \text{RCOOH}$$

$$\text{HDL-Cholesterol} + O_2 \xrightarrow{\text{PEG Cholesterol oxidase}} \Delta^4\text{-cholestenone} + H_2O_2$$

$$2H_2O_2 + 4\ \text{aminoantipyrine} + \text{HSDA} + H^+ \xrightarrow{\text{POD}} \text{Purple blue Pigment} + 4H_2O$$

HSDA = Sodium N (2 - hydroxy - 3- sulfopropyl) – 3,5-dimethoxyaniline

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R1-S-SR

Direction of Reaction	Increase
Wavelength A/B	583/659 nm
Test range	0-3.12 mmol/L (0-120 mg/dL)
Unit	mmol/L

Conversion factor: mmol/L x 38.66 = mg/dL

SERUM LDL CHOLESTEROL:

Method:

Homogeneous enzymatic colorimetric assay.

Principle:

Cholesterol esters and free cholesterol in LDL are measured on the basis of a cholesterol enzymatic method using cholesterol esterase and cholesterol oxidase in the presence of surfactants which selectively solubilize only LDL. The enzyme reactions to the lipoproteins other than LDL are inhibited by surfactants and a sugar compound. Cholesterol in HDL, VLDL and chylomicron is not determined.

Reaction:

$$\text{LDL-Cholesterol ester} + H_2O \xrightarrow{\text{Detergent cholesterol esterase}} \text{Cholesterol} + \text{Free fatty acid}$$

Cholesterol oxidase

LDL-Cholesterol + O_2 ⟹ Δ^4-cholestenone + H_2O_2

POD

$2H_2O_2$ + 4 aminoantipyrine + EMSA[a] + H_2O + H^+ ⟹ red purple pigment + $5H_2O$

a) N-ethyl-N-(3-methylphenyl)-N-succinylethylenediamine

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R1-S-SR
Direction of Reaction	Increase
Wavelength A/B	583/659 nm
Calu.first/last	33/69
Unit	mmol/L

Conversion factor: mmol/L x 38.66 = mg/dL

SERUM ALANINE AMINO TRANSFERASE(ALT):

Method:

Enzymatic assay according to International Federation of Clinical Chemistry (IFCC), but without pyridoxal-5´-phosphate

Principle:

ALT catalyzes the reaction between L-alanine and 2-oxoglutarate. The pyruvate formed is reduced by NADH in a reaction catalyzed by lactate dehydrogenase (LDH) to form L-lactate and NAD^+.

$$\text{L-Alanine} + \text{2-oxoglutarate} \xrightarrow{\text{ALT}} \text{pyruvate} + \text{L-glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^+$$

The rate of the NADH oxidation is directly proportional to the catalytic ALT activity. It is determined by measuring the decrease in absorbance at 340 nm.

Test Definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Kinsearch

Mode of reaction	R1-S-SR
Direction of Reaction	Decrease
Wavelength A/B	340/378 nm
Calu.first/last	39/64
Test range	0 – 700 U/L (0 – 11.7 µkat/L)
With postdilution	0 - 7 000 U/L (0 – 117 µkat/L)
Postdilution factor	10 recommended
Unit	U/L

SERUM ASPARTATE AMINO TRANSFERASE (AST):

Method:

Enzymatic assay according to the International Federation of Clinical Chemistry (IFCC), but without pyridoxal-5'-phosphate

Principle:

AST in the sample catalyzes the transfer of an amino group between L-aspartate and 2oxoglutarate to form oxaloacetate and L-glutamate. The oxaloacetate then reacts with NADH, in the presence of malate dehydrogenase (MDH), to form $NAD^{+.}$

AST

L-Aspartate + 2-oxoglutarate ⟹ oxaloacetate + L-glutamate

MDH

Oxaloacetate + NADH + H^+ ⟹ L-malate + NAD^+

The rate of the NADH oxidation is directly proportional to the catalytic AST activity. It is determined by measuring the decrease in absorbance at 340nm.

Test Definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Kinsearch
Mode of reaction	R1-S-SR
Direction of Reaction	Decrease
Wavelength A/B	340/378 nm
Calu.first/last	39/64
Test range	0 – 700 U/L (0 – 11.7 µkat/L)
With postdilution	0 - 7 000 U/L (0 – 117 µkat/L)
Postdilution factor	10 recommended
Unit	U/L

SERUM ALKALINE PHOSPHATASE (ALP):

Method:

Colorimetric assay in accordance with a standardized method

Principle:

In the presence of magnesium and zinc ions, p-nitrophenyl phosphate is cleaved by phosphatases into phosphate and p-nitrophenol.

$$\text{P-nitrophenyl phosphate} + H_2O \xrightarrow{\text{ALP}} \text{phosphate} + \text{p-nitrophenol}$$

The p-nitrophenol released is directly proportional to the catalytic ALP activity. It is determined by measuring the increase in absorbance at 409 nm**.**

Test Definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Kinsearch
Mode of reaction	R1-S-SR
Direction of Reaction	Decrease
Wavelength A/B	409/659 nm
Calu.first/last	41/64
Test range	0 – 1200 U/L (0 – 20 µkat/L)

With postdilution	0 - 6000 U/L (0 – 100 µkat/L)
Postdilution factor	5 recommended
Unit	U/L

SERUM GAMMA GLUTAMYL TRANSFERASE (GGT):

Method:

Enzymatic colorimetric assay

Principle:

Gamma-glutamyltransferase transfers the γ-glutamyl group of L-γ glutamyl 1-3-carboxy-4-nitroanilide to glycylglycine.

$$\text{L-γ-Glutamyl-3-carboxy-4-nitroanilide} + \text{glycylglycine} \xrightarrow{\text{GGT}} \text{L-γ Glutamyl-glycylglycine} + \text{5 amino-2 nitrobenzoate}$$

The amount of 5-amino-2 nitrobenozate liberated is proportional to the GGT activity in the sample. It is determined by measuring the increase in absorbance at 409 nm.

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Kinetic
Mode of reaction	R1-S-SR
Direction of Reaction	Increase
Wavelength A/B	409/659 nm
Calu.first/last	50/69
Test range	0 – 1200 U/L (0 – 20 µkat/L)
With postdilution	0 - 12000 U/L (0 – 200 µkat/L)
Postdilution factor	10 recommended
Unit	U/L

SERUM TOTAL BILIRUBIN:

Method:

Colorimetric assay

Principle:

Total bilirubin, in the presence of a suitable solubilizing agent, is coupled with a diazonium ion in a strongly acidic medium.

acid

Bilirubin + diazonium ion ⟹ azobilirubin

The color intensity of the red azo dye formed is directly proportional to the total bilirubin and can be determined photometrically.

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R1-S-SR
Direction of Reaction	Increase
Wavelength A/B	552/629 nm
Calu.first/last	33/46
Unit	µmol/L

Conversion factor: µmol/L x 0.0585 = mg/dL

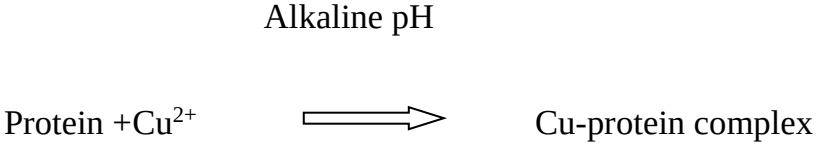
SERUM TOTAL PROTEIN:

Method:

Colormetric assay

Principle:

Divalent copper reacts in alkaline solution with protein peptide bonds to form the characteristic purple-colored biuret complex. Sodium potassium tartrate prevents the precipitation of copper hydroxide and potassium iodide prevents auto reduction of copper.



The color intensity is directly proportional to the protein concentration. It determined by measuring the increase in absorbance at 552 nm.

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	End point
Mode of reaction	R1-S-SR
Direction of Reaction	Increase
Wavelength A/B	552/659 nm
Calu.first/last	33/52
Test range	2 - 120 g/L (0.2 – 12 g/dL)
With post dilution	2 - 600 g/L (0.2 – 60 g/dL)

Post dilution factor	5 recommended
Unit	g/L

SERUM UREA:

Method:

Kinetic test with urease and glutamate dehydrogenase

Principle:

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

$$\text{Urea} + 2\,H_2O \xrightarrow{\text{Urease}} 2\,NH^{4}+ + CO_3^{2}-$$

$$NH^{4}+ + \text{2-oxoglutarate} + NAD \xrightarrow{\text{GLDH}} \text{L-glutamate} + NAD^{+} + H_2O$$

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

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Kinetic
Mode of reaction	R1-S
Direction of Reaction	Decrease
Wavelength A/B	340/409 nm
Calu.first/last	23/28
Test range	0 – 40 mmol/L (0 – 240 mg/dL)
With postdilution	0 - 400 mmol/L(0 – 2402 mg/dL)
Postdilution factor	10 recommended
Unit	mmol/L

Conversion factor: mmol/L x 6.006 = mg/dL urea

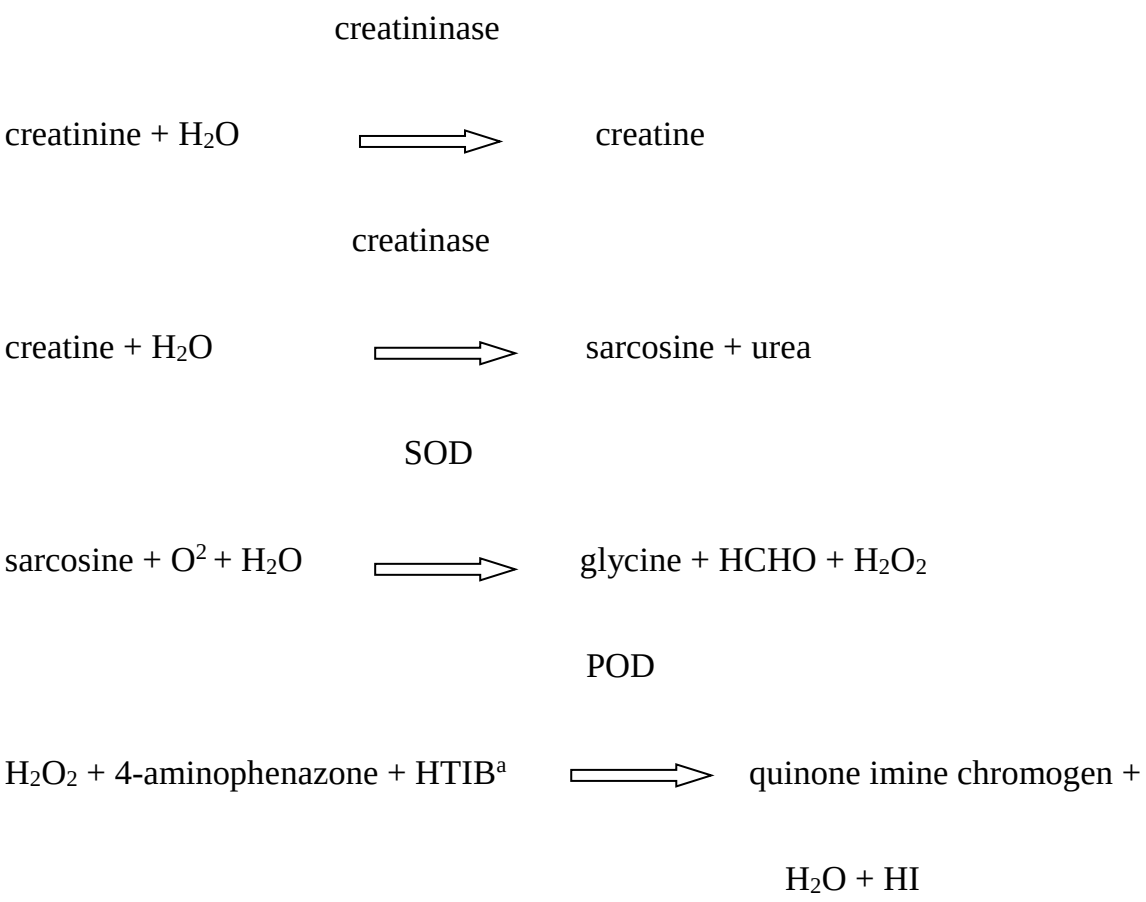
SERUM CREATININE:

Method:

Enzymatic colormetric method

Principle:

The enzymatic method is based on the established determination of hydrogen peroxide after conversion of creatinine with the aid of creatininase, creatinase, and sarcosine oxidase. The liberated hydrogen peroxide reacts with 4-aminophenazone and HTIB to form a quinone imine chromogen.



a) 2,4,6-triiodo-3-hydroxybenzoic acid

The color intensity of the quinone imine chromogen formed is directly proportional to the creatinine concentration. It is determined by measuring the increase in absorbance at 552 nm.

