Association of ABCA-1 gene Polymorphism with the risk of Ischemic Heart Disease in Local Population.

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Polymorphism
Coronary
Ischemic heart disease
ABCA1 gene

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FINAL APPROVAL

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A thesis submitted to Department of Bioinformatics and Biotechnology International Islamic University Islamabad as a partial fulfillment of requirement for the award of the Degree of MS Biotechnology.

DEDICATION

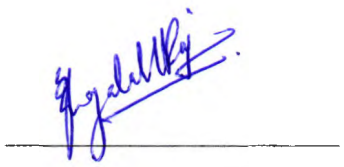
This work is dedicated to my parents whose love, prayers, hopes and encouragements have always been there for me.

"My Lord, have mercy upon them as they brought me up when I was small." ***(17:24)***

DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date: 10 - 10 - 2016



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God bless you all.

Ghazala Mushtaq Raja

LIST OF ABBRIVIATIONS

ABCA1	ATP Binding cassette transporter A 1
ARMS	Amplification Refractory Mutation system
CAD	Coronary Artery Disease
CERP	Cholesterol Efflux Regulatory Protein
CHF	Coronary Heart Failure
CVD	Cardiovascular disease
DALYs	Disability adjusted life years
eNOS	Endothelial Nitric oxide synthase
FHA	Familial Hyperalphalipoprotenemia
HDL	High density Lipoproteins
HF	Heart Failure
IHD	Ischemic heart disease
LCAT	Lecithin cholesterol acetyltransferase
LDL	Low density Lipoprotein
LTA	Lymphotoxin Alpha
MEF2A	Myocyte specific enhancer factor 2A
MI	Myocardial infarction
PCR	Polymerase chain reaction
SNP	Single Nucleotide Polymorphism
SR-BI	Scavenger Receptor class B type 1
YLL	Years of lives lost

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ABSTRACT

Cardiovascular diseases (CVDs) are a broad range of problems related to vasculature. Ischemic heart disease is the most prevalent type of CVDs. ATP Binding Cassette transporter A1 (ABCA1) gene polymorphisms have been known to contribute as one of the major genetic risk factor associated with the risk of IHD. In the light of cholesterol efflux, ABCA1 gene plays a vital role. The present study was conducted to check R1587K polymorphism in ABCA1 gene and find out its association with the risk of IHD in selected Pakistani population. The genotyping for ABCA 1 gene was done through Tetra-primer ARMS-PCR for 120 Ischemic Heart Disease patients and 100 healthy controls. All the genotypes for the polymorphism were in Hardy-Weinberg Equilibrium. Homozygous TT genotype of major allele was 9.09% in the patients and 10.98% in controls with p=0.850. Percentage of heterozygous CT genotype was higher in patient 39.39% as compared to controls i.e., 35.16% with p=0.775. However the minor allele or the risk allele genotype CC was higher than that of major/ancestral allele with the frequencies of 51.51% and 53.84 % in patients and controls respectively, yet again with p=0.950. These results showed non-significant association of respective SNP with the risk of IHD patients.

KEY WORDS: ABCA1 gene, ischemic heart disease, Cholesterol efflux, coronary artery disease.

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Cardiovascular diseases cover a wide range of disorders: diseases of the vasculature, diseases of the myocardium, diseases of the heart's electrical circuit, and congenital heart disease (Roger *et al.*, 2012). In 2001, one third of the deaths were caused by CVDs with about 85% of the mortalities occurring in low to middle income countries. The most prevalent CVDs include Ischemic heart disease, cerebro-vascular disease, hypertension, inflammatory heart disease, Rheumatic heart disease. Together these five major CVDs contribute to 16 million deaths per annum around the globe. The number is estimated to rise in future years in high income countries. It is notable that the number of CVD related deaths is much higher in some regions than the others.

When talking about CVDs, most of the attention is paid on environmental causes. In fact, major risk factors are considered to be those from individual's own life style and corresponding health states. For instance, smoking, physical activity, diet, obesity, cholesterol, high blood pressure, diabetes etc. CVDs comprise of a wide range of anomalies expanding from myocardial infarction (MI) to congenital heart disease, most of which are reported to be inherited. With growing evidence that CVDs have a considerable hereditary component, more emphasis is laid on the investigation of genetic predispositions which are a result of genetic mutations that alter a normal biological function of gene hence, increase the risk of disease.

A huge number of genetic polymorphisms have been studied regarding Cardio-Vascular diseases. Mutation in Low density lipoprotein receptor genes LDLR is known to be associated with severe Hypercholesterolemia (Garcia *et al.*, 2001). Mutations in MEF2A genes have been reported to be independent factor for myocardial infarction and coronary artery disease patients (Wang *et al.*, 2003). Certain SNPs in Lymphotoxin alpha (LTA) gene are known to be associated with high risk of MI (Ozaki et al., 2002). Mutations in endothelial Nitric oxide synthase eNOS gene is studied to be associated with coronary spasm that ultimately leads to ischaemic heart disease (Masafumi *et al.*, 1999). Apolipoprotein APOE gene polymorphism has been significantly associated with coronary artery disease (Asma *et al.*, 2015). ATP- binding cassette transporter A1

(ABCA1) gene polymorphisms is reported to be linked with High density lipoprotein cholesterol and risk of ischemic heart disease (Bodzioc *et al.,* 1999). Extensive genetic studies have been conducted around the globe to catch up to the core of CVDs. This study, however, will majorly focus on the association of ABCA1 gene polymorphisms with the risk of CVDs, Ischemic Heart Disease as a dominant type.

ABCA1 is a member of ATP Binding Cassette, a membrane transporter involved in the mobilization of various substrates across plasma membranes. ABCA1 gene encodes a 2261 amino acid, a trans-membrane peptide liable for the efflux of cholesterol and phospholipids across the cell membrane to lipid poor Apolipoprotein A-I participating in interaction with vesicular traffic machinery. It is an initial and crucial step in the reverse cholesterol transport (RCT) system (Klein *et al.,* 1999). ABCA1 functions as a cholesterol efflux pump in the cellular lipid removal pathway. Since the protein encoded by ABCA1 is a basic gatekeeper that influences intracellular cholesterol transport, it is also named 'cholesterol efflux regulatory protein' (CERP) (Brooks-Wilson *et al.,* 1999). Isotopic in situ hybridization mapping showed the location of ABCA1 gene to 9q22-q31 (Luciani *et al.,* 1994). The ABCA1 gene extends to 149 kb. It is reported that it contains 50 exons with 62 repetitive Alu sequences in the 49 introns (Santamarina *et al.,* 2000). The ABCA1 gene is reported to be highly polymorphic, a large number of sequence variations affecting HDL cholesterol (HDL-C) concentrations have been identified (Wang *et al.,* 2000).