Test definition:

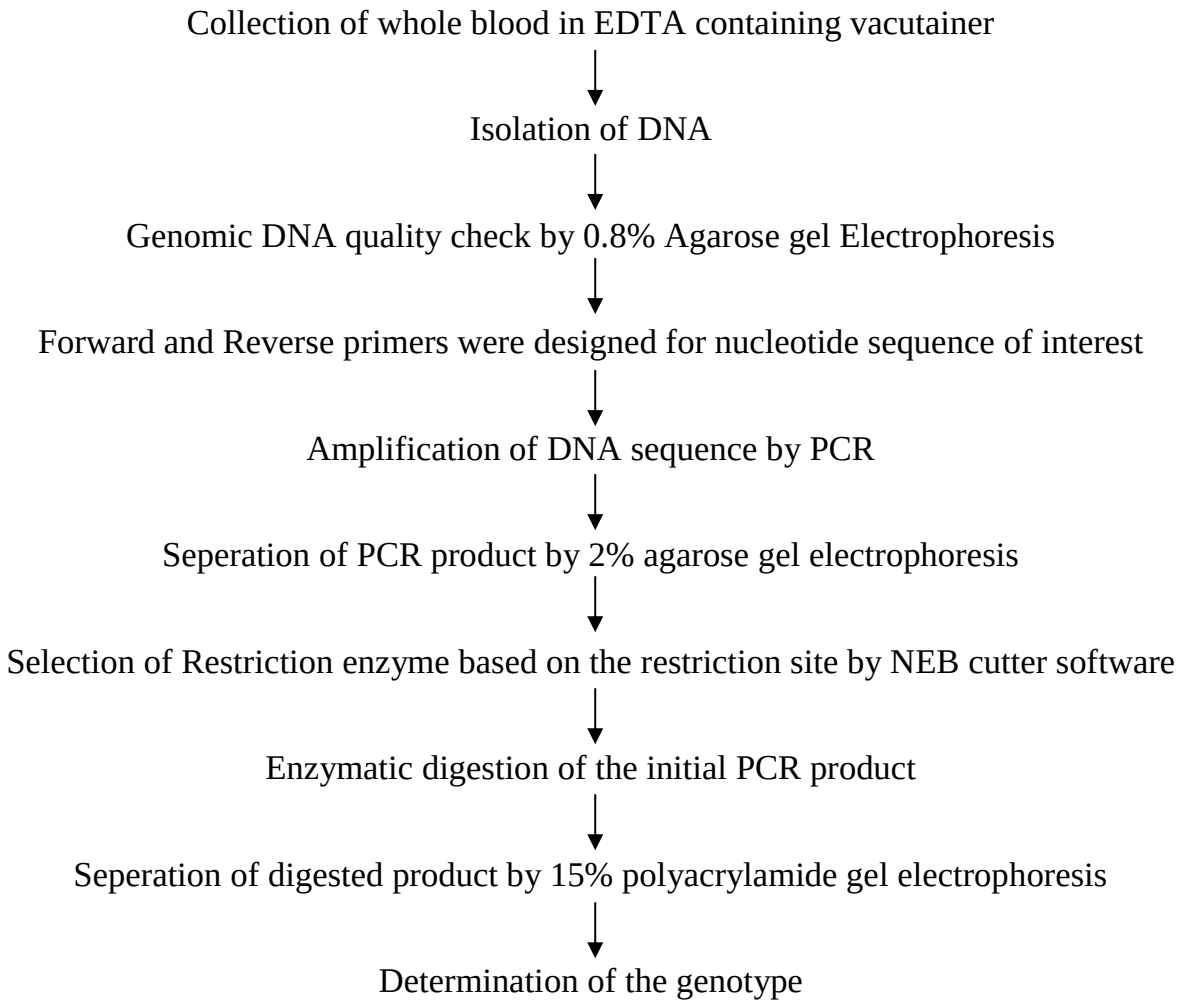
Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R1-S-SR
Direction of Reaction	Increase
Wavelength A/B	552/659 nm
Calu.first/last	35/65
Test range	0 – 2700 µmol/L (0 – 30.5 mg/dL)
With postdilution	0 - 27 000 µmol/L(0 – 305 mg/dL)
Postdilution factor	10 recommended
Unit	µmol/L

Conversion factor: µmol/L x 0.0113 = mg/dL

ABCA1 GENE POLYMORPHISM:

The single nucleotide polymorphisms in ABCA1 gene namely R219K(rs 2230806) and C69T(rs 1800977) were selected for the present study based on population genetics. The mutation in R219K polymorphism was (G→A) whereas for C69T it was (C→T).

The following flowchart presents the overall steps involved in determination of ABCA1 gene polymorphism.



ISOLATION OF DNA:

The blood samples stored at -80 degree Celsius were thawed and DNA was extracted using EZ-10 Column Blood Genomic DNA Minipreps kit(Bio Basic Inc.). The following are the steps involved in the DNA extraction.

300µl blood and 600µl of tributyl phosphine buffer are added in 2ml eppendorf tube

↓

Contents mixed using vortex and allowed to stand for 1 min for RBC lysis to complete

↓

Centrifuged at 8000rpm for 1 minute

↓

Supernatant is discarded and white pellet is obtained

↓

Pellet is washed twice with TE buffer by centrifuging at 8000rpm for 1 min

↓

20 µl of Proteinase K and 200 µl of Cell lysis buffer are added

↓

Mixed using vortex and incubated at 56 degree Celsius for 10 minutes

↓

200µl of 100% ethanol is added and mixed

↓

Entire content is transferred onto EZ-10 column in 2 ml collection tube

↓

Centrifuged at 10,000rpm for 2 minutes

↓

Flow through in the collection tube is discarded

↓

500µl of CW1 solution is added to the column and centrifuged at 10,000 rpm for 1 minute

↓

Flow through in the collection tube is discarded

↓

500µl of CW2 solution is added to the column and centrifuged at 10,000 rpm for 1 minute

↓

Flow through in the collection tube is discarded

↓

Column is placed in 1.5 ml eppendorf tube and 30µl of CE buffer is added to the column

↓

Centrifuged at 10,000 rpm for 1 min to elute DNA from the column

↓

Genomic DNA quality assessed by electrophoresis using 0.8% agarose gel

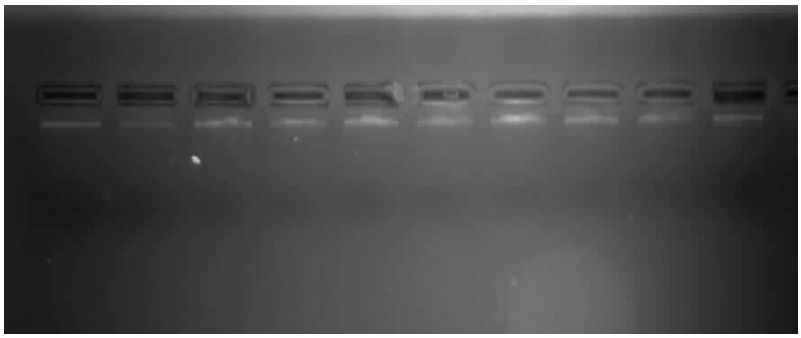


Figure: Photograph showing the extracted DNA of 10 samples run on ethidium bromide stained 0.8% agarose gel

AGAROSE GEL ELECTROPHORESIS

Reagents required:

1. TAE buffer 50 X stock:

- 24.2gmTris base
- 5.7ml Glacial Acetic acid
- 10ml 0.5M EDTA pH 8.0
- Dissolve in 100 ml Distilled water

2. TAE buffer 1X Tank buffer:

50X TAE buffer is diluted to 1X by adding water. For eg: 10ml 50X TAE + 490ml Water makes 500ml of 1X TAE. This is used for electrophoresis tank to run the gel.

3. DNA loading dye

4. Ethidium bromide.

5. Agarose powder.

Gel preparation:

- 30ml of 1X TAE buffer (based on gel template size) is taken in a conical flask and add 0.6 g agarose (0.8% gel) is added ,then the solution is heated well for the agarose to dissolve in it .
- Before that the gel template is sealed with cello tape and the well template is kept ready.
- Then, add 0.2µl ETBR solution is added to the agarose solution, mixed well and poured into the sealed gel template and the comb is placed to create wells.This is allowed to stand undisturbed for 15 minutes for solidification to occur.

Electrophoresis:

- 1X TAE buffer is poured in the tank (~600ml required) and the gel plate is kept inside the tank.
- Then 2µl of DNA loading dye and 8µl of DNA (isolated) are taken and mixed well and loaded into the wells carefully.

- The voltage is set at 65V and electrophoresis is run it for 30-45 minutes.

Gel Doc system was used to visually check the quality of DNA. The extracted genomic DNA was stored at -80 degree Celsius until further analysis.

POLYMERASE CHAIN REACTION:

The nucleotide sequence for the two SNPs of interest in ABCA1 gene was determined. Then forward and reverse primers for the flanking sequence of these nucleotide sequences were designed.

REQUIREMENTS:

- **R219K(rs 2230806):**

Forward primer: 5'CCT CTT GTG CTT GTC TCT CTT TGC ATG 3'

Reverse primer: 5' TTG GCT TCA GGA TGT CCA TGT TGG 3'

- **C69T(rs 1800977):**

Forward primer: 5' CAG CGC TTC CCG CGC GTC TTA 3'

Reverse primer: 5' CCA CTC ACT CTC GTC CGC AAT TAC 3'

- Taq DNA polymerase 2X master mix –Ampliqon (Composition – Tris-HCl pH 8.5,Ammonium sulphate, 3mM Magnesium chloride,0.2% Tween,0.4mM of each dNTPS,0.2units/µl Taq DNA polymerase,Inert red dye and stabilizer)
- DNA sample
- Milli Q water

Amplification by PCR was carried out in DNA Thermal Cycler. Reactions were performed with 1µmol concentration for the primers of R219K and

10µmol concentration for the primers of C69T. The total reaction volume was 20µl as follows:

- Forward primer - 1µl
- Reverse primers- 1µl
- Taq master mix - 10µl (as per recommended by the manufacturer)
- DNA - 0.8µl
- milliQ water - 7.2µl

The annealing temperature was standardized at 60ºC for primers of R219K and at 63.5ºC for primers of C69T.

PCR Programme:

- Initial denaturation - 94ºC for 2 minutes
- Denaturation - 94ºC for 30 seconds
- Annealing Temperature – 45 seconds
- Extension - 72ºC for 45 seconds

} 35 cycles

- Final extension - 72ºC for 2 minutes
- End / Hold - 4 ºC

PCR amplification products were confirmed by 2% agarose gel electrophoresis. The PCR product size was determined using Gel Doc system by comparing with 100 bp DNA ladder which was run alongside the sample. For SNP R219K the product size was found to be 211 base pairs whereas for SNP C69T the product size was 345 base pairs.

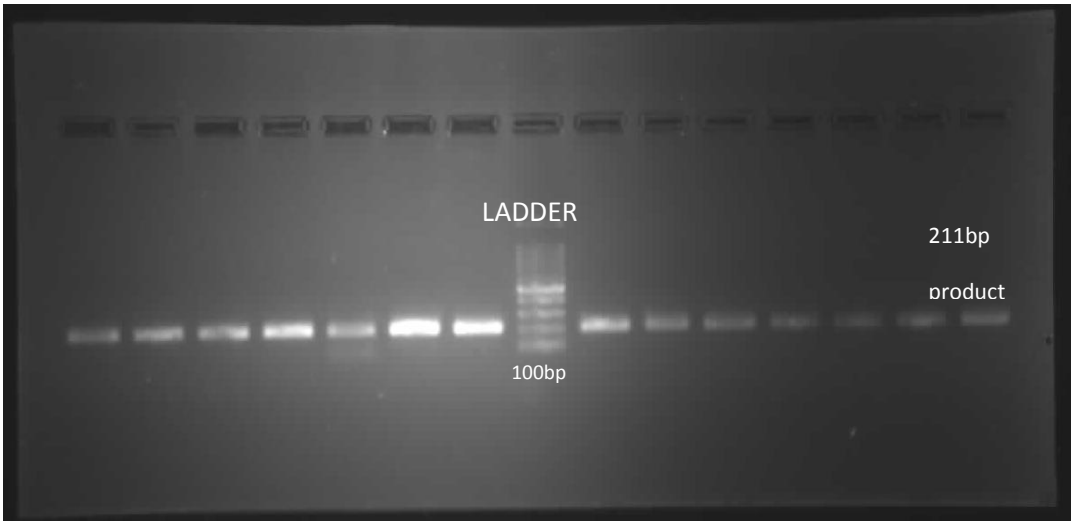


Figure: Photograph showing PCR product of SNP R219K with 211bp

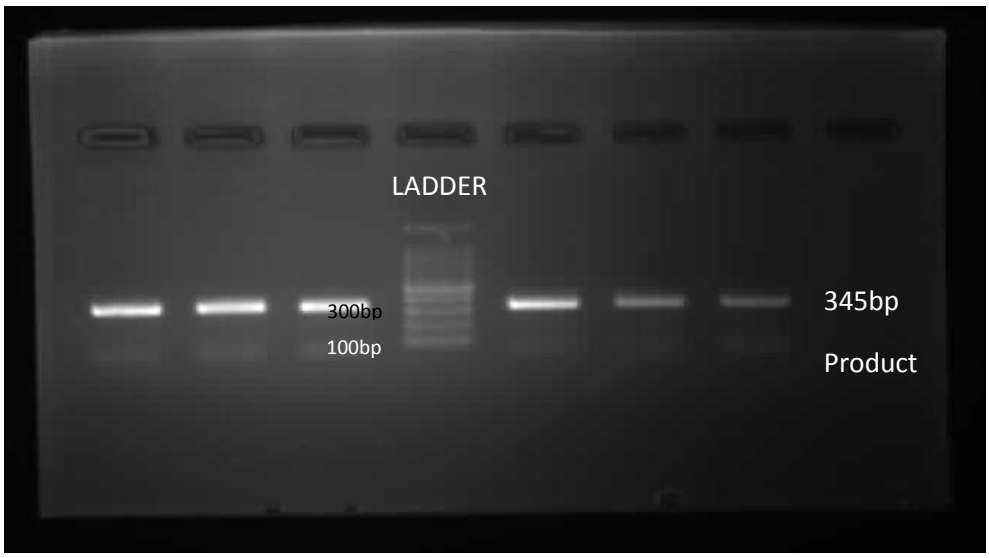


Figure: Photograph showing PCR product of SNP C69T with 345bp

RESTRICTION FRAGMENT LENGTH POLYMORPHISM:

Restriction enzyme digestion:

Requirements:

1. EcoNI (XagI) enzyme for SNP R219K
2. BsmAI enzyme for SNP C69T

3. 10X NE buffer
4. PCR product
5. Milli Q water

The initial PCR product was digested with restriction enzyme.

SNP R219K:

- PCR product of SNP R219K was digested with EcoNI(XagI)
- Incubation at 37^{o}C for one hour
- Inactivation at 65^{o}C for 20 minutes.

SNP C69T:

- PCR product of SNP C69T digested with BsmAI
- Incubation at 55^{0}C for one hour.
- No inactivation

The total reaction volume was 50 µl and the protocol is as follows:

Restriction enzyme - 0.8µl

DNA - 1µl

10X NE buffer - 5 µl

milliQ water - 43.2 µl

The mixture was centrifuged for few seconds before incubation.

Polyacrylamide Gel Electrophoresis:

For analysis of restriction fragments, 15% polyacrylamide gel was used. The composition of the gel for total volume of 10ml is as follows.

Acrylamide:Bisacrylamide(30:0.8) – 5 ml

1.5M Tris HCl of pH 8.8 - 2.5ml

10% Ammonium Persulphate - 100µl

TEMED - 5 µl

Distilled water - 2.3 ml

The polyacrylamide gel was run in Amersham electrophoresis system at 90V for 5 hours using 1X TAE buffer. Apart from the samples, 100 bp ladder and undigested PCR product were loaded into adjacent wells to identify the fragment size. The gel was stained using ethydium bromide and the fragments were visualized using Chemiluminescence Gel Doc System.

Identification of Genotypes:

Three genotypes were identified for both the single nucleotide polymorphisms.

R219K:

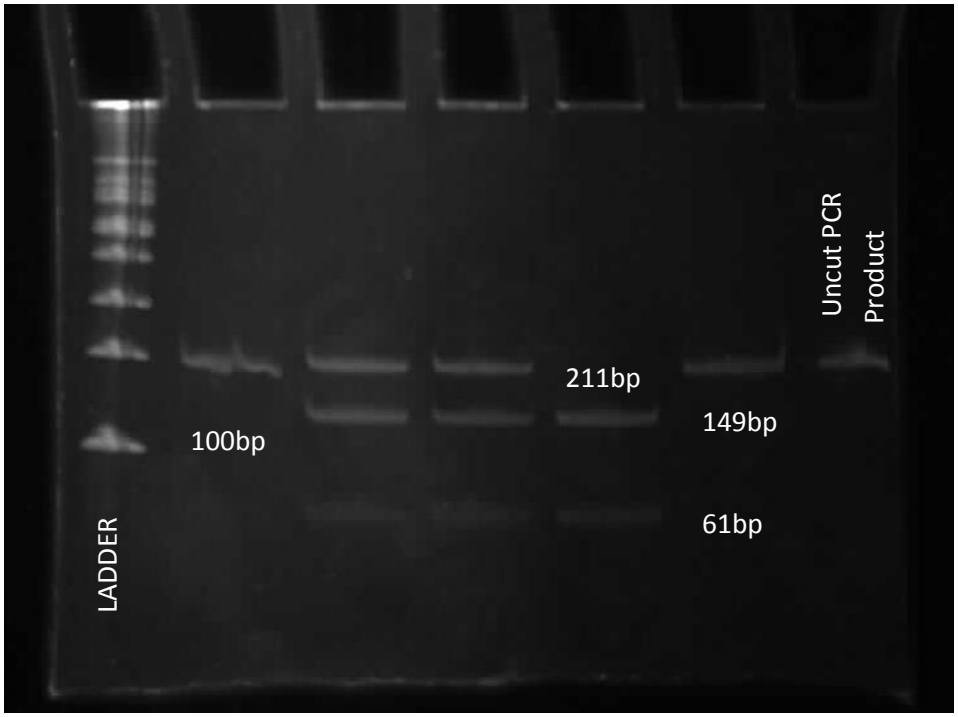


Figure: Photograph showing restriction fragments of SNP R219K

- Restriction did not occur when G allele(recessive allele) was present, yielding fragment of 211 bp(GG- homozygous wild type)
- Restriction occurred when A allele(dominant allele) was present, yielding fragments of 149bp and 61bp (AA –homozygous mutant)
- Restriction occurred when both A allele and G allele were present, yielding fragments of 211bp +149 bp + 61 bp(AG- heterozygous)

C69T:

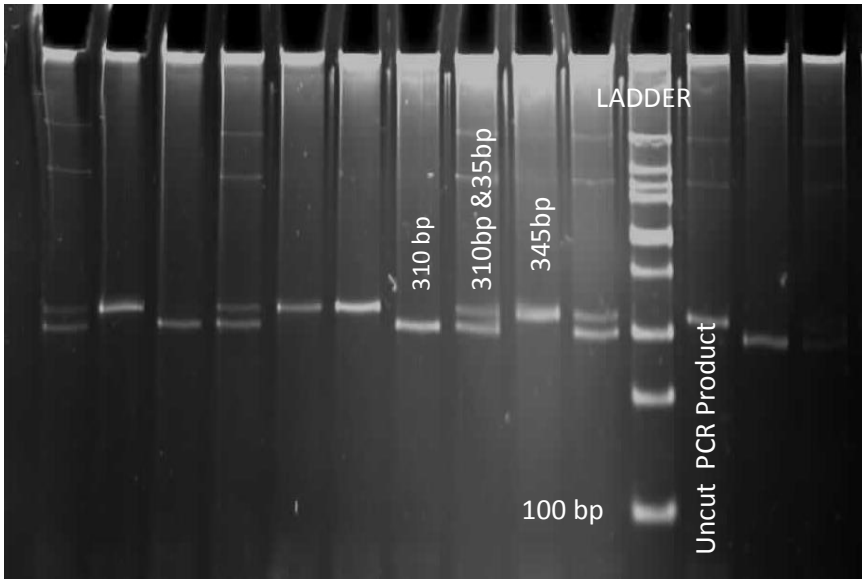


Figure: Photograph showing restriction fragments of SNP R219K

- Restriction did not occur when C allele(recessive allele) was present, yielding fragment of 345bp(CC-homozygous wild type)
- Restriction occurred when T allele(dominant allele) was present, yielding fragment of 310 bp (TT-homozygous mutant)
- Restriction occurred when both A allele and G allele were present, yielding fragments of 310 bp + 35 bp(CT – Heterozygous)

STATISTICAL ANALYSIS

STATISTICAL ANALYSIS:

The data obtained after the computing BMI, WHR, estimation of FPG, post prandial glucose, lipid profile, HbA1c and genotype analysis were statistically analyzed using IBM SPSS software version 24.

The data distribution was displayed by bar diagram, histogram and pie chart. To find any statistically significant difference between the two groups in the distribution of the demographic characteristics and measured parameters, independent t-test was used. Additive model of odds ratio was used to determine the effect of dominant allele. The dependence of categorical variable was tested with Chi Square test. Significance was assessed at 5% level.