Several SNPs in non-coding regions of ABCA1 that may be important for the appropriate regulation of ABCA1 expression (i.e., in the promoter, intron 1, and the 5-prime un-translated region) have been identified. Phenotypic effects of these SNPs in 804 Dutch men with proven IHD risk and prevalence have been studied. Common variation in non-coding regions of ABCA1 may significantly alter the severity of atherosclerosis, without necessarily influencing plasma lipid levels. (Zwarts *et al.,* 2002). R219K, V771M, I883M, E1172D and R1587K are the five known ABCA1 gene polymorphisms in early-onset IHD and matched control samples from northern Germany that have been studied. Only I883M was found to modulate susceptibility to IHD in the German population. This association between I883M and IHD is consistent with many other reports that have

provided with an increased IHD risk for carriers in other populations (Brousseau *et al.*, 2001 ; Tan *et al.*, 2003 ; Clee *et al.*, 2004). ABCA1 polymorphisms are also associated with varying levels of HDL-C in Pakistani individuals which require further investigations as ABCA1 polymorphisms may have a major role in the high incidence of cardiovascular disorders in South Asians (Danish *et al.*, 2007).

OBJECTIVES

The main objectives of the study are:

1. To investigate the association of R1587K C/T polymorphism in ABCA 1 gene with the risk of IHD in localized Pakistani population.
2. To find out the genotypic and allelic frequency of the R1587K C/T polymorphism associated with risk of IHD.

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 Cardiovascular Diseases (CVDs)

Cardiovascular diseases covers a wide range disorders: brain diseases and diseases of blood vessels, diseases of the myocardium, diseases of the heart's electrical circuit, congenital heart disease, in short, diseases of vasculature wherever it may lead in the body (Roger *et al.*, 2012).

2.2 Types of CVDs

CVDs generally involve diseases due to ***atherosclerosis*** (Mendis *et al.*, 2011). It is describes as a complex pathological process in the walls of blood vessels that develops over many years. In atherosclerosis, fatty material and cholesterol deposited inside the lumen of medium and large sized blood vessels called arteries. These deposits, termed as plaques, cause the inner surface of the blood vessels to become irregular and the lumen to become narrow, making it harder for blood to flow through. Blood vessels also become less flexible as a result. Eventually, the plaque can rupture the vessel causing the formation of a blood clot. If the blood clot develops in a coronary artery, it can cause a heart attack. If the clot develops in the brain, it can cause a brain-stroke. Diseases that may result due to atherosclerosis include:

- Ischemic heart disease (IHD) also referred to as Coronary artery disease that leads to Myocardial Infarction (MI) commonly known as heart attack (Wong, 2014, Bhatia *et al.*, 2010).
- Cerebro-vascular disease commonly known as stroke.
- Diseases of the Aorta and arteries often termed as aortic Aneurysms including Hypertension.
- Peripheral Vascular disease in which vessels carrying blood to various organs and limbs are blocked by plaques.

There are several other types of CVDs. Conditions like cardiac arrhythmias where disturbances in electrical conduction system of the heart occur. Cardiomyopathies calling for disorder of heart muscle are known. Congenital heart disease in which development

of heart muscle is compromised during fetus formation that may lead to conditions like, holes in septum of the heart, abnormalities in valves or heart chambers, is another form of CVD. Rheumatic heart disease, which is caused by damage to the heart muscle and heart valves from rheumatic fever, also adds to the list of CVDs.

2.3 Prevalence of CVD

They are responsible for over 17.3 million annual mortalities and are leading causes of death worldwide (Causes of death, WHO. 2008). According to Global Burden of Disease study 2004, likelihood of CVD mortality has been reported to increase up to 23.4 million by 2030 with CVD assumed to be the major cause of disability and death around the globe (World health organization, 2008).

Accounting for 29.3% of all deaths worldwide, cardiovascular diseases (CVDs) are the leading cause of mortality in the world (The World Health Report, 2004). In developed countries, ischemic heart disease (IHD) and cerebro-vascular disease are together responsible for 36% of all deaths (World Health Report, 2003). Moreover, the mortality and burden resulting from CVDs are rapidly increasing in developing regions and population growth, ageing and globalized lifestyle changes combine to make CVD an increasingly important cause of morbidity and mortality (Abegunde *et al.*, 2007). CVDs encompass a broad range of disorders, including diseases of the vasculature, diseases of the myocardium, diseases of the heart's electrical circuit, and congenital heart disease (Roger *et al.*, 2012).

In addition to deaths, CVD is reported to cause a large non-fatal global disease burden such as angina all over the world. It is estimated that this disease burden caused up to 11.8% of global DALYs (Disability Adjusted Life Years) in 2010. However, major diseases within this group include IHD (5.2%) followed by cerebro-vascular disorders (4.1%) and hypertensive disease (0.6%). In Europe, 13.8% of the total DALY burden has been assigned to IHD, 7.1% to cerebro-vascular disease and 0.8% to hypertensive disease (Murray *et al.*, 2013).

2.4 Ischemic Heart Disease (IHD)

On top of the list of CVDs, goes Ischemic heart Disease. Ischemic heart diseases (IHD), such as myocardial infarction (MI) and heart failure (HF) remain among the most important health challenges worldwide (World health report, 2007). MI, due to IHD, is currently one of the major contributors to the development of HF (Pfeffer *et al.*, 1990). In a multicenter heart failure treatment trial, comprising over 20,000 patients, IHD was the underlying cause in 68 % of cases (Gheorghiade *et al.*, 1998).

IHD is a resultant of blockage or narrowing of the coronary arteries due to accumulation of plaque over the inner walls of lumen. A severe blockage can result in death of cardiac cells and tissues. It is basically the most common type of cardiovascular diseases and may include stable and instable angina, myocardial infarction (MI) or sudden cardiac death (Wong, 2014).

2.5 Prevalence of IHD

Annually, about 15.6 million people die estimated up to 30% global mortality rate due to CVDs. IHD is reported to be the leading cause of death, with 13.3 % of total deaths followed by cerebro-vascular disease with 11.1%. Around 12.9 million people have died around the world in 2010 because of IHD and other forms of strokes. This is the increase from one in five deaths worldwide 20 years earlier (Lozano *et al.*, 2013).

Absolute deaths in past twenty years due to CVDs and IHD have increased by 31.2% and 34.9%, respectively. IHD has risen in rank from fourth to first with the increase of 28% according to years of lives lost (YLLs). IHD has also risen in position among top 10 causes of DALYs (Murray *et al.*, 2013).

IHD, contributing to 26.6% of all mortalities, turned out to be the largest single cause of death according to global trends in the 2010 Global Burden of Disease study. IHD being the most prevalent particular cause of CVD, accounting for 45% of deaths around the world and 54.6% in Europe due to cardiovascular disorder (Institute for health Matrix and evaluation, 2013).

2.6 Symptoms

Breathlessness, ankle swelling, and fatigue are the characteristic symptoms and signs of heart failure but may be difficult to interpret, particularly in elderly patients, in obese, and in women. Fatigue is also an essential symptom in heart failure. The origins of fatigue are complex including low cardiac output, peripheral hypo-perfusion, skeletal muscle deconditioning, and confounded by difficulties in quantifying this symptom. Peripheral edema, raised venous pressure, and hepatomegaly are the characteristic signs of congestion of systemic veins (Butman *et al.*, 1993).