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RESULTS

RESULTS:

A total of 100 subjects were recruited in this study namely 50 type 2 diabetes cases and 50 non-diabetic healthy controls. In our study, the primary aim was to investigate the association of anthropometric characteristics, demographic factors and risk factors in type 2 DM with ABCA1 polymorphism R219K(rs2230806) and SNP C69T (rs1800977) among the study subjects.

The distribution of demographic characters of the study population including age, sex, BMI, waist-hip ratio(WHR) are given in figures 1,2,3 &4. With respect to age and sex, both the parameters were found to be equally distributed among cases and controls implying that the study groups were well matched for both the parameters.

The means of BMI, WHR, systolic and diastolic blood pressure were higher in control groups than in patients. The BMI of the subjects in both the groups was found to be clustered around 27 reflecting that most of the study population fell into the overweight category.

The values of the t-test done to compare the means of these two groups' age , BMI and WHR is shown in Tables 1,2&3. This shows that there is no significant difference between the two groups with regard to age, BMI and WHR at the time of sampling. This suggests that the two groups are harmonized well with respect to these parameters.

In our study it was found that the means of FPG and HbA1c were found to be greater in patients than healthy controls. However while considering the lipid

profile, total cholesterol and HDL-c mean were almost equal in both the groups but TGL and LDL-c were higher among patients. The means of parameters like urea, creatinine, serum protein,serum bilirubin, SGPT, SGOT, ALP,GGT did not show any statistical significance among case and controls.

Out of the 50 subjects with diabetes mellitus, only 19 (38%) of them had a positive family history. More than three fourth of the cases, 88% (N=44) were on Oral Hypoglycemic Agents (OHA). Furthermore, the genotype analysis of SNP R219K(rs2230806) and SNP C69T (rs1800977) of ABCA1 gene did not have statistically significant association with type 2 DM patients. While considering the association of various anthropometric and clinical parameters of patients with both SNPs, there was no significant statistical difference except for age and HbA1c which were statistically significant (P value 0.018 and 0.019 respectively) between the genotypes of SNP R219K.

FIGURE 1: Age distribution among study groups

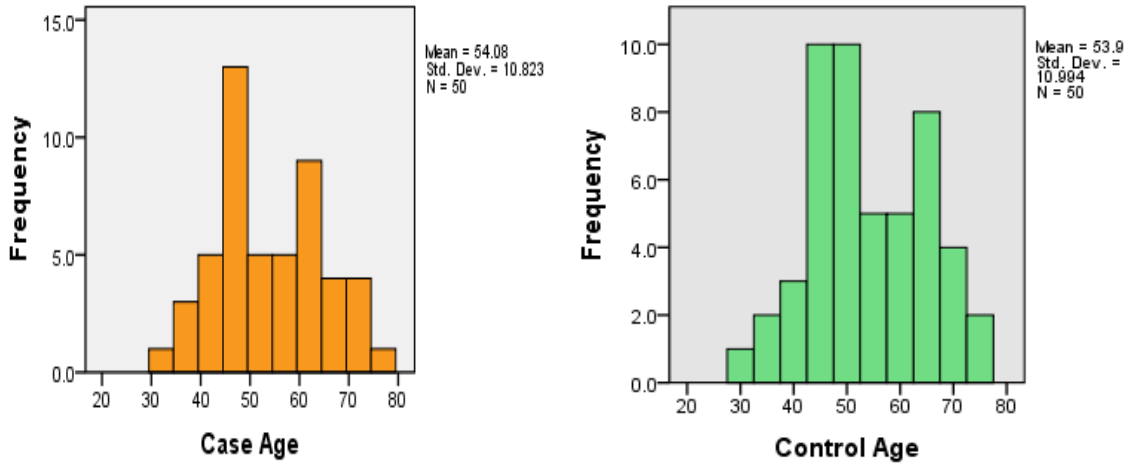


FIGURE 2: Age distribution among study groups

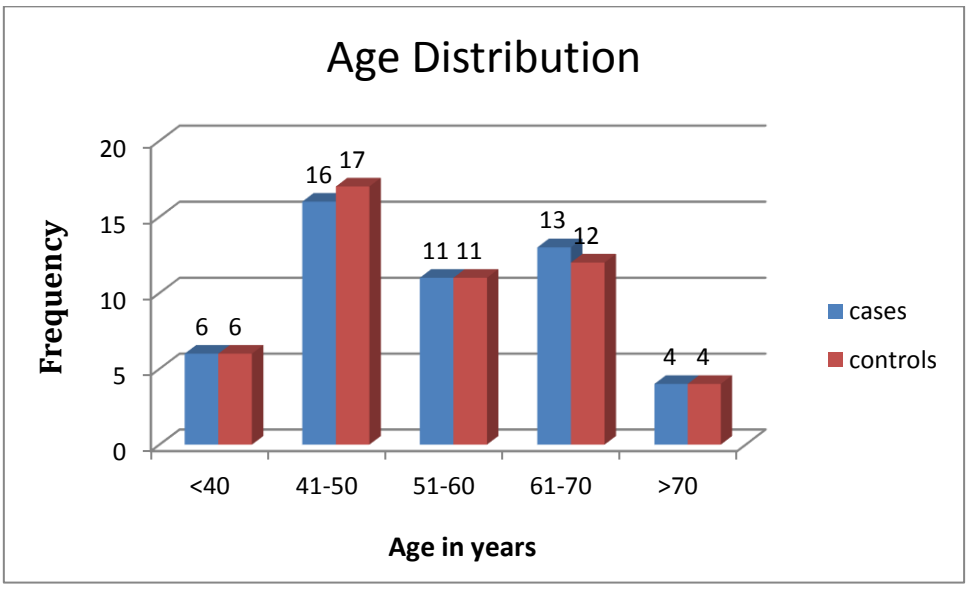


TABLE 1: Clinical and biochemical characteristics among study groups

Parameters	Type 2 DM N=50 (Mean±SD)	Controls N=50 (Mean±SD)	P value
AGE(in years)	54.08±10.82	53.90±0.99	0.934
BMI(kg/m^2)	26.510±3.4847	27.476±2.6307	0.121
WHR	0.90±0.08	0.92±0.08	0.450
FPG(mg/dl)	137.36±32.68	100.78±8.80	0.001
HbA1c(%)	7.64±1.50	5.68±0.37	0.001
TC(mg/dl)	186±40.14	186.8±28.01	0.949
HDL(mg/dl)	41.44±8.6	42.28±10.11	0.657
TGL(mg/dl)	133.40±48.18	130.30±48.10	0.711
LDL(mg/dl)	127.46±32.9	116.26±21.3	0.046
Creatinine(mg/dl)	0.75±0.16	0.80±0.18	0.198
Urea(mg/dl)	22.6±6.16	22.5±6	0.935
Total Bilirubin(mg/dl)	0.60±0.26	0.59±0.24	0.782
SGPT(IU/L)	23.36±7.97	21.94±8.59	0.394
SGOT(IU/L)	22.26±8.39	22.18±7.55	0.96
ALP(IU/L)	72.10±19.37	79.12±17.20	0.058
GGT(IU/L)	29.06±19.69	29.08±12.81	0.133
Total Protein(g/dl)	7.21±0.69	7.46±0.43	0.052

FIGURE 3: Sex distribution among study groups

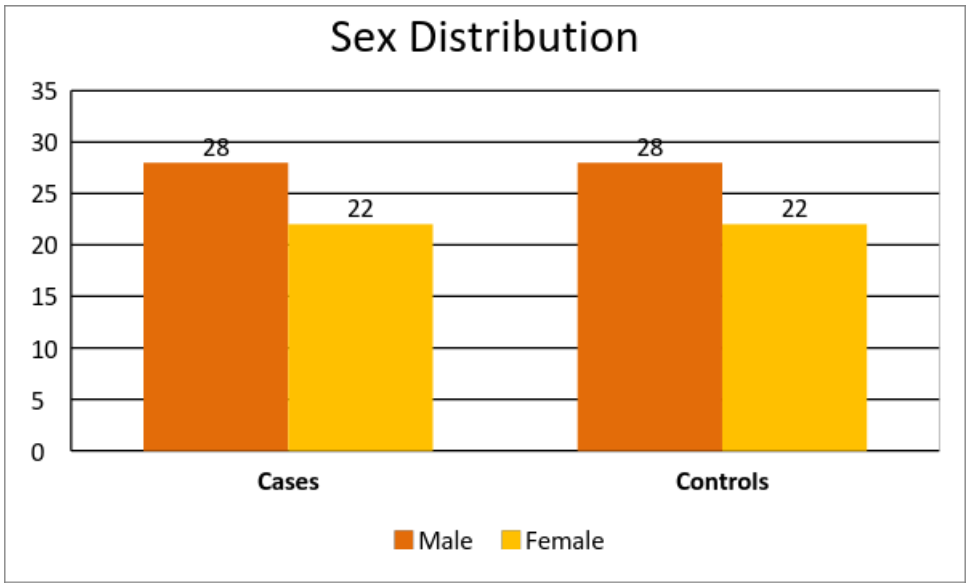
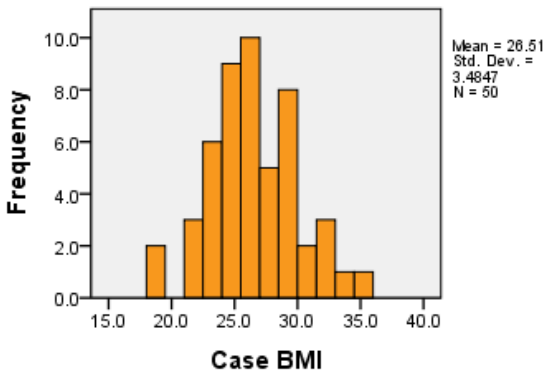


FIGURE 4: BMI distribution among study groups:



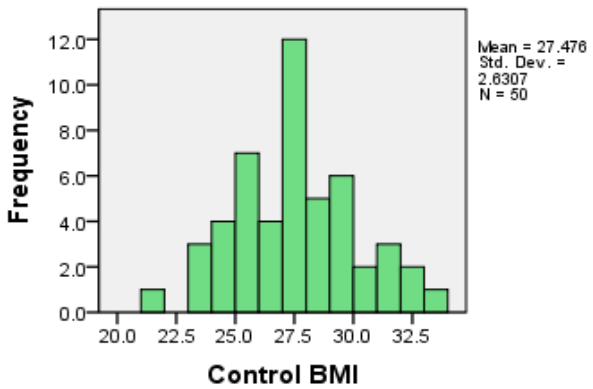


TABLE 2: Comparison of BMI among study groups

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
BMI	Equal variances assumed	3.472	.065	-1.564	98	.121	-.9660	.6175
	Equal variances not assumed			-1.564	91.159	.121	-.9660	.6175

TABLE 3: Comparison of WHR among study groups

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
WaistHipRatio	Equal variances assumed	.008	.931	-.758	98	.450	-.01280	.01688
	Equal variances not assumed			-.758	97.869	.450	-.01280	.01688

FIGURE 5: Distribution of fasting plasma glucose levels among study groups

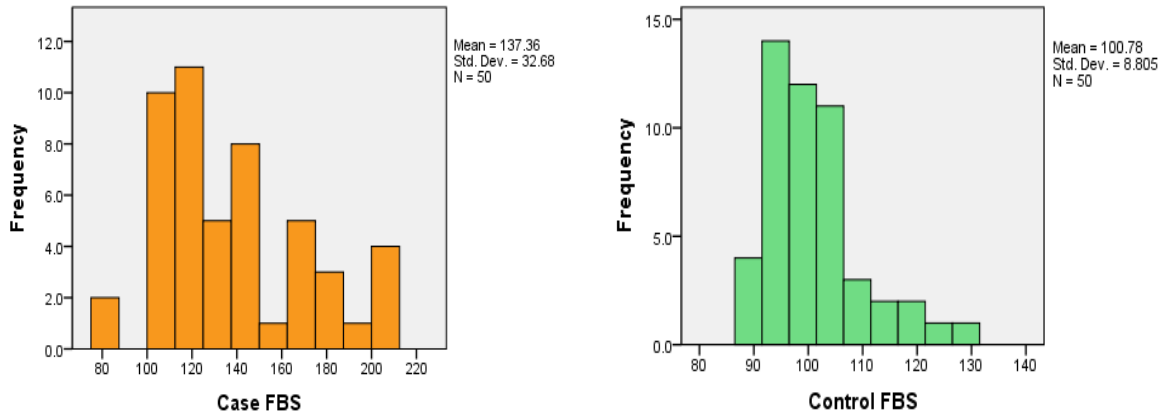


TABLE 4: Comparison of fasting plasma glucose among study groups

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
FBSmgdl	Equal variances assumed	53.381	.000	7.642	98	.000	36.5800	4.7865
	Equal variances not assumed			7.642	56.076	.000	36.5800	4.7865

FIGURE 6: Distribution of HbA1c levels among study groups

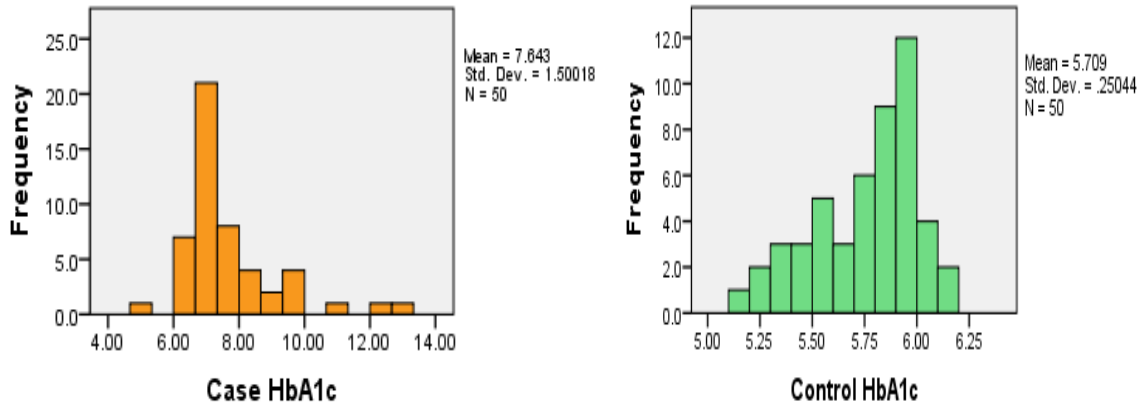


TABLE 5: Distribution of HbA1c levels among study groups

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
HbA1c	Equal variances assumed	32.446	.000	8.568	98	.000	1.87400	.21871
	Equal variances not assumed			8.568	55.125	.000	1.87400	.21871

FIGURE 7: Distribution of total cholesterol levels among study groups

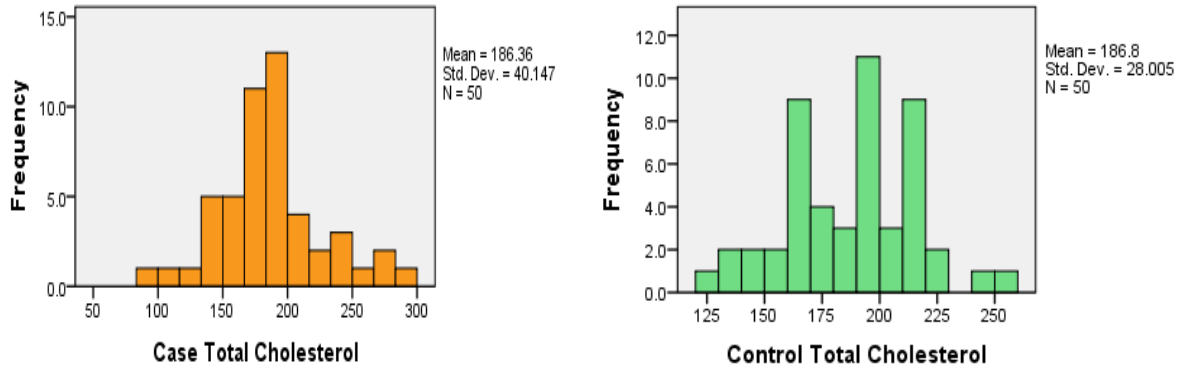


TABLE 6: Comparison of total cholesterol levels among study groups

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
TotalCholesterol	Equal variances assumed	2.148	.146	-.064	98	.949	-.4400	6.9225
	Equal variances not assumed			-.064	87.557	.949	-.4400	6.9225

FIGURE 8: Distribution of HDL-c levels among study groups

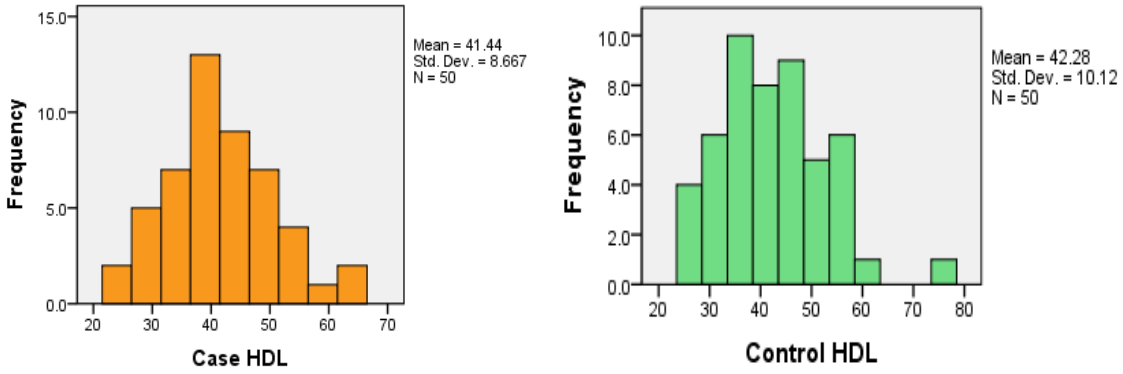


TABLE 7: Distribution of HDL-c levels among study groups

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
HDL	Equal variances assumed	1.279	.261	-.446	98	.657	-.8400	1.8842
	Equal variances not assumed			-.446	95.736	.657	-.8400	1.8842

FIGURE 9: Distribution of triglyceride levels among study groups

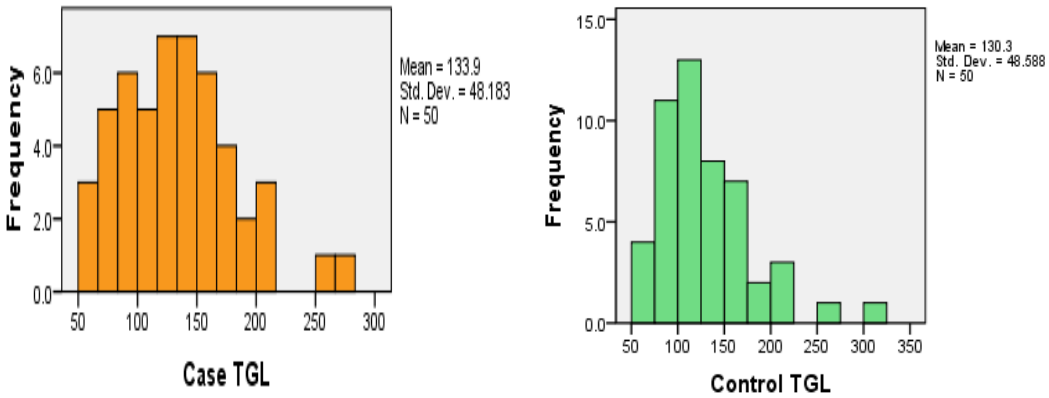


TABLE 8: Comparison of triglyceride levels among study groups

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
TGL	Equal variances assumed	1.460	.230	1.080	98	.283	24.3200	22.5163
	Equal variances not assumed			1.080	58.959	.284	24.3200	22.5163

FIGURE 10: Distribution of LDL- c levels among study groups

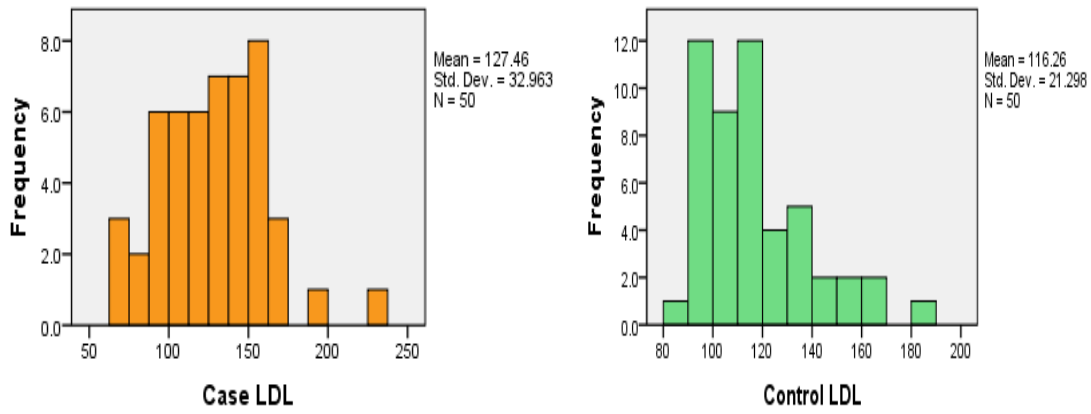


TABLE 9: Comparison of LDL- c levels among study groups

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
LDL	Equal variances assumed	7.638	.007	2.018	98	.046	11.2000	5.5501
	Equal variances not assumed			2.018	83.838	.047	11.2000	5.5501

TABLE 10: Comparison of clinical and biochemical characteristics between male and female among cases

Type 2 diabetes mellitus Patients	Male (Mean ±SD)	Female (Mean ±SD)	P value
BMI(kg/m^2)	26.79±2.82	27.08±3.16	0.795
WHR	0.91±0.11	0.91±0.07	0.847
FPG(mg/dl)	112.35±23.6	120.16±30.8	0.371
HbA1c(%)	6.75±1.86	6.69±1.36	0.890
TC(mg/dl)	178.78±28.4	187.84±35.16	0.364
HDL-c (mg/dl)	41.28±9.04	38.62±9.46	0.543
TGL(mg/dl)	122.85±44.8	133.6±48.82	0.442
LDL-c(mg/dl)	121.77±27.7	121.8±29.6	0.983
Duration of disease (months)	17.89±6.13	15.2±6.03	0.232

TABLE 11: Comparison of renal function &liver function parameters among study groups