2.7 Diagnosis

Hematological and biochemical tests like complete blood count (Hb, leukocytes, and platelets), S-electrolytes, S-creatinine, S-glucose, S-hepatic enzymes, and urine analysis are routine diagnostic evaluation of patients with IHD. Coronary angiography is majorly considered in patients with acute CHF and in patients with severe heart failure and myocardial ischemia. Electrocardiographic changes are common in patients suspected of having heart failure whether or not the diagnosis proves to be correct making it an important diagnostic method for IHD. Chest X-ray as part of the initial diagnostic work-up in heart failure is useful to detect cardiomegaly and pulmonary congestion. Echocardiography also referred to as echo, is the preferred method for the documentation of cardiac dysfunction at rest (Karl *et al.*, 2005).

Electron-beam computed tomography (EBCT) is another modern and highly sensitive technique for detecting coronary artery calcium and is being used with increasing frequency for the screening of asymptomatic people to assess those at high risk for the diagnosis of obstructive IHD in symptomatic patients (Paolo *et al.*, 2005).

CMR is a versatile, highly accurate, and reproducible imaging technique for the assessment of left and right ventricular volumes, global function, regional wall motion, myocardial thickness, thickening, myocardial mass, and cardiac valves (Thomas et al., 1990).

2.8 Causes/Risk Factors

2.8.1 Clinical/Anthropometric Factors

When talking about IHD, most of the attention is paid on clinical and anthropometric and clinical causes. Major risk factors are considered to be those from individual's own life style and corresponding health states. 57% of CVD deaths and overall 19% of total global mortality rate can be linked to risk factors like malnutrition, high blood pressure, diabetes, lack of physical activity, physical inactivity, obesity, low fruit and vegetable intake, high cholesterol. Tobacco is another key risk factor accounting for almost 10% of CVD (World health Organization, 2009). According to 2010 Global Burden of Disease study, high blood pressure and tobacco use are leading risk factors for global disease burden causing 9.4 million deaths and 6.3 million deaths respectively. Similar are the highly contributing risk factors for IHD DALYs where 53% cases are due to high blood pressure, 41% is due to lack of significant diet, 31% is due to tobacco smoking and 31% due to lack of physical activity or physical inactivity (Lim *et al.,* 2012).

2.8.2 Genetic Factors

The understanding of the genetic contribution to ischemic heart disease (IHD), the most common cause of death in developed countries, is very limited (Murray *et al.,* 1997). Rare mutations in the heterozygous or homozygous state are known to cause familial hypercholesterolemia (Goldstein *et al.,* 2001); however, the major genetic contribution to IHD is generally considered to be conferred by common DNA polymorphisms. Alternatively, rare mutations independent of plasma cholesterol levels may have a considerable impact on IHD risk (Wang *et al.,* 2003). We know that IHDs comprise of a range of conditions extending from myocardial infarction to congenital heart disease, most of which are heritable and that a lot of effort has been invested in understanding the genes and specific DNA sequence variants that are responsible for this heritability. With the growing evidence that IHD has a considerable hereditary component, more emphasis is laid on the investigation of genetic predispositions which are a result of genetic mutations that alter a normal biological function of gene hence, increase the risk of disease.

2.9 Association of Various genes with IHD Risk

A huge number of genetic polymorphisms have been studied regarding Cardio-Vascular diseases. Mutation in Low density lipoprotein receptor genes LDLR is known to be associated with severe Hypercholesterolemia (Garcia et al., 2001). Mutations in the coding sequence of the LDL receptor gene (LDLR) may considerably reduce or abolish the function of the LDL receptor and lead to an important rise of circulating LDLC, which in turn is associated with a commensurate increase in CHD risk. More than 700 different mutations of LDLR responsible for familial hypercholestrolemia have been reported, some of them clustered in particular populations (Austin *et al.*, 2004).

Mutations in MEF2A genes have been reported to be independent factor for myocardial infarction and coronary artery disease patients (Wang *et al.*, 2003). Certain SNPs in Lymphotoxin alpha (LTA) gene are known to be associated with high risk of MI (Ozaki et al., 2002). Mutations in endothelial Nitric oxide synthase eNOS gene is studied to be associated with coronary spasm that ultimately leads to ischemic heart disease (Masafumi *et al.*, 1999). Apolipoprotein APOE gene polymorphism has been significantly associated with coronary artery disease (Asma *et al.*, 2015). ATP- binding cassette transporter 1 (ABCA1) gene polymorphisms have been associated with High density lipoprotein cholesterol and the risk of IHD (Bodzioc *et al.*, 1999). Enormous genetic studies have been conducted worldwide to catch up to the core of IHD. This study will majorly focus on the association of ABCA1 polymorphisms with ischemic heart disease in Pakistani population.

2.10 ABCA1 Mechanism of action.

The ATP-binding cassette transporter A1 (ABCA1) influences the initial steps in the formation of high-density lipoprotein (HDL) and the cholesterol efflux across cell membranes to acceptor molecules in the plasma. Disturbance in both functions can play an important role in the development of atherosclerosis and IHD (Yancey et al., 2003).

2.10.1 HDL metabolism and reverse cholesterol transport

The liver secretes lipid-poor ApoA-I, which quickly acquires cholesterol via the hepatocyte ABCA1 transporter. Lipid-poor ApoA-i also promotes the efflux of free cholesterol from macrophages via ABCA1. Lecithin cholesterol acetyltransferase (LCAT) esterifies free cholesterol to cholesteryl esters to form mature HDL that promotes cholesterol efflux from macrophages via the ABCA1 transporter and from other peripheral tissues. Mature HDL can transfer its cholesterol to the liver directly through Scavenger receptor B1 (SR-BI, with subsequent uptake by the liver through the LDLR. Hepatic cholesterol can be excreted directly into the bile as cholesterol or after conversion to bile acids, reabsorbed by the intestine and is ultimately excreted in the feces. HDL can be remodeled by lipases such as hepatic lipase and endothelial lipase, which hydrolyze HDL triglyceride and phospholipids, respectively (Duffy *et al.*, 2009).