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Creatinine	Equal variances assumed	1.336	.251	-1.295	97	.198	-.046	.035
	Equal variances not assumed			-1.296	96.602	.198	-.046	.035
Urea	Equal variances assumed	.742	.391	.082	98	.935	.1000	1.2176
	Equal variances not assumed			.082	97.932	.935	.1000	1.2176
BiliTot	Equal variances assumed	.072	.789	.277	98	.782	.0140000000000100	.0504777178565596
	Equal variances not assumed			.277	97.368	.782	.0140000000000100	.0504777178565596
BiliDirect	Equal variances assumed	4.001	.048	-2.064	98	.042	-.0280000000000100	.0135646599663351
	Equal variances not assumed			-2.064	95.066	.042	-.0280000000000100	.0135646599663351
BiliIndirect	Equal variances assumed	1.114	.294	.934	98	.353	.0420000000000100	.0449843510205900
	Equal variances not assumed			.934	95.988	.353	.0420000000000010	.0449843510205900

TABLE 11(contd..) : Comparison of liver function parameters among study groups

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
SGPT	Equal variances assumed	.731	.395	.856	98	.394	1.4200	1.6582
	Equal variances not assumed			.856	97.460	.394	1.4200	1.6582
SGOT	Equal variances assumed	2.095	.151	.050	98	.960	.0800	1.5940
	Equal variances not assumed			.050	96.981	.960	.0800	1.5940
ALP	Equal variances assumed	.807	.371	-1.916	98	.058	-7.0200	3.6644
	Equal variances not assumed			-1.916	96.640	.058	-7.0200	3.6644
GGT	Equal variances assumed	5.529	.021	1.516	98	.133	4.9800	3.2843
	Equal variances not assumed			1.516	82.225	.133	4.9800	3.2843
ProteinTotal	Equal variances assumed	15.794	.000	-2.179	98	.032	-.2520	.1156
	Equal variances not assumed			-2.179	81.809	.032	-.2520	.1156

TABLE 12: Comparison of genotypes of C69T polymorphism among study groups

Crosstab

Count

		CASECONTROL		Total
		1.0	2.0	
C69T	1.0	9	5	14
	2.0	15	16	31
	3.0	26	29	55
Total		50	50	100

C69T 1.0 –TT

C69T 2.0 – CC

C69T 3.0 – CT

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	1.339[a]	2	.512
Likelihood Ratio	1.355	2	.508
Linear-by-Linear Association	.929	1	.335
N of Valid Cases	100		

.

0 cells (.0%) have expected count less than 5. The minimum expected count is 7.00.

TABLE 13: Comparison of genotypes of C69T polymorphism among study groups(Additive model)

C69T	Cases	Controls	Total
TT	9	5	14
CT + CC	41	45	86
Odds ratio – 1.975			
95% CI – 0.612 to 6.380			
P value – 0.255			

TABLE 14: Comparison of genotypes of C69T polymorphism among study groups(Additive model)

C69T	Cases	Controls	Total
CT + TT	35	34	69
CC	15	16	31
Odds ratio – 1.098			
95% CI – 0.4703 to 2.563			
P value – 0.8288			

FIGURE 11: Distribution of C69T genotype among study groups

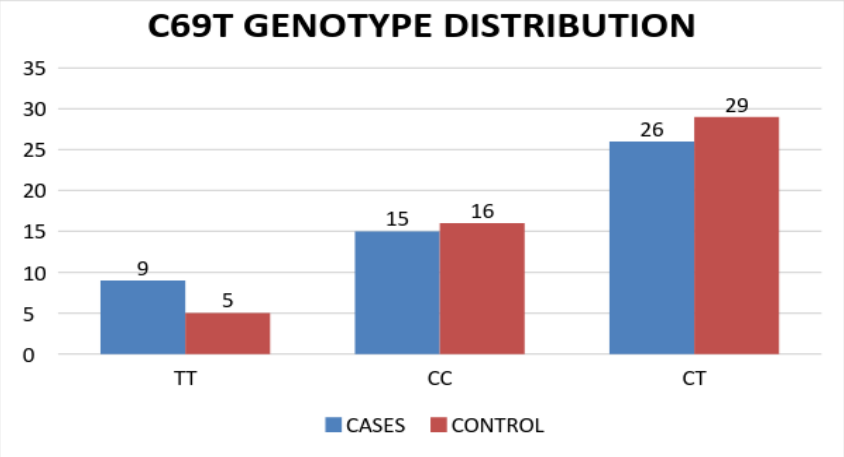


TABLE 15: Comparison of genotypes of R219K polymorphism among study groups

Crosstab

Count

		CASECONTROL		Total
		1.0	2.0	
R219K	1.0	7	4	11
	2.0	21	16	37
	3.0	22	30	52
Total		50	50	100

R219K 1.0 - AA

R219K 2.0 - GG

R219K 3.0 - AG

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.725[a]	2	.256
Likelihood Ratio	2.742	2	.254
Linear-by-Linear Association	2.593	1	.107
N of Valid Cases	100		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.50.

TABLE 16: Comparison of genotypes of R219K polymorphism among study groups(Additive model)

R219K	Cases	Controls	Total
AA	7	4	11
AG+GG	43	46	89
Odds ratio – 1.872			
95% CI – 0.512 to 6.848			
P value – 0.338			

TABLE 17: Comparison of genotypes of R219K polymorphism among study groups(Additive model)

R219K	Cases	Controls	Total
AA+ AG	29	34	63
GG	21	16	37
Odds ratio –1.064			
95% CI – 0.287 to 1.972			
P value – 0.301			

FIGURE 12: Distribution of R219K genotype among study groups

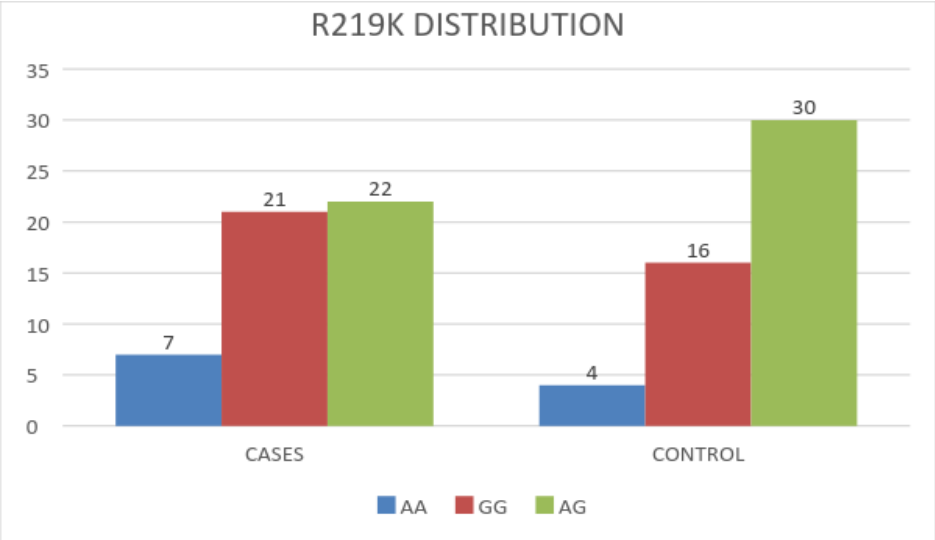


TABLE 18: Comparison of allele frequencies of C69T polymorphism among study groups

Crosstab

Count

		PATIENTS		Total
		1	2	
C69T	1	56	60	116
	2	44	40	84
Total		100	100	200

C69T 1 – C allele

C69T 2 – T allele

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.328[a]	1	.567		
Continuity Correction[b]	.185	1	.667		
Likelihood Ratio	.329	1	.567		
Fisher's Exact Test				.667	.334
Linear-by-Linear Association	.327	1	.568		
N of Valid Cases	200				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 42.00.

b. Computed only for a 2x2 table

FIGURE 13: Distribution of alleles of C69T among study groups

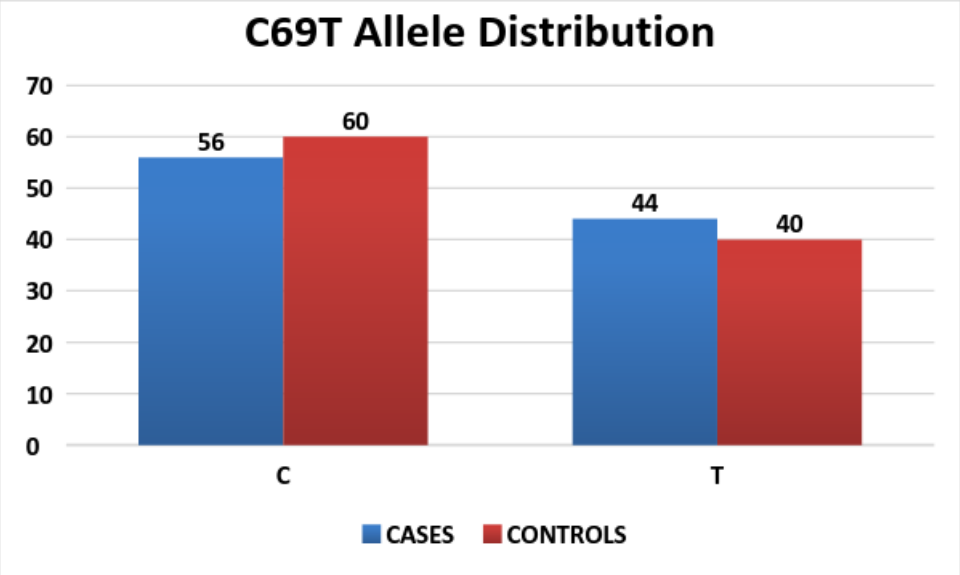


FIGURE 14: Distribution of alleles of R219K among study groups

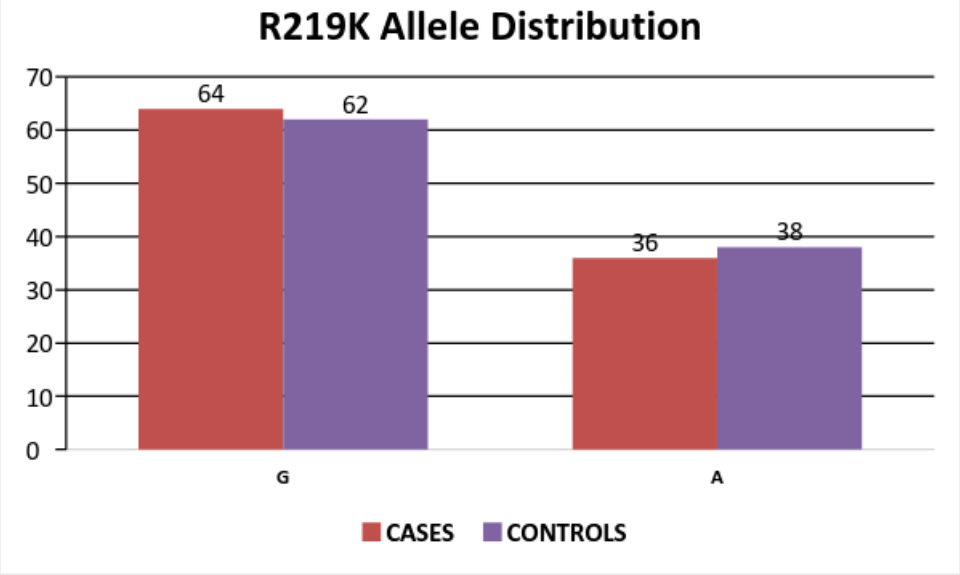


TABLE 19: Comparison of allele frequencies of R219K polymorphism among study groups

Crosstab

Count

		PATIENTS		Total
		1	2	
R219K	1	64	62	126
	2	36	38	74
Total		100	100	200

R219K 1 – G allele

R219K 2 – A allele

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.086[a]	1	.770		
Continuity Correction[b]	.021	1	.884		
Likelihood Ratio	.086	1	.770		
Fisher's Exact Test				.884	.442
Linear-by-Linear Association	.085	1	.770		
N of Valid Cases	200				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 37.00.

b. Computed only for a 2x2 table

TABLE 20: Sex distribution of R219K genotype among study groups

Crosstab

Count

		R219K		Total
		1.0	2.0	
SEXCODE	1.0	8	49	57
	2.0	3	40	43
Total		11	89	100

Sex code 1.0 - Male

Sex code 2.0 - Female

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.247[a]	1	.264		
Continuity Correction[b]	.630	1	.427		
Likelihood Ratio	1.303	1	.254		
Fisher's Exact Test				.343	.216
Linear-by-Linear Association	1.235	1	.266		
N of Valid Cases	100				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 4.73.

b. Computed only for a 2x2 table

TABLE 21: Sex distribution of C69T genotype among study groups

Crosstab

Count

		C69T		Total
		1.0	2.0	
SEXCODE	1.0	5	52	57
	2.0	9	34	43
Total		14	86	100

Sex code 1.0 - Male

Sex code 2.0 – Female

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3.009[a]	1	.083		
Continuity Correction[b]	2.084	1	.149		
Likelihood Ratio	2.988	1	.084		
Fisher's Exact Test				.144	.075
Linear-by-Linear Association	2.979	1	.084		
N of Valid Cases	100				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 6.02.

b. Computed only for a 2x2 table

TABLE 22: Comparison of family history among cases

	Among Cases N=50	
Family History	No Of Patients	Percentage
Present	19	38%
Absent	31	62%

FIGURE 15: Comparison of family history among cases

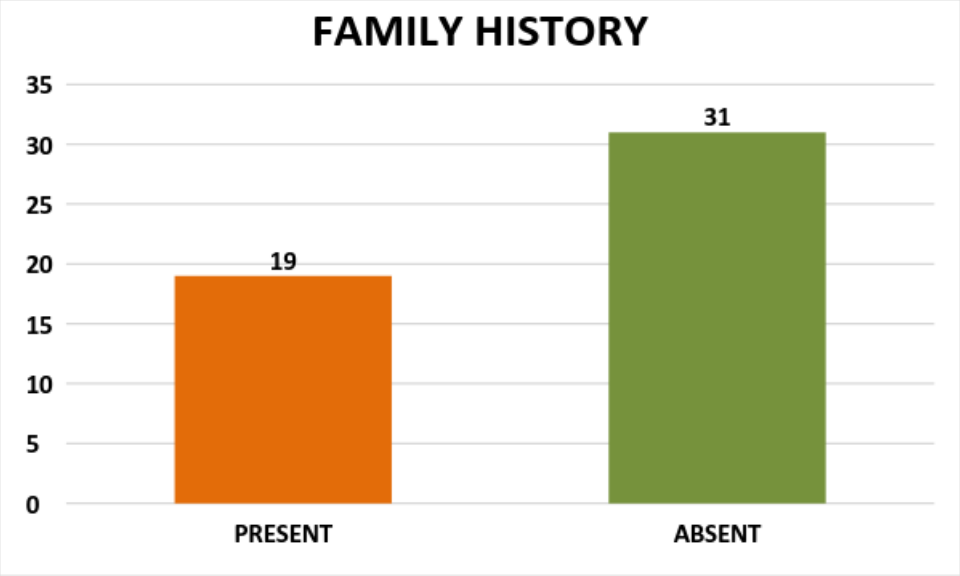


TABLE 23: Comparison of drug history among cases

	Among Cases N=50	
Treatment	No Of Patients	Percentage
OHA+ Insulin	4	8%
OHA Only	44	88%
Insulin Only	2	4%

FIGURE 16: Comparison of drug history among cases

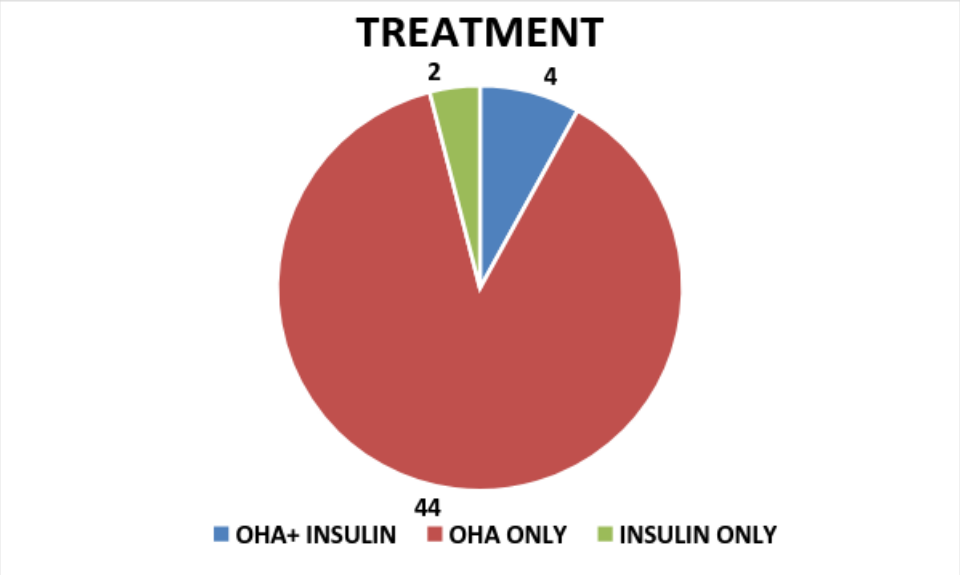


TABLE 24: Comparison of clinical and biochemical parameters of C69T genotype among cases

C69T	Mutant (Mean ±SD)	Non-Mutant (Mean ±SD)	P value
BMI(kg/m^2)	26.79±2.82	27.08±3.16	0.795
WHR	0.91±0.11	0.91±0.07	0.847
FPG(mg/dl)	112.35±23.6	120.16±30.8	0.371
HbA1c(%)	6.75±1.86	6.69±1.36	0.890
TC(mg/dl)	178.78±28.4	187.84±35.16	0.364
HDL-c (mg/dl)	43.28±9.04	41.62±9.46	0.543
TGL(mg/dl)	122.85±44.8	133.6±48.82	0.442
LDL-c(mg/dl)	121.77±27.7	121.8±29.6	0.983
Duration of disease (months)	17.89±6.13	15.2±6.03	0.232

TABLE 25:Comparison of drug history of C69T genotype among cases

	C69T	
Treatment	Mutant	Non Mutant
OHA+Insulin	1	3
OHA	7	37
Insulin	1	1
P Value - 0.139		

FIGURE 17: Comparison of drug history of C69T genotype among cases

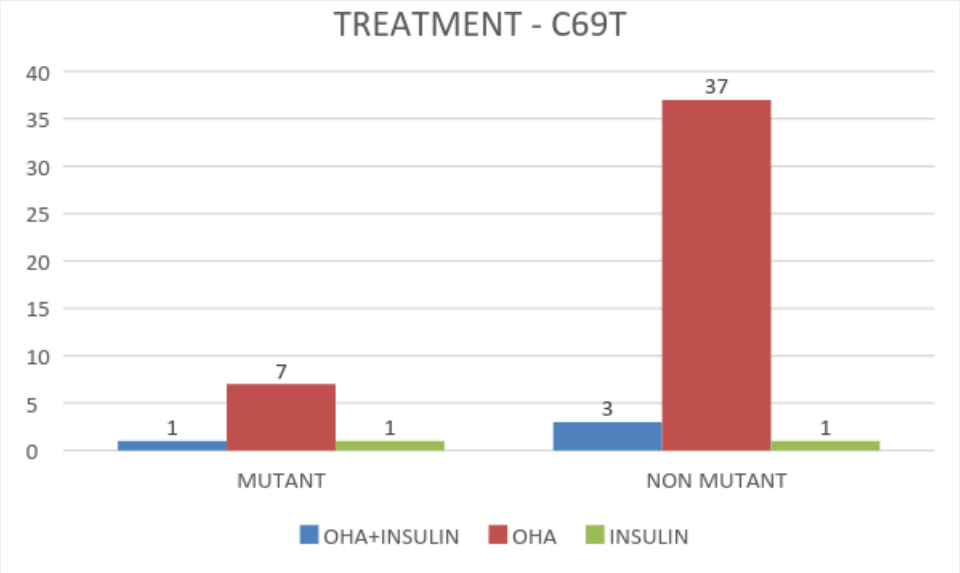


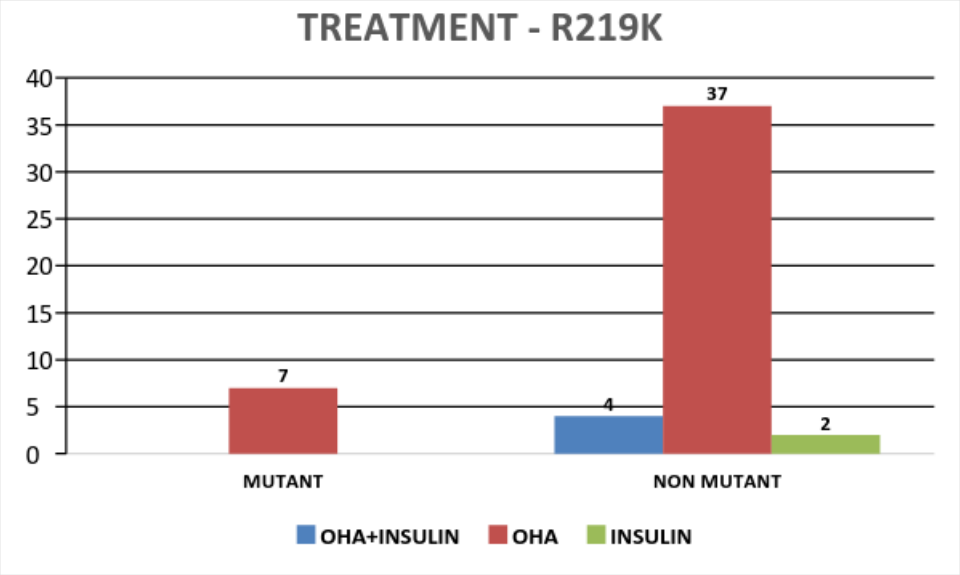
TABLE 26: Comparison of clinical and biochemical parameters of R219K genotype among cases

R219K	Mutant (Mean ±SD)	Non-Mutant (Mean ±SD)	P value
BMI(kg/m^2)	27.3±3.86	26.95±3.02	0.730
WHR	0.90±0.05	0.91±0.08	0.756
FPG(mg/dl)	126±25.36	118.2±30.62	0.121
HbA1c(%)	7.65±2.3	6.58±1.26	0.019
TC(mg/dl)	183.27±45.27	186.98±55.56	0.738
HDL-c (mg/dl)	41.27±7.87	41.93±9.58	0.827
TGL(mg/dl)	127±45.82	132.73±48.74	0.712
LDL-c(mg/dl)	114.36±27.7	122.78±28.25	0.352
Duration of disease (months)	14.86±6.03	15.81±6.79	0.704

TABLE 27: Comparison of drug history of R219K genotype among cases

	R219K	
Treatment	Mutant	Non Mutant
OHA+Insulin	0	4
OHA	7	37
Insulin	0	2
P Value - 0.574		

FIGURE 18: Comparison of drug history of R219K genotype among cases



DISCUSSION

DISCUSSION:

The global prevalence of type 2 DM has increased exceptionally making it important to carry out research, to assess the risk factors and the multigenetic factors associated with type 2 DM. Type 2 DM is becoming a major public health problem mainly because of its complications[5]. One of the foremost complication due to type 2 DM is atherosclerosis, making it to be considered as cardiovascular risk equivalent.