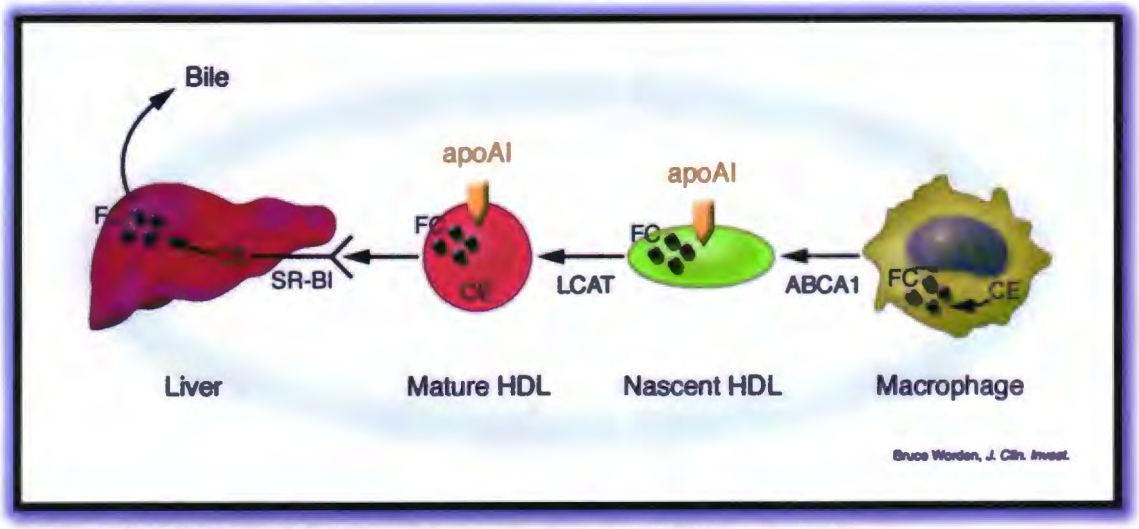
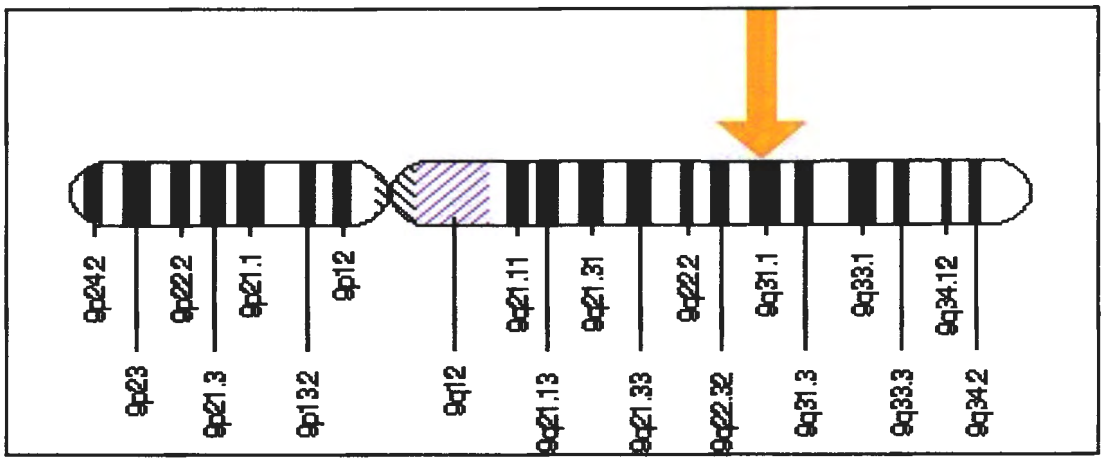


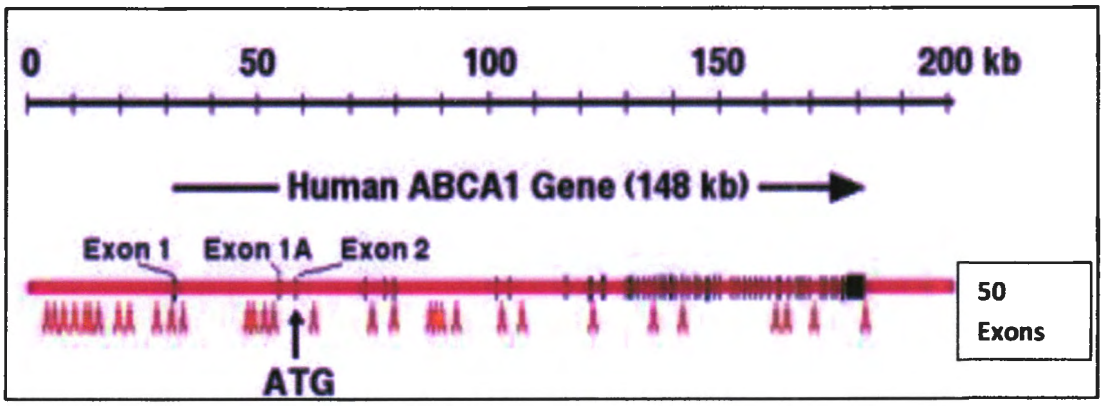
Figure 2.1 HDL metabolism and reverse transport (Pajukanta, 2004)

2.11 ABCA1 gene

ABCA1 is a member of ATP Binding Cassette membrane transporters that are involved in the mobilization of various substrates across plasma membranes. The gene encodes a 2261 amino acid trans-membrane peptide responsible for the efflux of cholesterol and phospholipids across the cell membrane to lipid poor Apo A-I involving interaction with vesicular traffic machinery, a process which is an initial and essential step in the reverse cholesterol transport system (Klein *et al.*, 1999). ABCA1 functions as a cholesterol efflux pump in the cellular lipid removal pathway. Since the protein encoded by ABCA1 is a key gatekeeper influencing intracellular cholesterol transport, it is also termed as 'cholesterol efflux regulatory protein' (CERP) (Brooks *et al.*, 1999). Isotopic in situ hybridization mapping showed the location of ABCA1 gene to 9q22-q31 (Luciani *et al.*, 1994). The ABCA1 gene spans 149 kb and contains 50 exons. 62 repetitive Alu sequences in the 49 introns have been identified (Santamarina *et al.*, 2000).The ABCA1 gene is highly polymorphic and to-date a large number of sequence variations have been identified, many of them affecting HDL cholesterol (HDL-C) concentrations (Wang *et al.*, 2000).



(a)



(b)

Figure 2.2. (a) Location of ABCA-1 gene. **(b)** Gene specifications.

2.12 Association of Genetic Variants of ABCA 1 with IHD.

Heterozygous mutations in ABCA1 have been identified as one cause of familial hypoalphalipoproteinemia (FHA) (Clee *et al.*, 2000). FHA is known to be associated with almost 50% reduction in HDL, normal levels of LDL, and elevated risk of premature coronary artery disease (CAD). In both diseases, low HDL is associated with an increased risk of atherosclerosis development despite normal FHA or even very low LDL levels (Hovingh *et al.*, 2005). Common single nucleotide polymorphisms (SNPs) in the ABCA1 gene are present in the general population and some have been associated with changes in HDL-C and cardiovascular risk (Singaraja *et al.*, 2003).

Various SNPs have been studied in ABCA 1 gene. G1051A (R219K, rs2230806), a G to A substitution, at position 1051 in exon 7 has resulted in the change of lysine to argenine at residue 219. The presence of the A allele has been associated with a lowered risk for IHD in certain European and American populations (Cenarro *et al.*, 2003). But there are no associations seen between the genotype and risk of IHD in Japanese studies (Takagi *et al.*, 2002). The genotype's effect on HDL-C also remains controversial, with some studies showing an association between the genotype and higher levels of HDL-C (Woll *et al.*, 2005). And there are others that show no effect (Brousseau *et al.*, 2001).

A study conducted on German population, association between I883M and IHD is consistent with several previous reports that have demonstrated an increased IHD risk for carriers in other populations (Brousseau *et al.*, 2001).

Study conducted on the population of US have shown that polymorphisms in promoter regions −191G/C and −565C/T variants were associated with the lower risk of IHD without affecting HDL-C concentration (Benton et al., 2007).

In a Norwegian population study, Variants R219K, V399A, V771M, V825I, I883M, E1172D, and R1587K have been reported as SNPs. Except for V399A, these nonsynonymous SNPs were observed in two or more patients included in this study. In addition, R219K, V771M, V825I, I883M, E1172D, and R1587K have been found in

similar frequencies in patients with low or high HDL cholesterol levels (Frikke *et al.*, 2004).

Allelic variants in the ABCA1 gene have been shown to be associated with IHD in various population samples indicating that the gene is an important player in the development of disease, but there are also studies that have quite insignificant associations. However, many of the reported findings remain unprecedented with reported results being contradictory as well. We therefore have conducted a study to find out how this particular polymorphism R1587K can relate to the risk of IHD in our local Pakistani population.

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Study Approval

The study was approved by Department of Bioinformatics and Biotechnology and Ethical Review Committee of International Islamic University Islamabad (IIUI), Pakistan and Institute of Biomedical and Genetic Engineering (IBGE), Islamabad before conducting the research.

3.2 Sampling

About 120 Ischemic Heart Disease (IHD) patients were ascertained from PIMS and KRL hospital Islamabad, after the clinical evaluation and diagnosis made by the clinicians at the Departments of Cardiology at both hospitals along with 100 healthy control subjects. The patients were evaluated by personal information, family history, clinical, laboratory investigations and IHD related complications of the patient; all information was collected with the help of properly designed questionnaires. The patients were informed about the objectives of the study and consent form had been signed by them. Then 5 ml blood was drawn from each participant and collected in Acid Citrate Dextrose (ACD) vacutainers that serve as anti-coagulant and preservative (BD Franklin Lakes NJ USA). The samples were assigned specific identity numbers and were stored at 2-8°C till DNA extraction.

3.3 Genomic DNA Extraction

The genomic DNA from all of the samples was isolated by following standard organic methods which recruits two chief organic chemicals namely Phenol and Chloroform (Sambrook and Russell, 2001).

5ml blood of the IHD patients and controls was collected in ACD vacutainer which contained 2.5ml acid-citrate-dextrose solution so that the final volume turns to 7.5ml.

The DNA extraction was performed in strict accordance to the following steps:

Day 1

1. The samples were transferred to 50 ml centrifuge tubes and 3X volume of cell lysis buffer (KHCO3, NH4Cl and 0.5M EDTA) was added. Tubes were placed in ice for 30 min.
2. Then the samples were centrifuged at 1200rpm for 10minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130).
3. The supernatant was discarded and the pellet at the base of the tube was re-suspended.
4. If the pellet was reddish then again 10ml of cell lysis buffer was added. The sample tubes were centrifuged at 1200rpm at 4°C for 10 minutes
5. The supernatant was discarded and the pellet was re-suspended.
6. To the pellet, 4.75ml of STE and 250µL of 10% SDS were added.
7. To the above mixture 10 µL of proteinase K enzyme (20mg/ml) (Fermentas, Lithuania) was added and samples were incubated at 55°C for overnight in shaking water bath (Orbit Shaker Bath, Lab-Line, USA).

Day 2

8. The samples were extracted with 5ml (equal quantity) of equilibrated phenol (pH=8), agitated for 10 minutes and kept on ice for 10 minutes. Spin at 3200rpm for 30min at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130), removed the supernatant with 1ml cut tip into the separate labeled 15ml centrifuge tubes.
9. To the samples 5ml of chilled chloroform:isoamyl alcohol (24:1) was added and agitated for 10 minutes and kept on ice for 10 minutes. Samples were spun at 3200rpm for 30 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130).The supernatant was separated with cut tip into the separate labeled 15ml centrifuge tubes.

10. To the isolated supernatant added 500µL of 10M ammonium acetate and 5ml of chilled Isopropanol and agitated until DNA precipitates as visible white threads. The samples were then placed overnight at -20°C (or for 15 minutes at -70°C).
11. The samples were then centrifuged at 3000rpm for 60 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130). The supernatant was discarded and the DNA pellet was loosened by tapping the 15ml centrifuge tube.
12. Then the loosened DNA pellet was washed with 5ml of chilled 70% ethanol 3200rpm for 40 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130).
13. The supernatant was discarded and the DNA pellet was dried.
14. The dried pellet was dissolved in TE (10mM Tris; 1mM EDTA) buffer (the volume buffer added according to the size of the pellet).
15. The complete DNA dissolving in TE buffer was achieved by incubating the sample tube in shaking water bath at 55°C (Orbit Shaker Bath, Lab-Line, USA).
16. The DNA samples were then transferred to 1.5 ml eppendorf tubes and stored at 4°C

3.4 Composition of the solutions used in DNA Extraction

3.4.1 Cell-lysis buffer

This buffer is composed of three chemicals as:

i. KHCO3 (Potassium Carbonate) 1gm/L
ii. NH_4Cl (Ammonium Chloride) 8.29gm/L
iii. 0.5 M EDTA (Ethylene Diamine Tetra Acetate) 0.34gm

Cell lysis buffer cleaves the cell membranes and exposes the chromatin material of the nucleated cells.

3.4.2 STE buffer (Sodium chloride-Tris-EDTA Buffer)

This buffer provides a saline environment to the newly exposed chromatin material

i. 3M NaCl (Sodium Chloride) 33.3ml
ii. 1M Tris-HCl buffer (pH8.0) 4.0ml
iii. 0.5M EDTA (pH8.0) 2.0ml

The above reagents were mixed and volume was made up to 1 liter with dH_2O.

3.4.3 DNA Dissolving Buffer

This buffer is used for dissolving the DNA and is composed of

i. 10mM Tris-HCl (pH8.0)
ii. 0.1mM EDTA

3.4.4 SDS solution

10 % solution of sodium dodecyl sulfate or sodium lauryl sulfate helps in protein degradation.

3.4.5 Chloroform-Isoamyl Alcohol

This solution is made in 24:1 v/v for 500 ml total volume as:

i. Chloroform 480ml (24)
ii. Isoamyl alcohol 20ml (1)

3.5 Assessment of Quality and Quantity of DNA

DNA was quantified using Nanodrop (Thermo USA).

Optical density (OD) ratio for each sample was calculated as:

OD=Absorbance at 260nm / Absorbance at 280nm

The ratio from above formula should lie between 1.7-1.9 for good quality DNA.

The concentration of DNA samples was calculated as:

DNA concentration (µg/ml) =Absorbance at 260nm × dilution factor ×correction factor

3.5.1 Working Solution of DNA

40ng/µl working solution of DNA was prepared from the stock DNA solution by using the following formula:

$$C1V1=C2V2$$

3.5.2 Reconstitution of primers

Lyophilized primers were mixed with calculated amount of deionized water to make a stock solution of 100µM of each primer. From these 100µM stock primer solutions, 20µM working solutions for each primer were prepared by taking 20µl stock solution and 80µl of distilled water. PCR amplifications were performed by using 20µM primer solutions.

3.6 Primer for rs2230808

To find out the genetic determinants of complex diseases like IHD, analyses of SNPs work as a dynamic discipline. Such type of analyses and studies can be apprehended through rapid, convenient high-throughput and cost effective methodologies for SNP genotyping. One such SNP genotyping method is ARMS PCR which recruits two primer pairs to amply the two different alleles of a SNP in a single PCR (Shu *et al.*, 2001). The amplification-refractory mutation system (ARMS), also known as allele-specific polymerase chain reaction (ASPCR) or PCR amplification of specific alleles, is a simple, rapid, and reliable method for detecting any mutation involving single base changes or small deletions. ARMS is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample and will not amplify the non-target allele. Following an ARMS reaction the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele.

The targeted and allele specific amplification of rs2230808 SNP in ABCA1 gene was done through Tetra-primer ARMS PCR. Upstream and downstream sequence of *rs2230808* was obtained from Human Genome at *Ensemble Genome Browser* (http://asia.ensembl.org/Homo_sapiens/Variation/Explore?r=9:104800023-104801023;v=rs2230808;vdb=variation;vf=1609969). Allele Specific Tetra primer ARMS PCR primers for *rs2230808* polymorphism in the ABCA-1 gene was designed at the following internet link:

http://cedar.genetics.soton.ac.uk/public_html/primer1.html

The detail of primers with allele specifications is given below in Table 3.1

Table 3.1 Sequence of primers used for genotyping of rs2230808

Primer	Sequence 5'- 3'
Forward inner primer (FI) (T Allele)	AGACAGCGGTTTACCTTGACATTATGTT
Reverse inner primer (RI) (C Allele)	GAAGATTTATGACAGGACTGGACACAAG
Forward outer primer (FO)	CAGATGAGGGAACTGAGGTTTAGATAGG
Reverse outer primer (RO)	AATTTCAGTGAACAAGGTAGTGGCAT

3.7 PCR analysis

After the optimization with reproducible results, all the samples were amplified for target DNA fragment spanning the rs2230808 SNP. The PCR reaction was done for each sample in final volume of 25µl containing 2.5µl of 10X PCR buffer without $MgCl_2$ (Fermentas, Lithuania), 1.5µl of 25mM MgCl2 (Fermentas, Lithuania), 1µl 2mM dNTPs (Fermentas, Lithuania), 0.2µl of 5U/µl *Taq* DNA polymerase enzyme (Fermentas, Lithuania), 1µl of 20µM of each forward and reverse outer primers, 1µl of 20µM of each forward and reverse inner primers, 7µl of 40ng/ µl human genomic DNA and volume of the reaction was made up to 25µl with autoclaved deionized water. The initial and final concentrations of the reagent used in PCR are also shown in the table 3.2.

Table 3.2 Concentrations of reagents used for PCR

S.no	Reagents with initial concentration.	Reagents with final concentration.	Quantity used in µl
1	d.H2O	---	7.8 µl
2	10x PCR buffer	1x buffer	2.5 µl
3	25 mM MgCl2	1.5 mM MgCl2	1.5 µl
4	100% DMSO	5 % DMSO	1 µl
5	2.5mM dNTPs	200µMdNTPs	1 µl
6	5U/µlTaq DNA polymerase	0.5U Taq DNA polymerase	0.2 µl
7	20µM Forward outer primer	1.0 µM	1 µl
8	20µM Reverse outer primer	1.0 µM	1 µl
9	20µM Forward inner primer	1.0 µM	1 µl
10	20µM Reverse inner primer	1.0 µM	1 µl
11	40ng Sample Genomic DNA	ng/µl	7 µl
---	Total reaction volume	---	25 µl

3.8 Optimization and thermal profiling of PCR

The above reaction was carried out in 96 well containing thermal cycler (Thermo Electron Corporation, Mill ford, USA) at the following optimized thermal stages, steps and cycles as shown in table 3.3.

Table 3.3 PCR stages and Cycles

Stages	Steps	Temperature	Time	Cycles
Stage 1	Step 1	94 °C	4 Minutes	01 Cycle
Stage 2	Step 1	94 °C	45 Seconds	35 Cycles
	Step 2	58 °C	45 Seconds	
	Step 3	72 °C	45 Seconds	
Stage 3	Step 1	72 °C	10 Minutes	01 Cycle

3.9 Analysis of PCR products by using Agarose Gel Electrophoresis

Analysis of PCR product was performed by using 2.5% agarose gel through electrophoresis.

Following reagents were used in the process of agarose gel electrophoresis:

1. 10x Tris-Borate- Ethylene diamine tetra acetic acid (TBE) buffer
2. 6X Gel loading dye (Fermentas, Lithuania)
3. 10mg/ml Ethidium Bromide
4. Agarose (Promega)
5. 100bp DNA Ladder (Ferments Lithuania).

1. Preparation of 10 TBE buffer stock

a) Tris-base (promega) = 107.8gm/liter
b) Boric acid (promega) = 55.02gm/liter

c) EDTA (Ethylene diamine tetra acetic acid) (Bio Rad) = 9.04gm/liter

All reagents were dissolved in 800ml deionized water using magnetic stirrer and finally 1000ml volume was made in measuring cylinder and stirred to mix well.

2. Preparation of 2% agarose gel

For 20cm×20cm agarose gel pouring plate 300 ml of 2% agarose was made as:

a) Ion free fine agarose (Promega) = 6gm

b) 10 X TBE = 30ml

c) Deionized water = 270ml

d) Ethidium bromide (10mg/ml) = 5µl

Mix 6gm agarose, 30ml 10X TBE and 270ml deionized water in Pyrex bottle and heat in oven with loose bottle cap until agarose boils to mix in liquids. Add 5µl Ethidium bromide in the agarose gel solution and shake to mix. Set the 20cm×20cm gel plate with combs at suitable distance and pour the gel solution. The gel is polymerized in 30-45 minutes.

3.9.1 Gel electrophoresis and gel documentation

Before loading the sample in the gel, 5µl 6X gel loading buffer (Fermentas, Lituania)was added in each sample. Then after the addition of the gel loading buffer, samples were loaded on agarose gel. In the first well of gel, 100bp DNA marker was loaded as size reference for amplified target DNA. The gel was run for 40 minutes using the Maxicell EC360-M electrophoretic gel system (EC Apparatus Corporation, St. Petersburg, Florida, USA) at 200 constant volts by using BioRad Power Pack 3000 (BioRad, USA) in 1X TBE buffer. DNA bands were visualized under UV illumination and photographed using Syngene gel documentation system (GeneGenius, Syngene, UK).

3.10 Statistical analysis:

The statistical analysis was done by managing and interpreting the data in SPSS (version 14.0, USA). The following statistical tests were performed for the data.

3.10.1 Hardy-Weinberg Equilibrium:

The frequency of each allele of rs2230808 was calculated by Hardy-Weinberg Equilibrium equation:

$$p^2 + 2pq + q^2 = 1$$

The allele frequency of the study groups was calculated by using the following formula:

$$\textit{Allele Frequency} = \frac{\textit{No. of that allele in study group}}{\textit{Total number of alleles}}$$

The expected H-W frequencies for the two alleles C and T; three genotype CC, CT and TT were calculated to determine any significant deviation of the observed allele and genotype frequencies of rs2230808 in IHD and control group using an internet source (http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html).

3.10.2 *Chi* square test and *p* value

To find the association of the rs2230808 genotype and allele with the IHD, the *p* value and *Chi* square test was performed by using statistical program SPSS (Version 14.0) and as described by Preacher (Preacher, 2001). A *p* value < 0.05 was considered as statistically significant whereas *p* value > 0.05 was taken as non-significant.

CHAPTER 4

RESULTS

4. RESULTS

120 IHD patients and 100 controls with their age and gender matched, participated in current study. The IHD patients were assessed for certain clinical and social parameters such as, gender, age, marital status, smoking status, Diabetes, Hypertension, Family history of disease, lipid profiles (Cholesterol, HDL, LDL and triglycerides) and BMI via questionnaires. These registered parameters for IHD participants of the given study are summarized in table 4.1.

4.1 Demographic Characteristics

The results were analyzed which showed that patients enrolled in study included 72% males and 28% females. Mean age of total patients was 56.71 with standard deviation 12.30. Almost all patients were married and only two out of 120 were unmarried. 56% of these patients were smokers whether it is heavy or light smoking. 32.5% were diabetic and 33% turned out to be hypertensive. Family members of 42% were also known to have IHD history (Table 4.1).

4.2 Clinical Parameters

Lipid profiles were evaluated with mean cholesterol being 150.15 with standard deviation 45 mg/dl, HDL being 39 with standard deviation 13.24 mg/dl, LDL being 83.75 with standard deviation 37.20 mg/dl and Triglycerides being 151.94 with standard deviation 79.69mg/dl. The mean Body mass index (BMI) was 25.17 with standard deviation 5.15 kg/m^2 (Table 4.1).

Table 4.1 Demographic characteristics and clinical parameters of Ischemic Heart disease Patients.

Parameters	**IHD Patients (n=120)**	
Gender	Male	72%
	Female	28%
Mean age	56.71 ± 12.30 years	
Smokers	56%	
Hypertensive	33%	
Diabetics	32.5%	
Family History	42%	
Cholesterol (mg/dl)	150.15 ± 45.0	
HDL (mg/dl)	39 ± 13.24	
LDL (mg/dl)	83.75 ± 37.20	
Triglycerides (mg/dl)	151.94 ± 79.69	
BMI (kg/m2)	25.17 ± 5.15	

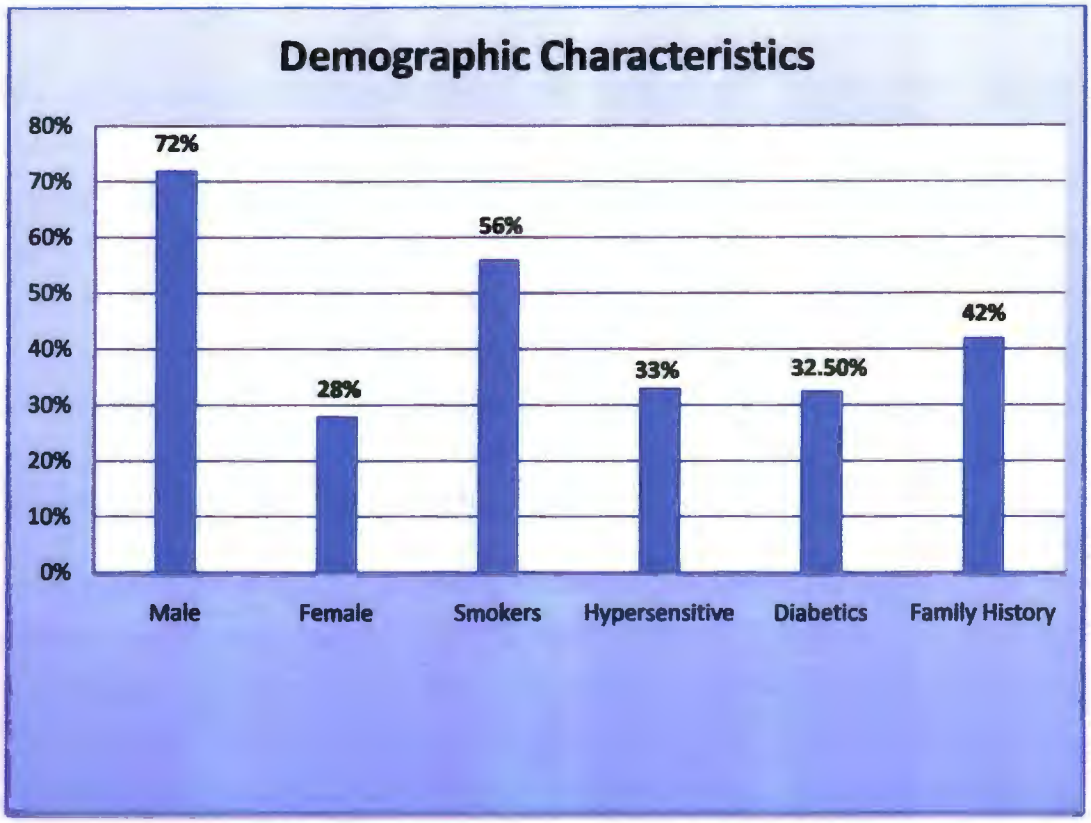


Figure 4.1 Demographic characteristics of IHD Patients.

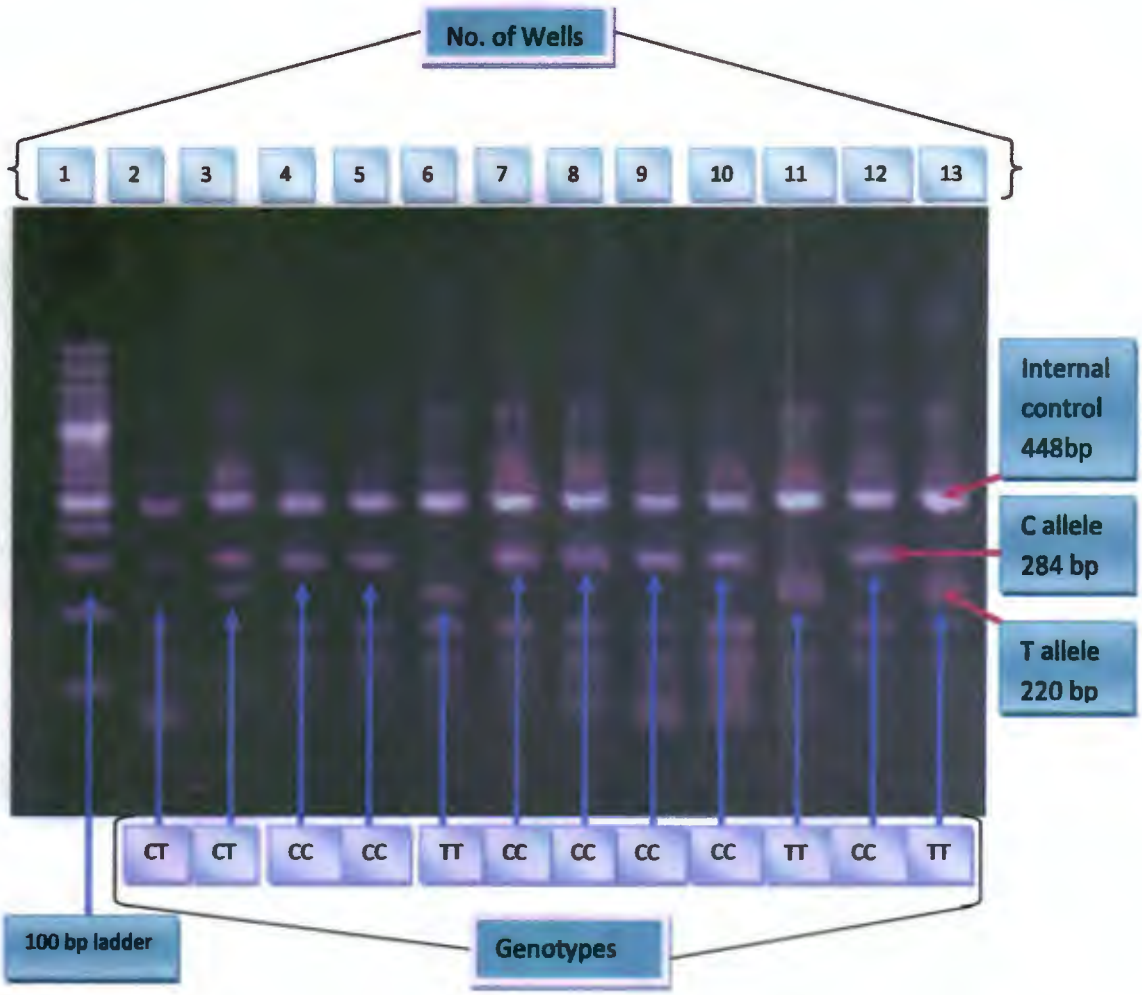


Figure 4.2 ARMS-PCR gel image of ABCA 1 R1587K (rs2230808) genotype.

Table 4.2 Genotypic Distribution of ABCA 1 R1587K (rs2230808) Polymorphism in IHD Patents and Controls

Group	**ABCA1 T/C genotypes percentage**		
	TT	**CT**	**CC**
Patient (n= 120)	9.09	39.39	51.51
Expected H-W genotype	8.29	41	50.71
Control (n= 100)	10.8	35.16	53.84
Expected H-W genotype	7.87	40.37	51.75
***p*-value**	**0.950**	**0.775**	**0.850**

Significant value: $p<0.05$

Table 4.3 Allele frequencies of T and C allele of ABCA 1 R1587K (rs2230808) polymorphism in IHD Patients and Controls

Groups	**Allele Frequencies**	
	C	**T**
Patients	28.79	71.21
Controls	28.06	71.94
p-value	0.890	0.933

Significant value: $p < 0.05$

CHAPTER 5

DISCUSSION

5. DISCUSSION

In the line of current study, we assessed the association of ABCA-1 gene polymorphism, R1587K (T/C) with the risk of IHD in local Pakistani population. Data analyses of our study showed insignificant results to subordinate current polymorphism with the risk of disease.

It had been perceived in one of the European study that K carriers of R1587K (RK, KK) had lesser HDL-C concentration in an allele dose-dependent tendency as allied with the non-carriers. The R1587K genotype persisted in a substantial analysis of HDL-C, when observed in numerous reversion explorations, comprising numerous parameters e.g. age, body mass index, smoking, and triglycerides as covariates. (Clee *et al.,* 2001).

It had been observed in German population, that the R1587K variant showed a gradual reduction in HDL-C in women in heterozygotes and homozygotes and this reduction was also observed in men (Frikke *et al.,* 2004). No statistically significant results were found in American population between R1587K variant with the lipid profile (Mantaring *et al.,* 2007). An alternative American study presented that after the treatment with the fenofibrate, KK genotype of R1587K was linked with considerably amplified minor HDL and also with enlarged HDL element concentrations (Tsai *et al.,* 2010). A study on Greek subjects showed the change in Low density lipoprotein-C and TGs concentration, between heterozygous RK and homozygous RR genotypes. It was observed that conferring to gender the blood lipid intensities did not appear to differ in genotypes of R1587K polymorphism (Kolovou *et al.,* 2011).

Numerous studies e.g. German, Greek, American, and various European population have delivered with diverse altitudes of relationship of ABCA 1 gene polymorphisms with the possibility of deranged Lipid profiles, some of which are substantial, while others being in-significant.

Our main concern was also to study the association of ABCA-1 gene polymorphism with the danger of ischemic heart disease in the light of high density lipoprotein levels. Due to numerous limitations in our study we are not yet at the level of totally associating

relevant polymorphism with ischemic heart disease in this specific area. With only 120 patients, our sample size was comparatively very small. Many patients were reluctant towards research and hesitated to provide clinical data, lipid profiles for not all of them were ready to pay for the tests just for study. Lipid profiles that we achieved to accomplish were not very much unstable in our current study, though main concern of ABCA1 is with lipid profiles, majorly high density lipoprotein. Maximum number of patients who delivered the data had normal level of lipid profiles with the mean high density lipoprotein being 39mg/dl with standard deviation 13.24mg/dl portraying inconsistent results with ABCA1 dysfunction, however various studies accompanied in other countries have shown association of ABCA1 gene with decrease levels of HDL. The region we covered had patients mostly belonging to North Punjab and KPK and not the whole country which is also a restrictive factor as far as environmental and cultural influences are concerned.

Conclusion and Future Recommendations

The possibility of ischemic heart disease is amplified if the level of high density lipoprotein decreases or the level of low density lipoprotein increases resulting in Atherosclerosis. The action of HDL as being cardio-protective has been accredited to play its potential part in reverse cholesterol transport (RCT), assembling the transport of cholesterol from the peripheral tissues to liver. About 50% of the plasma HDL-C variability is observed by certain genetic factors (Breslow *et al.,* 1995, Prenger *et al.,* 1992). So we can emphasize more on lipid profiles of patients by also paying them the cost for the tests while reviewing this specific polymorphism or we can also examine more SNPs in ABCA-1 gene other than R1587K. A few other genes like APOE gene, LDLR gene, MEF2A gene as stated before, had revealed the association with cardiovascular situations, therefore we can also take them under attention in Pakistani population. Increasing the sample size may help in achieving substantial results. Meta analysis studies can also be conducted from around the country, or from nearby regions, say around Asia for instance.

CHAPTER 6

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6. REFERENCES

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