According to the current evidence, it is believed that both environmental and genetic factors have almost equal contribution for the development of type 2 DM in an individual[46]. Several susceptibility genes linked with the metabolism of lipids are being investigated to assess their relationship with type 2 DM. One such gene is the ABCA1 gene, which is associated with HDL-c metabolism.

ABCA1 is a protein involved in reverse cholesterol transport of HDL-c metabolism.This pathway is recognized to protect against the development of atherosclerosis by clearing excess cholesterol from arterial cells[50].Polymorphism in ABCA1 gene causes HDL-c deficiency leading to accumulation of cholesterol in the tissue macrophages and the resultant atherosclerosis[51].

Our study was done to find the association of ABCA1 gene polymorphism with type 2 DM. Newly diagnosed type 2 DM patients(n=50) and non-diabetic healthy controls (n=50) were recruited for our study. The association of the genotype with demographic and biochemical characters of the cases were also studied.

Figure 1 shows the age distribution among the study groups. The mean age±SD (years) for the cases was 54.08±10.82 whereas for controls it was found to be

53.09±0.99. The difference between the mean age was not statistically significant(P>0.05) as shown in Table 1. This indicates that the two groups were matched well with respect to age. Sex distribution of the patients was found to be almost equal among the two groups (Figure 3).There was no statistical difference in BMI and WHR between cases and controls (P>0.05) as shown in Tables 2 & 3 respectively.

As per ADA criteria, individuals with HbA1c levels of 5.7%-6.4% fall into the prediabetes category whereas HbA1c>6.5% is diagnostic of type 2 DM. In the present study, the mean ±SD(%) values of HbA1c for cases and controls were 7.64±1.50 and 5.68±0.37 respectively. This clearly shows a noteworthy variation which was reinforced statistically (P<0.05) as shown in Table 5. In addition to this the FPG levels among cases and controls had a significant difference with P<0.05(Table 4).

In the present study when the means of TC,TGL and LDL-c were assessed between the cases and controls, TC and TGL did not demonstrate any statistical difference(Tables6&8).Nevertheless, LDL-c showed a significant difference(P<0.05) between the cases and controls as depicted in Table 9.

In individuals with type 2 DM, clearance rate of LDL-c is reduced. This is because LDL-c binding to the receptor is under the stimulation by insulin.Hence due to the insulin resistance in type 2 DM there is a decrease in clearance of LDL-c thereby promoting dyslipidemia associated with type 2 DM[52].

According to NCEP ATP III guidelines, the optimal level for HDL-c is >50 mg/dL for both men & women. In our study, the mean±SD (mg/dL) of HDL-c levels

among the cases and controls were 41.44±8.6 and 42.28±10.11 respectively(Table1). This shows that HDL-c is below the optimal level in both cases and controls. Also, the mean±SD(mg/dL) of HDL-c was computed for males and females of cases and controls separately(Table 10). The mean HDL-c levels of males and females in case group were 41.28 mg/dL and 38.62 mg/dL respectively. In control group, the mean was 42.6 mg/dL and 39.9 mg/dL among the males and females respectively. Hence there is a decrease in HDL-c the levels among the cases when compared to the controls. But this difference was not statistically significant($P>0.05$) which might be due to the small sample size.

When the levels of HDL-c decreases, it is considered as a risk factor for the development of cardiovascular complications of type 2 DM [44,45,49]. This decreased levels of HDL-c is promoted through the action of CETP which enhances the formation of TGL- rich HDL-c , thereby making it a very good substrate for hepatic lipase, an enzyme involved in HDL-c catabolism[53]. Due to this decrease in HDL-c levels and chronic inflammatory state of type 2 DM, HDL-c becomes pro-oxidant, pro-inflammatory and pro-thrombotic in nature. Thus, inspite of strict glycemic control and medications prescribed by the healthcare physicians, there is increased risk for the development of cardiovascular disease in type 2 DM which is evidenced by increased mortalities and morbidities.

Our study results shows that the means of liver function and renal function parameters, namely urea, creatinine, serum protein, serum bilirubin, SGPT, SGOT, ALP, GGT did not have statistical difference when compared among cases and controls(Table11).

Genotype Analysis:

In the current study, we chose two SNPs of ABCA1 gene namely R219K and C69T based on the population genetics and the clinical studies by Polin et al[44].,Udita Singh et al[45]., in which the presence of these two SNPs were found to be associated with type 2 DM among Malaysian and central Indian population respectively.

ABCA1 gene encodes a vital protein regulating cholesterol efflux from the cells to HDL-c. Many studies have reported that genetic variations in ABCA1 gene is associated with decreased serum HDL-c levels[47].This is because,it is speculated that ABCA1 and lipid bilayer bind cooperatively with apoA-I and deliver the intracellular cholesterol for nascent HDL-c formation[43]. Therefore a genetic variation in ABCA1 gene might disrupt the function of the protein resulting in decreased formation of HDL-c.

Our study results shows that there is a difference in the frequency of distribution of both C69T and R219K genotypes among the study groups. The genotypes of SNP R219K were AA(homozygous mutant),AG(heterozygous mutant) and GG(homozygous wild type) whereas the genotypes of SNP C69T were TT(homozygous mutant),CT(heterozygous mutant) and CC(homozygous wild type).

The frequency of R219K genotypes was AA(7&4),GG(21&16) and AG(22&30) among cases and controls respectively. Similarly, the genotype distribution of C69T was TT(9&5),CC(15&16),CT(26&29) among cases and controls respectively. Thus our study results show that there is a noteworthy increase in the distribution of the homozygous mutant (AA&TT) among the cases, which was in

accordance with studies conducted by Polin et al[44] and Alharbi K K et al[46]., which demonstrated their association with decreased levels of HDL-c.

In our study, eventhough this difference in genotype frequency could not be substantiated statistically(P>0.05) the reason might be the small sample size(n=50 in each group) which was not large enough to demonstrate statistical difference(Table 12 &13). On increasing the sample size, there is a high likelihood of obtaining statistically significant results which will prove the association of ABCA1 gene polymorphism with type 2 DM.

Additionally, additive model for odds ratio was used to determine whether the presence of the dominant allele alone(A allele in R219K and T allele in C69T) is enough to cause pathological changes in HDL-c metabolism in type 2 DM. By this model, our study results showed that OR >1 (Tables 14,15,17&18) implying that the presence of the dominant allele is a risk factor for the development of decreased levels of HDL-c in type 2 DM. This result is in concordance with the studies conducted by Polin et al[44] and Alharbi K K et al[46].

In our study, when the allele frequencies of C69T and R219K were computed, it revealed no significant difference amid the study groups(P >0.05) as shown in Tables 19 & 20 respectively. The difference in sex distribution was assessed for C69T and R219K and it was not found to be statistically significant(P>0.05) as displayed in Table 21 & 22 respectively.

The association between the two SNPs and various parameters like age, BMI, WHR, FPG, HbA1c and lipid profile was assessed in the present study. There was no substantial association(P>0.05) found for these parameters and the genotype distribution(Tables 25 & 27). This was in accordance with the studies done by K K Alharbi et al[46] and Ergen et al[49].

Most of the patients were on oral hypoglycemic agents (N=44 ie 88%) hence we studied the association of the modality of treatment with the genotypes. But, it was found to be insignificant(P>0.05) for both the SNPs namely C69T and R219K(Table 26 & Table 28 respectively).

The limitation with our study was the small sample size. Inspite of the presence of noteworthy difference in genotype distribution among cases and controls, the difference was not evident statistically due to the small sample size. The previous studies done to demonstrate the association of ABCA1 gene polymorphism in type 2 DM were done with a minimum sample size of 300. Hence, by increasing the sample size there is a high likelihood of obtaining statistically significant results.

CONCLUSION

CONCLUSION:

1. There is a difference in distribution of the genotypes of SNPs, C69T and R219K of ABCA1 gene among subjects with type 2 DM and non- diabetic healthy controls. But, this difference in genotype frequency could not be substantiated statistically due to small sample size. On increasing the sample size, there is a high probability of obtaining statistically significant results which will provide a strong evidence for the association of ABCA1 gene polymorphism in type 2 DM.
2. The presence of dominant allele (A allele in R219K and T allele in C69T) is a risk factor for the development of decreased HDL-c levels in type 2 DM. This shows the association of ABCA1 gene polymorphism in type 2 DM.
3. Allele frequencies of ABCA1 gene polymorphism (R219K and C69T**)** were not significantly different amidst type 2 DM patients and non-diabetic healthy controls.
4. There is no significant association between ABCA1 gene polymorphism (R219K and C69T**)** and clinical and biochemical characteristics like BMI, WHR, FPG , HbA1c and lipid profile among cases.

SUMMARY

SUMMARY:

Type 2 Diabetes Mellitus is a complex polygenic disorder with derangements in carbohydrate, lipid and protein metabolism. Eventhough the number of studies describing the epidemiology of diabetes over the last 20 years has been extraordinary, diabetes mellitus still remains an enigma due to its multifactorial etiology. Development of dyslipidemia with elevated levels of LDL-c and low levels of HDL-c concentration is associated with type 2 DM.

ABCA1 is a subfamily of proteins , involved in transportation of phospholipids and cholesterol from the intracellular compartments to lipid-poor apolipoproteins. ABCA1 gene is located on the chromosome 9 region q31.1. ABCA1 gene polymorphisms have been associated with reduction in the serum levels of HDL-c, along with alterations in the quality of HDL-c.

This study was done to find the association of the polymorphism of ABCA-1 gene with type 2 DM since it is accompanied by dysregulation in lipid metabolism.The study design was a case control study and each group consisted of 50 subjects. Analysis of genotype was performed by PCR-RFLP method and the biochemical parameters like FPG, HbA1c, lipid profile were analyzed by autoanalyzer.

The findings of our study showed that there was noteworthy difference in the genotype distribution of both C69T and R219K among cases when compared with the controls. However, the difference was not statistically evident, the reason for which might be due to the small sample size. Our study results showed that the presence of dominant allele alone(A allele in R219K and T allele in C69T) is a risk factor for the development of pathological changes in HDL-c metabolism in type 2 DM. This reflects that there is an association between ABCA1 gene polymorphism and type 2 DM which can be reinforced by conducting the study with a large sample size.

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ANNEXURE

Data Collection Tool:

1) Serial No:
2) IP Number / OP Number:
3) Age:
4) Sex:
5) Height:
6) Weight:
7) BMI:
8) Waist circumference:
9) Hip circumference:
10) Waist- Hip ratio:
11) BP:
12) History of illness:
    a. Duration:
    b. Oral drugs: 1.Yes 2. No
    c. Insulin: 1.Yes 2.No
13) Family History:
14) Biochemical Investigations:
    a. Fasting plasma glucose:
    b. HbA1c:
    c. Total cholesterol:
    d. HDL cholesterol:
    e. TGL:
    f. LDL cholesterol:
    g. RFT:
    h. LFT: