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Relationship Between ABO Blood Groups and Lipid Profile Level in Adult Residents of Mirpur Azad Kashmir

by

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Dedicated to my Late Mother

CERTIFICATE OF APPROVAL

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Abstract

Cardiovascular diseases including coronary artery disease CAD and other heart disease have been proved as the major cause of mortality all over the world. Dylipidemia have been accepted as a major risk factor of cardiovascular disease but the influence of different blood types has not been extensively and mechanistically investigated, specially in the region of Mirpur Azad Kashmir where prevalence of cardiac disease is increasing day by day. This study determines various parameters of lipid profile among different ABO blood phenotypes in this region. Present study includes 250 randomly selected subjects. Among of them 62 belongs to blood type, 78 of blood type B, 26 of blood type AB and 84 of blood type O. The subject were male (133) and female (117) ranging from 23 to 88 years old who gave consent to participate in the study. Blood samples were obtained for serum lipid profile and ABO blood typing. By the following standard techniques, the results represented that there was no significant difference ($p > 0.05$) in the serum mean total cholesterol, triglyceride, low density lipoproteins and high density lipoprotein level in different ABO blood phenotypes. However results implicated that the blood type A was more likely to be at risk due to the presence of high values of TC, TG and LDL as well as lower value of HDL. So it is suggested that to investigate CVD in Mirpur Azad Kashmir should take into account the blood group status of patients.

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Abbreviations

ASCVD	Atherosclerotic Cardiovascular Disease
apo A	Apolipoprotein A
apo B	Apolipoprotein B
APOC2	Apolipoprotein C2
APO45	Apolipoprotein 45
ABCA1	ATP-binding Cassette Transporter A1
CAD	Coronary Artery Disease
CE	Cholesterol Esters
CD Nomenclature	Cluster of Differentiation
CHD	Cardiac Heart Disease
CoA	Coenzyme A
CETP	Cholesteryl Ester Transfer Protein
DEL	D Elution
DAG	Diacylglycerides
FUT 1	Galactoside 2-Alpha-L-Fucosyltransferase 1
FUT2	Galactoside 2-Alpha-L-Fucosyltransferase 2
FPLC	Fast Protein Liquid Chromatography
GPIHBP1	Glycosylphosphatidyl Inositol Anchored High Density lipoprotein binding Protein 1
GPD1	Glycerol-3-Phosphate Dehydrogenase 1
GGE	Gradient Gel Electrophoresis
HDN	Hemolytic Disease of the New Born
HTR	Hemolytic Transfusion Reaction
HDL-c	High- Density Lipoprotein Cholesterol

HMG-CoA	3-Hydroxy-3-Methylglutaryl CoA
HMGR	HMG-CoA Reductase
HPLC	High-Performance Liquid Chromatography
IDL	Intermediate- Density Lipoprotein Cholesterol
LDL-c	Low-Density Lipoprotein Cholesterol
LPL	Lipoprotein Lipase
LP(a)	Lipoprotein A
LTIP	Lipid Transfer Inhibitor Protein
LMF1	Lipase Maturation Factor 1
LPS	Lipopolysaccharide
LBP	Lipopolysacharid Binding Protein
LCAT	Lecithin-Cholesterol Acyltransferase
MAG	Monoacyle Glycerides
MCP-1	Monocyte Chemoattractant Protein-1
PL	Phospholipid
PSF-AH	Platelet Activating Factor Acetyl Hydrolase
PLTP	Phospholipid Transfer Proteins
PON1	Paraoxanase 1
RBCs	Red Liood Cells
rHDL	Reconstituted HDL
RCT	Reverse Cholesterol Transport
SNPs	Single Nucleotide Polymorphisms
SCA	Sickle Cell Anemia
SAA	Serum Amyloid A
SdLDL	Small Aense LDL
SIP	Sphingosine-1 Phosphate
TC	Total Cholesterol
TG	Triglycerides
TAG	Triacylglycerols
tRNA	Transfer RNA
VLDL	Very Low-Density Lipoprotein Cholesterol

NMR	Nuclear Magnetic Resonance
VCAM-1	Vascular Cells Adhesion Molecule-1
ICAM-1	Intercellular Adhesion Molecule-1
OxLDL	Oxidized LDL

Chapter 1

Introduction

Human ABO blood group system plays a significant role in transfusion and transplantation medicine. Discovery of ABO blood group antigens along with their structure and function is the result of deep research of more than a century (Storry and Olsson, 2009). Two antigens A and B are responsible for determination of this blood group and are present on the surface of erythrocytes (Agomuoh et al., 2006). In fact these antigens are two antigenically different structures and represent themselves on cell surface as glycan units of mucin glycoproteins (Storry & Olsson, 2009; Eastlund, 1998).

ABO gene is responsible for glycosyl transferase enzyme production which involve in the attachment of a sugar onto a carbohydrate structure known as H antigen. ABO and H antigen are primarily represented on glycoproteins and glycolipids by small carbohydrate epitopes by H gene which is involved in the production of the H antigen substrate, act as precursor of A and B antigen. This glycosyl transferase enzyme activity is missing in O blood type due to loss of function mutation (Storry & Olsson, 2009). ABO blood group system was discovered by Landsteiner in 1900 lead to open the ways of other systems of classifications as well as determination of relationship between ABO blood group system and various diseases (Landsteiner, 1900).

The word association means that a specific disease occurs in individuals of specific blood group more often than would be expected by chance. Although various

investigations have been carried out to demonstrate the relationship between ABO blood groups and occurrence of specific disorders along with markers of some diseases but the facts of such associations are remained controversial and many of them are still not fully explored. The clinical importance of ABO blood group system is not only confined to transfusion and transplantation events but also its reciprocal relationship to various diseases has been the focus of researchers.

1.1 Previous Attempts

Several investigations have been conducted to explore the relationship between ABO blood types and systemic diseases like gastric cancer (El Hajj, 2007), periodontal diseases (Demir, 2007), thrombotic vascular diseases (Franchini & Mannucci, 2014), malarial infections (Singh, 2015) Maxillofacial deformities (Gheisari et al., 2008), pancreatic cancer, cholera, peptic ulcer, type II diabetes mellitus (Waseem et al., 2012), and various cardiovascular diseases (Qureshi & Bhatti , 2003; Reilly et al., 2011).

Various studies have reported that specific diseases are more commonly found in specific blood types i.e. blood group O phenotype has higher chances of tuberculosis, A and O phenotype have more affinity for plasmodium vivax (Malarial protozoan) infection and phenotype B shows more association with plasmodium falciparum (Malarial protozoan) infections (*http* : *//www.dadamo.com/pdf/Dadamo−Disease.pdf*). Several investigations have proved that ABO blood types, especially O-phenotype having individuals have higher chances of cardiovascular incidents due to more association with cardiovascular risk factors (Ketch, 2008; Fang et al., 2006; Nixon , 2004; Kaur, 2012). It has also been found in some studies that A blood group phenotype have more affinity for cardiovascular diseases and O phenotype is more protective against atherogenic factors (Stakisaitis et al., 2002). Hence it is evident from all these examples that a specific disease has chances to be more likely present in specific blood type than others. It may be due to genetic predisposition or may be the result of immunological interaction in different ABO

blood phenotypes against different diseases. To date, strong association of cardiovascular diseases and lipid profile with genetic predisposition has been found, thus quantification and differentiation of possibilities of cardiac problems in specific blood group phenotypes as compared to others may be possible.

Cardiovascular diseases include coronary artery disease CAD and other heart diseases and have been proved as the major cause of mortality all over the world (Mendis et al.,2011). According to world health organization out of 56.9 million deaths all over the world in 2016, about 15.2 million deaths were due to Ischeamic heart disease and stroke. So cardio vascular diseases (CVD) have been proved as a leading cause of mortality worldwide for the last 15 years (WHO, 2019). According to WHO Non-communicable diseases (NCD) country profile 2014, Pakistan is facing about 50% NCD (WHO/NCD country profile 2014) and about 38.7% individuals had facing Ischemic heart diseases and hypertension (Naseem et al 2016). Lipids and lipoproteins which play significant role in metabolism have seek attention in case of association with coronary heart disease (Omorogiuwa and Ozor 2015). Dyslipidemia have been accepted as a major risk factor of cardiovascular diseases. (Hatmi et al., 2007 ; Klop et al., 2013). Risk factors related to cardiovascular disease are increased with the increase in total serum cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and serum triglycerides (TGs) but these risk factors are inversely related to serum high-density lipoprotein cholesterol (HDL-C) thus it has been considered that HDL-C plays a protective role against heart diseases and stroke as being involved in reverse cholesterol transport.

Another clinically the much significant blood group system is the Rhesus factor (Rh) based blood group system. It is the largest blood group (with 49 antigens) of all blood group systems described so far. This blood group is represented by a huge group of antigens which express theselves on the membrane of red blood cells (Avent & Reid, 2000). Which may responsible for ammonia transport (Marini et al., 2000) or transport of CO_2 (Endeward et al., 2008; Kustu & Inwood, 2006), or play a structural role in red blood cell membrane and may not involve in transport (Westhoff, 2004; Westhoff & Wylie, 2006). Two major Rhesus proteins RhD and

RhCE carry Rh-antigens and their variations are related to the changes in their amino acid sequences.

Difference between Rh positive and Rh negative phenotypes is due to the presence or absence of RhD protein in the membrane of RBCs. Unlike many other cell proteins it is an unusual red blood cell protein due to be lacking completely in many humans. Clinically the Rh blood group is more important because of the Rh antigens which are highly immunogenic and most of the Rh antibodies are the potential source to cause hemolytic transfusion reaction (HTR) and hemolytic disease of new born (HDN)(Filbey et al., 1995; Bowman, 1997).

Usually lipid profile includes assessments of total cholesterol, high density lipoproteins, low density lipoproteins and triglyceride. Now a days multichannel chemistry analyzers are commonly being used to assess these parameters. Among them total cholesterol and triglycerides are measured by enzymatic techniques while HDL-c can be determined by the use of precipitation technique, immunotechniques or by polymers and detergents. Non precipitation methods are preferably considered to separate HDL from other Lipoproteins because they can provide more accurate results as well as easily acclimatized to automated chemistry analyzer without any manual interference. LDL-c can be measured by using Friedwald equation as well as it can be directly assessed by homogenous enzymatic techniques or selective precipitation techniques. HDL-c and LDL-c are two most common test prescribed by physicians to assess the risk for atherosclerotic cardiovascular disease (ASCVD) in patients.

Triglycerides are the common example of fats in the living body. They take up and store excess energy from diet. An elevated triglyceride level along with high LDL (bad) cholesterol or low HDL (good) cholesterol is releted with fatty buildups inisde the artery walls, which enhances the risk of heart attack and stroke. Serum triglycerides (TGs) collectively form a most variable component among all the lipids which are being measured in routine clinical practice. Elevated triglycerides level is known as hypertriglyceridemia. Although the hypertriglyceridemia can be classified in multiple ways but more common and useful classification scheme includes:

1. Mild to moderate hypertriglyceridemia

2. Severe hypertriglyceridemia (Hegele et al., 2014)

Hypertriglyceridemia is considered as a cardiovascular disease (CVD) risk factor (Benn et al.,2012; Varbo et al.,2014). Serum triglycerides (TGs) number should be below 150 mg/dl.

Serum total cholesterol includes two major forms, one is the free cholesterol and other is cholesteryl ester. Most commonly circulated fraction of cholesterol in the blood is the cholesteryl ester. In the plasma about 70% of total cholesterol is esterified by different fatty acids. Cholesterol is included in heterogenous group of fats and is the principle sterol found in the living beings which is needed to synthesize cell membranes and to regulate the fluidity of membranes across a range of temperatures (Lohe et al., 2010). But cholesterol level of individuals can be affected by more saturated fat content in diet; genetic inheritance and variety of metabolic defects i.e. type II diabetes mellitus. Above or below the normal range of serum cholesterol level is critical. Various disorders co-relate with non-optimal cholesterol level especially elevated level of cholesterol in the blood is more critical as being an accelerator or amplifier in case of atherosclerosis which leads into peripheral vascular disease, stroke and heart attack (Nseka et al., 2005) Increased concentration of blood cholesterol is known as hypercholesterolemia which has been proven as risk factor of CHD/CVD by various epidemiological studies (Mills et al., 2008). The normal mean serum cholesterol should be $118.4 \pm 32\ mg/dl$ whereas serum cholesterol level above or below the normal range is of concern.

Mammals possess a comprehensive system for transportation of lipids, specifically of triglycerides and cholesterol throughout in the body. Much of the lipid transport is regulated by highly complex network of plasma lipoproteins.Approximately all lipoproteins carry small fractions of cholesterol, triglyceride, phospholipid and protein. Cholesterol travels through the blood on these “lipoproteins” which include very low-density lipoproteins (VLDL), low-density lipoproteins (LDL or “bad cholesterol”) and high-density lipoproteins (HDL or “good cholesterol”). These lipoproteins carry cholesterol throughout the body.

LDL is considered as major cholesterol transporter in man and is more vulnerable to oxidation as a consequence considered as major risk factor of coronary artery disease (CAD) and hence initiation and progression of atherosclerotic plaque development also increases (Goldstein & Brown, 2015). So LDL particles are considered as bad cholesterol because LDL particles pose a risk for cardio-vascular disorders.Retained LDL particles in the endothelial cells of the vessel walls cause the initiation of inflammatory process which can lead to atherosclerosis.Maximum range of LDL-c should be confined within 130 mg/dl and if someone does not have heart disease, blood vessel disease, or diabetes, then it should be no more than 100 mg/dl.

As compare to VLDL and LDL, HDL performs its physiological function by picking cholesterol from tissues, specifically from walls of blood vessels and dropped into liver, where this cholesterol is either metabolized or excreted from living body by the process of reverse cholesterol transport (RCT) (Duffy & Rader, 2005; Azzam & Fessler, 2012). HDL is thus termed as good cholesterol as they can remove cholesterol and TGs out of arterial walls, minimize macrophage aggregation, and thus help in prevention and regression of atherosclerosis over weeks or even years, therefore helping prevent cardiovascular disease, stroke(s), and other complicated vascular diseases. HDL-c should be higher than 55 mg/dL for females and 45 mg/dL for males.

1.2 Problem Statement

Various parameters of lipid profile such as total cholesterol (TC), triglycerides (TG) and LDLs have been proved as risk factors for atherosclerosis cardiovascular disease (ASCVD) throughout the world. Association of lipid profile with ABO blood types has been studied in different populations of various regions of the world. This association had not been studied yet in the population of Mirpur Azad Kashmir, so there is a need to investigate the correlation of lipid profile with different ABO blood types of adult residents of this region.

1.3 Objectives

Elucidative studies have been carried out for a longtime to associate the lipid profile with ABO blood groups, in different parts of the world because lipid profile is strongly associated with atherosclerosis and cardiovascular risk factors. Results of previous investigations did not produce consistent results to elaborate the association of ABO blood groups distribution and CVD risk factors in various parts of the world (Capuzzo et al., 2016). Besides this, most of the association studies have included the selected subjects which are confirmed CVD patients. We have decided to plan simple random sampling to study the association between ABO blood group and lipid profile among individuals visited for routine checkup including healthy volunteers to buildup an effective strategy for primary prevention of risk factors of CVD in population of Azad Kashmir.

1.4 Scope

Lack of investigations with respect to the association of lipid profile and ABO blood groups in Azad Kashmir region especially in adult's residents of Mirpur was considered as a Lacuna to be filled. Therefore, this study was designed to investigate the association between ABO blood groups and lipoprotein profiles of adult residents of Azad Kashmir region.

1.5 Hypothesis of Study

Prevalence of ASCVD in adult males and females residents of Mirpur Azad Kashmir in relation to different blood phenotypes will be studied.

Chapter 2

Literature Review

2.1 ABO Blood Group System

Human ABO blood group system plays a significant role in transfusion and transplantation medicine. Discovery of ABO blood group antigens along with their structure and function is the result of deep research of more than a century (Storry & Olsson, 2009). In the early 20th century, an Austrian scientist Karl Landsteiner reported a visible agglutination and hemolysis, when six sera (took from five colleagues and sixth one his own) got mixed with their saline suspension of erythrocytes separately (Landsteiner, 1900). Three phenotypes of the ABO blood group system were identified as a result of this experimentation along with first genetic polymorphism in man. These early discovered blood groups were A, B and C (renamed as O) (Storry & Olsson, 2009). The fourth blood group, AB was discovered in 1902, by Decastello & sturti (Decastello & Sturli, 1902).

2.1.1 Antigens

Two glycans, antigen A, $N-$acetylgalactosamine ($GalNAc\alpha 1{-}3(Fuc\alpha 1{+}2)Gal\beta 1$) and Antigen B, galactose ($Gal\alpha 1-3(Fuc\alpha 1+2)Gal\beta 1$) are responsible for the variety of phenotypic expressions related to ABO blood grouping and are the products of the two alleles named A and B of the same nucleotide sequence responsible for two different glycosyltransferase activities (Storry & Olsson, 2009).

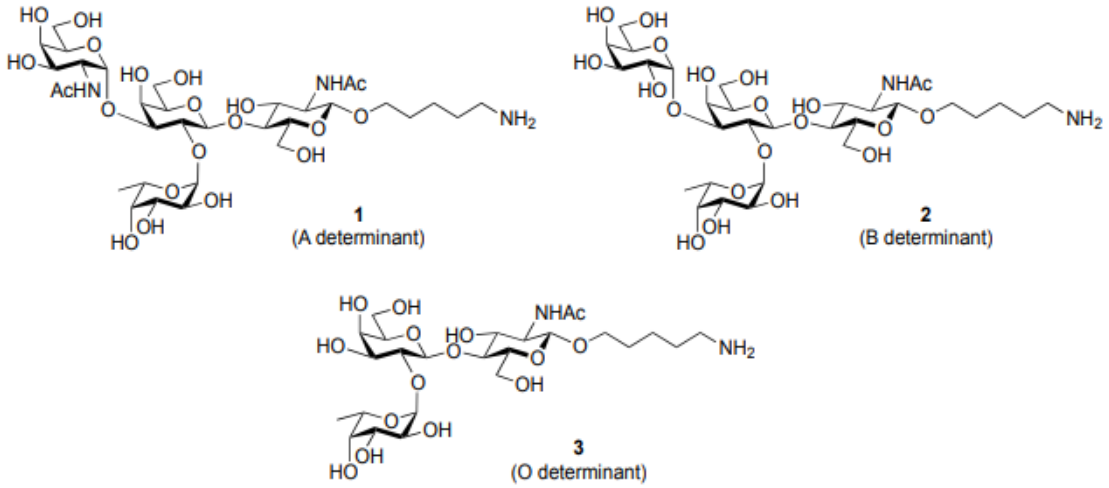

FIGURE 2.1: Structure of the target of ABO blood group antigens (Hara et al., 2013).

Antigen A and antigen B are produced by the addition of either N-acetylgalactose amine or galactose respectively, to the precursor H antigen. Blood group O is the result of absence of both antigens which inturn the consequence of a frameshift mutation at the N-terminus of the related enzyme (Yamamoto et al., 1990). These antigens usually produce their expression on RBCs surface glycosphingolipids or glycoproteins along with many other cells and tissues such as skin, gastrointestinal, bronchiopulmonary and urinogenital epithelium tissue (Le Pendu et al., 2001). Besides these antigens ABH antigens are also found in variety of secretions and body fluids such as saliva, breast milk and semen etc. (Seltsam et al., 2003). Approximately 2 million ABO blood group antigens are expressd on each erythrocyte while these antigens have been adsorbed by other blood cells i.e. T cells, B cells and platelets from plasma.

2.1.2 Genetic and Molecular Basis

Gene of the ABO blood group system is found on the terminal end of the long arm of chromosome number $9(9q34.1 - 9q34.2)$ and as being much larger in size, it is composed of 7 exons which span 18 kilobases at this locus (Yamamoto, 2004), having size stretching from 28 to 688 base pairs and with 5 introns having 554 to

12982 base pairs (Yamamoto et al., 1990; Yamamoto et al., 1995; Bennett et al., 1995).

Loss of enzymatic activity in O allele (Storry & Olsson, 2009) is due to the deletion in exon 6. Basically, human ABO blood group system is determined by three allelic forms of the same gene,among them two alleles A and B alleles are co-dominant and being recessive, O allele is silent. Genes of ABO blood group system inherit as Mendalian traits.

ABO gene is responsible for glycosyltransferase enzyme production which involve in the attachment of a sugar on to a carbohydrate structure known as H antigen. ABO and H antigens are primarily represented on glycoproteins and glycolipids by small carbohydrate epitopes and hence expression of ABO antigen is controlled by H gene which is involved in the production of the H antigen substrate, act as precursor of A and B antigen (Storry & Olsson, 2009).

2.1.3 The H Locus (FUT 1)

Expression of A and B antigen is primarily depends on H antigen so as being precursor it must be present for the synthesis of A and B antigens. H locus (FUT 1) is responsible for the synthesis of enzyme which catalyzes the synthesis of H antigen and is present on chromosome 19 at 19q 13.3 and it consists of three exons which spread over about 5 kilobases of genome, and encodes fucosyltransferase which is responsible for H antigen synthesis on erythrocytes (Dean, 2005).

Individuals having either dominant alleles (H/H) or even have single dominant allele can produce H antigen but homozygous recessive (h/h) individuals do not have H antigen with missing A and B antigen on their red blood cells. These individuals possess anti$-A$ and anti$-B$ antibodies in their serum along with anti$-H$ antibodies. This phenotype with $H-$deficient erythrocytes is rare and is called "Bombay phenotype" (Oh) known by the city in which it was first discovered. Bombay phenotypes are produced as a result of point mutations in the $FUT1$ gene (Kelly et al., 1994; Kaneko et al., 1997; Reid et al.,2012; Koda et al 1997). Individuals with such phenotype live healthy life but remain ever at a risk of acute

hemolytic reaction due to antibodies in their serum, if they ever needed any blood transfusion. The risk can be avoided by using only blood products from a donor having same phenotype.

2.1.4 The Se Locus (FUT2)

Se locus is found on chromosome 19 at 19q 13.3 and it consists of two exons which spread over 25 kilobases of genome (Dean, 2005). Se locus is responsible to encode an enzyme called fucosyltransferase which expresses itself in the epithelium of various secretory tissues in the body such as salivary glands, respiratory epithelium, gastrointestinal tract (Dean, 2005). This enzyme catalyzes the synthesis of H antigen in various bodily secretions. These individuals are called "Secretors" and must have at least single copy of the Se gene which encodes the functional enzyme with homozygous Se/Se or heterozygous Se/se genotype. They possess A or B antigens in their secretions (Hosoi, 2008), while non-secretors are homozygous recessive (se/se) for this allele and are unable to synthesize a soluble form of H antigen and in turn missing A or B antigen in their secretions.

2.1.5 ABO Antibodies

ABO antibodies are naturally occurring immunoglobulins and are found universally. An inverse relationship is found between the presence of A and B antigen on erythrocytes and anti-A and anti-B antibodies in sera of individuals. As being highly reactive, they are of much clinical importance in transfusion medicine because a wrong or mismatched blood transfusion or organ transplant can lead to hyper-acute immune response and death (Sazama, 1990; Williamson et al., 1999).

Landsteiner reported this inverse relationship as that the serum of an individual possessed antibodies to the missing antigen such as individuals with phenotype A possessed an A antigen on their erythrocytes and anti-B in their sera which agglutinated erythrocytes of B phenotype and sera of individuals with B phenotype had anti-A antibodies which caused agglutination of A phenotype RBCs (Landsteiner, 1900). Blood type O did not posses any antigen on the surface of RBCs but its

serum contained both anti-A and anti-B antibodies. If an individual having blood type O is transfused with blood type other than O, anti-A and anti-B antibodies which are naturally present in the serum of the recipient bind with the corresponding antigens of the transfused erythrocytes,eventually lead to complement fixing and severe hemolysis with intravascular coagulation which in turn can causes Shock, acute renal failure and death AB phenotype holder persons possessed both A and B antigens and did not have any type of antibodies in their sera. So on the basis of their erythrocytes agglutination patterns, individuals could classified as A, B, AB and O phenotypes. Safe blood transfusion have been made possible and adverse transfusion reactions due to ABO antibodies during blood transfusion should be prevented by routine blood grouping along with cross matching of blood types and products of donors and recipients but clerical error can be a curse of wrong blood transfusion and death (Sazama, 1990; Williamson et al., 1999).

TABLE 2.1: ABO human Blood groups their antigens, antibody and compatible blood groups transfusion.

Blood Group	Genotype	Antigen found	Antibody Present	Compatible blood group
A	I^AI^A *or* I^A i	A	B	A and O
B	I^BI^B *or* I^B i	B	A	B and O
AB	I^AI^B	AB	None	A, B, AB and O
O	ii	None	AB	O

The ABO antibodies are basically isohaemaglutinins (Mishra et al., 2014). Mostly they are IgM type and are not found in new born. They are produced few months after birth and were initially known as naturally occurring antibodies because of the concept that their production does not involve any antigenic stimulation but this belief have been proven wrong. It is now known that bacteria present in digestive tract and in environment stimulate the production of these naturally occurring antibodies i.e. entrobacteriaceae have been demonstrated to have the structures on their lipopolysaccharide coats, just like ABO antigens. (Springer,1971; Daniel –Johnson et al., 2009).

Probably all infants have proper isohemagglutinin content in their sera at the age of about one year (Cooling, 2011) because endogenous production of anti$-A$ and anti$-B$ antibodies can start at the age of about $3-6$ months. Titers of these

antibodies are progressively increased and reach to its maximum level at the age of $5-10$ years whereas ABO antigens on erythrocytes of the foetus can be reported at 5th to 6th week of pregnancy. These A and B antigens on erythrocytes become increased with increasing age and its normal expression can be reported at the age of $2-4$ years.

2.1.6 Subgroups of ABO Blood Types

ABO subgroups have been differentiated due to the decreased sites for antigen attachment and hence the decreased amount of antigens on the erythrocytes. More importantly A subgroups frequency is greater than the frequency of B subgroups (Beattie, 1972).

Major subgroups of A are A1 and A2. Red blood cells of both $A1$ and $A2$ individuals more frequently interact with anti-A antibodies in routine identification tests. However, to distinguish A1 from A2 serologically anti$-A1$ lectin from Dolichos biflorus seeds can be used which agglutinate $A1$ red blood cells and not of $A2$ (Landsteiner & Levine, 1930). About 80% individuals having A or AB phenotype are identified as $A1$ or $A1B$ while 20% are either $A2$ or $A2B$. RBCs of individuals having weak subgroups of A are serologically non-reactive or show weak interaction with anti$-A$ antiserum as compare to $A2$ individuals (Cartron et al., 1974). Subgroups weaker than A2 are not found more commonly and can be distinguished by decreased amount of antigen A on erythrocytes with inversely increased activity of H antigen. These rarely found A subgroups include A int, A3, Ax, A end, Am and A el. In which last four subgroups cannot be identified by routine blood typing test (Heier et al., 1994).

B subgroups, have been reported as more less common as compare to A subgroups and can be differentiated by the decreasing amount of B antigen as in the case of A subgroups including B3, Bx, Bm and B el in decreasing order (Hosoi, 2008).

2.2 Rh Blood Group System

The Rh (Rhesus factor based) blood group system of man is represented by a group of antigens expressed on the membrane of Red blood cells (Avent & Reid, 2000) which may responsible for ammonia transport (Marini et al., 2000) or transfport of CO_2 (Endeward et al. 2008; Kustu & Inwood 2006), or play a structural role in red blood cell membrane and may not involve in transport (Westhoff & Wylie 2006). Functional variation of Rh antigen related to this blood group system is the consequence of gene mutations such as insertion, deletion, gene conversion or single nucleotide polymorphisms (SNPs) in Rh-genes (RhD and RhCE) (Flegel, 2011; Mouro et al., 1993; Colin et al., 1991). D−negative phenotype is the consequence of homozygous deletion of complete RhD gene (Wagnar & Flegel, 2000). While D−positve blood type is the result of single or double (homozygous) RhD gene.

Rhesus factor based blood group is clinically the most important blood group system. It is the largest blood group (with 49 antigens) of all blood group systems described so far. The large number of Rh−antigens is the attribute of the complexity in genetics. The Rh-antigens are present on two Rhesus proteins named RhD and RhCE and their variations are the result of the changes in their protein sequences (Matassi et al., 1999). In CD nomenclature, they are denoted as $CD240D$ and $CD240CE$. As compare to other blood group representing proteins, Rh-proteins are located on the erythrocyte membrane and their intermediate precursors.(Flegel & Wagner, 2004).

The D−antigen was the first Rhesus antigen to be discussed in 1939, just after its discovery. $D-$positive persons were named as $Rh-$positive. After some years of antigen discovery, a quantitative variant with weak expression of D antigen (Wagner et al., 1999) was discovered and known as "Du" in 1946. This variant is now termed as "$weakD$" and clinically as well as diagnostically is much significant. The fact that D antigen has also its qualitative variants, have been described about 66 years before. The persons having partial D variant are Rh-positive and can be able to produce anti-D antibodies.

2.2.1 Molecular and Genetic Basis of Rh-Phenotypes

The locus for Rh gene is located on the short arm of chromosome $1(1p34.1 - 1p36)$(Cherif-Zahar et al., 1991). It contains two genes RhD and RhCE. The RhD and RhCE genes are structurally similar and the product of duplication of a common gene ancestor (Matassi et al., 1999). RhCE was the first Rh gene discovered in 1990 while RhD gene was discovered just after two years.

More than 170 alleles of RhD gene have been reported till now, but the site has not been completely explored still. The most common of all European partial D alleles, DNB was discussed in 2002 (Wagner et al., 2002). Knowledge about the development of two Rh genes on chromosome 1 has been more strengthen by the comparative description of both human genome project and Mammal Genome Project in 2002. (Wagner & Flegel, 2002).

Single Rh gene is present in most mammals which locate on the chromosome as according to the human RhCE gene position. RhD gene was formed during the evolution of mammals by the duplication of ancestral Rh gene. Many modern humans do not have RhD gene because of the RhD deletion (Wagner & Flegel, 2000) during the evolution of hominids and this haplotype is considered as the major cause of D-negative phenotype in all human populations.

Rh alleles can be classified on the basis of molecular structure they possess. Major causes of formation of all these alleles are nonsense, missense or frame shift mutations as a result of point mutations i.e. SNPs.

2.2.2 Molecular Basis

RhD and RhCE are two structurally similar proteins which differ only by 36 out of 417 amino acids, by which they are formed. Both have twelve segments inside the membrane of red blood cell and six extra cellular loops, while amino and carboxyl terminals are found inside the cell.

2.2.3 D-negative Phenotype

Difference between Rh positive and Rh negative phenotypes is due to the presence or absence of RhD protein in the membrane of RBCc. Unlike many other cell proteins it is an unusual red blood cell protein due to be lacking completely in many humans (Colin et al., 1991). Strong antigenicity of the RhD protein is due to its particular genetic ability.

2.2.4 D-antigen Variants

Some other D negative phenotypes have been reported on the basis of changes in D-antigen which in turn are the product of series of changes in RhD protein baside from lack of this protein. These RhD alleles are differentiated as weak D, partial D and DEL on the basis of their molecular structure and phenotypic differences. (Wagner et al., 1999).

2.2.5 Partial D

In the membrane of Red blood cells, the RhD protein traverses many times in such a way that a small portion of the amino acid sequence remains exposed at the surface. Any substitutional change even in single amino acid in a part of the protein present at the surface of the RBCs membrane as a result of point mutation will produce partial D phenotype and as a result of this amino acid substitution D antigen can miss its single epitopes. So new antigen can be formed (Cartron, 1994; Tippett et al.,1996). Hence partial D phenotype includes various subgroups known as D categories. Rh gene locus possess specific structure which facilities gene conversions (Wagner et al., 2001). A hybrid Rhesus allele will be formed as a result of insertion of some homologous exons of the RhCE into the RhD gene resulting in related hybrid protein expression. In this way some of D categories will be formed. This type of changes usually occurred in the amino acids present on the surface of RBCs.

2.2.6 Weak D

If replacement occurs in amino acids present within the membrane of RBCs or inside the cell i.e in cytoplasm, weak D phenotype will be formed. (Wagner et al., 1999). As a result of these substitutions RhD protein will not be integrated into the membrane and eventually, quantitative reduction of the D antigen. No $anti-D$ immunization will occur because this change is not qualitative.

2.2.7 DEL

Weak expression of D antigen is particularly represented as DEL (previously known as Del) with minimum copies of D antigen (less than 22) per RBCs (Kulkarni et al.,2006; Körmöezi et al.,2005) because it could be demonstrate only by elution. Antibodies are removed from RBCs in elution to demonstrate them in elute. As compare to weak D, molecular changes are more severe as they hinder to some extent but not entirely prevent the integration into the membrane. Almost 30% of the persons of East Asia with D negative phenotype have DEL allele RhD (Wagner et al., 2001; Shao et al., 2002).

2.2.8 The C/c and E/e Antigens

Variety of substitutional changes in the amino acid sequence at different positions in RhCE protein will produce the clinically more important Rhesus antigens C, c, E and e. These antigens are produced as consequence of protein polymorphisms and more oftenly a protein has two variants which are differentiated by only single amino acid i.e, the Rhesus antigen E and e. Allele RhCE having amino acid proline at 226 position expresses the E antigen, while RhCE allele having alanine at the same position expresses the e antigen (Flegel & Wagner, 2004). RhCE alleles have similar difference in relation to antithetical C and c antigens.

2.2.9 Clinical Significance of Rh Antibodies

Clinically the Rh blood group is more important because of the antigens Rh which are strongly immunogenic and most of the Rh antibodies are the potential source to cause hemolytic transfusion reaction (HTR) and hemolytic disease of new born (HDN) (Filbey et al., 1995; Bowman, 1997). Lack of D antigen will produce anti-D and if these anti-D antibodies encounter the D-antigen on erythrocytes of transfused blood(Urbaniak and Robertson,1981), causing HTR or on fetal RBCs causing HDN, that is why, routine Rh blood typing must be needed for blood donors, recipients and of pregnant females.

Most antigens located on RBC, differ from one another by only one are two amino acids while D antigen varies that of C/c and E/c antigens by 36 amino acids. such huge difference of amino acids is the major cause of great potential of Rh antigen to stimulate immune response (Westhoff, 2004).

Most of the antibodies produced in response of Rh antigens are 1gG type, which are the potent cause of HTR and HDN, aside from 1gG, there are also 1gM type antibodies involved in hemolytic reactions which are naturally occurring and are the examples of alloantibodies.

All anti-C,anti-D, anti-E and anti-e antibodies have been reported to involve in hemolytic transfusion reactinos, especially delayed reactions (Daniels, 2002) but anti-D is the major cause of HDN which causes fetal death. The prevalence of HDN due to RhD incompatibility have been decreased due to the introduction of anti-D immunoglobulin's along with careful monitoring of at risk pregnancies but cases of RhD alloimmunization cannot be controlled and it is still remained a major cause of HDN (Urbaniak & Greiss, 2000) Rh alloantibodies which cause severe HDN include anti-C (Wagner et al., 2002; Wagner & Flegel, 2002), which is of much clinical importance after D-antigen. Mild HDN caused by anti- c (Wagner et al., 1999) anti-E (Shao et al., 2002) and anti-e (Chapman and Waters, 1981). Although determination of RhD status of blood donors and recipient has reduced the risk of transfusion reactions due to anti-D but other Rh antigens sensitization

can cause serious results during transfusion, especially in individuals having sickle cell anemia (SCA).

2.3 Lipoproteins

Mammals possess a comprehensive system for transportation of lipids, specifically of triglycerides and cholesterol through the body. Much of the transport is regulated by very complex network of plasma lipoproteins which includes low density lipoproteins (LDL) and high density lipoproteins (HDL) as its important components.

These lipoproteins can be differentiated on the basis of their size, density and chemical composition whereas their size and density are inversely related i.e HDL have small size and high density and VLDL have large size and very low density (Dashti et al., 2011; Queiroz et al.. 2010; Rezaee et al., 2006).

Plasma lipoproteins are principally involved in homeostasis and are structurally composed of two major parts as an inner core of lipids and an outer layer of phospholipids and apolipoproteins. Any disturbance in their physiological function may cause dyslipidemia, hyperchloresterolemia, hyperlipidaemia, hypertriglyceridaemia which further causes severe pathophysiological problems such as obeisity, type 2 diabetes and atherosclerotic cardiovascular disease (ASCVD) (Compos et al.,2001; Lee et al., 2003).

LDL is considered as major cholesterol transporter in man and is more vulnerable to oxidation as a consequence considered as major risk factor of coronary artery disease (CAD). (Brown and Goldstein, 1997; Goldstein & Brown, 2015). Cholesterol rich apo B containing lipoproteins retained and accumulated at the sites in arterial intema having tendency for plaque formation (Skålén et al., 2002; Tabas et al., 2007). Infact, all apo B containing lipoproteins having diameter less than 70nm such as LDL, VLDL, IDL and apolipoprotein (a) continuously enter and exit the arterial intema (Tabas et al., 2007). Chances to retain LDL in arterial intima are increased with increased level of circulating LDL-c and hence initiation

and progression of atherosclerotic plaque development also increases. (Goldstein & Brown, 2015).

As compare to VLDL and LDL, HDL performs its physiological function by picking cholesterol from tissues, specifically from walls of blood vessels and dropped into liver, where this cholesterol is either metabolized or excreted from body by a process of reverse cholesterol transport (RCT) (Lewis & Rader, 2005; Azzam & Fessler, 2012).

2.4 LDL and its Composition

Basically LDL particle is a cholesterol–rich, triglyceride-poor particle. Its core is composed of estrified cholesterol, triglycerides and phospholipids along with their fatty acid tails while surface layer of the paticle is hydrophilic and is formed by phospholipids and free cholesterol along with hepatically derived apo B 100 which impart stability to the particle and also involes in packaging of the LDL particle. Like other proteins in the circulation, the LDL particle behaves as carrier for several insoluble particles like free fatty acids which are loosely attached to these particles (Phillips et al., 2005). Specifically the particle shows more affinity for lipoprotein lipase which facilitates the particle to attach with endothelial surface. LDL are the lipoprotein fractions having density range from 1.006 to 1.063 g/ml (Havel et al., 1955) which can easily be separated by different laboratory techinques. VLDL and IDL are also included in this range so LDL particles specifically have a density range from 1.019 to 1.063 g/ml (Havel et al., 1955).

2.4.1 LDL as Risk Factor for ASCVD

It is well accepted, on the basis of genetic, exprerimental and clinical studies that increased level of LDL-c is a major risk factor for CHD (Graham et al., 2012; Executive summary of NCEP, 2001; Sniderman et al., 2012; Sniderman & Kwiterovich, 2013; Brunzell et al., 2008). Elevated level of LDL-c promotes the

development of ASCVD and risk of various cardiovascular events such as myocardial infarction, ischeamic stroke and coronary mortality is directly related to as much as longer exposure of elevated LDL-c so in recent years, the major target in the treatment of dyslipidaemia is the LDL-c (Catapano et al., 2017; Stone et al., 2014) whereas HDL-c is not being recommended as primary treatment target in recent demonstrations because treatment strategies related to elevating $HDL-C$ and cardiovascular risk reduction have been failed (Ali et al., 2012). On the basis of well accepted evidences from randomized controlled prospective primary and secondary prevention clinical trials with anti-atherosclerotic drugs like statin, treatment guidelines recommended low LDL level as a principle strategy for CHD risk reduction (Executive summary of NCEP, 2001; Sniderman et al., 2012; Sniderman & Kwiterovich, 2013; Brunzell et al., 2008; Stone et al., 2014 (Part-B); Baigent C. 2010). By the 50% reduction of $LDL-Level$ through high dose of anti-atherogenic drugs like statin in patients with CHD is the key therapy recommended by US guidelines (Stone et al., 2014 (Part B)). On the other hand reduction of LDL-c level to less than 1.8 mmol/L (Less than 70 mg/dl) or up to 50% of the base line LDL-c with 1.8-3.5 mmol/L (between 70-135 mg/dl) is recommended by European guidlines (Catapano et al., 2017). Both these recommendations help us to consider the atheroprotective effect of reduced LDL-c level. LDL-c has been known as a key biomarker for LDL level/concentration for many years but LDL quantity is also measured by LDL particle number (LDL-P) as an alternative measure which is directly determined by nuclear megnatic resonance spectroscopy or determined by apo B lipoprotein concentrations. (Jeyarajah et al., 2006; Cromwell & Barringer, 2009).

2.4.2 LDL Sub-Classes and Methods of their Identification

Ultracentrifugation is the process by which plasma LDL profile can be analysed (Berneis et al., 2005; Krauss et al., 1980; Gofman et al., 1949). Plasma LDL concentration can also be analyzed by gradient gel electrophoresis (GGE) (Ensign et al., 2006) in which LDL particles can be separated on the basis of density or size. Other methods have also been used to analyze the particle size, charge

or chemical properties include nuclear magnetic resonance (NMR) (Otvos et al., 1992; Witte et al., 2004), high-performance liquid chromatography (HPLC) with gel filteration columns (Okazaki et al., 2005), dynamic light scattering (O'Neal et al., 1998; Sakurai et al., 2010), ion mobility analysis (Caulfield et al., 2008; Musunuru et al., 2009) and homogenous assay analysis (Hirano et al., 2003). Among these methods ultracentrifugation and gradient gel electrophoresis with specific modifications are widely being used for LDL determination.

To get quantitative results rapidly, tube gel electrophoresis for analysis of LDL subfraction has been used by some authors (Hoefner et al., 2001; Banuls et al., 2012) but to develop a standard procedure for analysis, more studies are required because different methods of analysis of LDL subclasses give different results and difficult to determine that which technique should be recommended to get most accurate results and suitable for clinical use.

2.4.3 Subclasses of LDL

LDL can be fractionated into different sizes with the help of GGE to get two major categories of particles, patern A and pattern B in which pattern B is also known as small dense LDL (sdLDL) (Krauss et al., 1980).

By using above discussed method in various demonstrations, LDL particles have been classified into 3 or 4 subclasses such as large ($LDLI$), intermediate ($LDL-II$), small ($LDL-III$) and in some demostrations very small ($LDL-IV$) LDLs (Hiryama & Miida 2012 , Berneis et al., 2005).

$LDL-III$ and $LDL-IV$ are being termed as small dense LDL (sdLDL). Serum of patients with atherosclerosis specially contain sdLDL which is responsible to increase their atherogenisity due to its susceptibility to chemical modification (Steingerg, 1989; Orekhov et al., 1991).

All the fractions of LDL have different atherogenisity, as demonstrated in recent studies that sdLDL is more atherogenic than larger LDL subfractions because it have more ability to penetrate the arterial walls and enhance their potential for cholesterol to develop atherosclerotic plaque as well as the prolonged circulation

of sdLDL can be more athergenic due to its more potential for modification in plasma (Ivanova et al., 2017). But LDL classification on the basis of different analytical procedures does not have uniformity so clinical results obtained from different methods should be compared carefully.

2.4.4 Lipoprotein (a)

Lp(a) is a well known variant of LDL which is covalently bonded with apo B nad its concentration is different in different populations and is highly determined by inherited variation of DNA sequence. (Kathiresan, 2009). Lp(a) has affinity to bind with phospholipids and potentially involved in phospholipid transportation and atherogenicity (Enkhamaa et al., 2011). It may physiologically involve in thrombosis because Lp(a) particles are structurally homologous with plasminogen which is pro-enzyme involved in fibrinolysis (Koschinsky, 2004; Koschinsky, 2005). Lp(a) is a strong candidate for an association between plaque formation and stenosis and is a risk factor for events associated with thrombosis such as atherosclerotic occlusion, because structurally it is a combination of pro-atherogenic factor and anti-fibrinolytic factor (Berglund L 2004). Its interference with Annexin A5 binding to a pro-coagulant phosphatidyl serine has been reported in recent years. Annexin 5 plays an important role in anticoagulation on the endothelial surface and hence Lp(a) enhance thromabosis in another way (Fu et al., 2010).

2.4.5 LDL Cholesterol and Progression of ASCVD/CHD

When LDL cholesterol penetrates into the walls of coronary artery through its endothelium, atherosclerotic plaque formation is initiated. Here in this site, LDL particles are oxidized and modification of LDL associated apo B Lipoprotein takes place. This modified apolipoprotein B is recognized by scavenger receptors present on macrophages, which take up this modified apo B. After being endocytosed it converted the macrophages into foam cells. Hereby these lipid-rich foam cells cause inflammation which in turn leads into proliferation and collagen production by smooth muscle cells of arterial intima. This plaque may detached and become

mobilized and finally ruptured causing thrombotic occlusion and subsequently leading to ACS (Goldstein & Brown, 2015).

Physiological importance of LDL cholesterol in relation to monocytes and endothelial cells was studied on isolated endothelial cells as well as LDL importance in relation of pathophysiology of CHD have been demonstrated by using animal cells (Ross, 1999). Moreover, various epidemiological studies have been conducted to demonstrate the direct relationship of elevated LDL cholesterol and development and progression of CHD without dependence of sex, body mass index and diabetes (Sarwar et al., 2009). Although the association between LDL cholesterol level and CHD risk is seen in people of any age group but has been specially noticed in young's with great strength. Particularly this association is demonstrated by a log-linear relationship i.e a strong positive relationship has been noticed in elevated LDL cholesterol, where as in low LDL cholesterol, this relation-ship is weaker (Sarwar et al., 2009). Direct and visual intravascular ultrasound demonstrations revealed the association between coronary plaque resizing and level of LDL cholesterol (Von Birgelen et al., 2003 , Von Birgelen et al., 2004). A strong positive association of annual coronary plaque resizing and LDL cholesterol level has been reported in a demonstration of 60 patients, with 75mg /dL LDL $-$c level which predict about the plaque size stability (Von Birgelen et al., 2003).

2.5 HDL

2.5.1 Size and Shape

High density lipoprotein (HDL) is protein enriched, dense lipoprotein particle having small size as compare to other types of lipoproteins, ranging the size between 8 to 10 nm and density range between 1.063 to 1.21g/ml (Kontush & Chapman 2012; Havel et al., 1955). HDL particles are discoidal or quasi-spherical, plurimolecular and pseudomicellar complexes.

2.5.2 Chemical Composition

HDL particle mainly composed of polar lipids and apolipoproteins with lots of antioxidant molecules, i.e. paraoxanase (PON1), Lecithin-cholesterol acyltransferase (LCAT) along with variety of other enzymes, acute-phase proteins and a small amount of non-polar lipids (Heinecke, 2009). Lipid content of HDL particle contains variety of lipids such as cholesterol, triglycerides, phospholipids, cholesterylesters which from $(40-60)\%$ part of HDL while remaining part of molecule is composed of proteins such as apo $A-I(70\%), apoA-II(20\%)$ and other apolipoprotiens and enzymes (10%) (Asztalos & Scheafer, 2003 a, b; Shceafer et al., 2010). Normally apo AI performs its function by interacting with tissues via ATP-binding cassette transporters (ABCA-I) after detaching from HDL particles, as a result new HDL particles are formed in liver and interstine (Lund-Katz & Phillips, 2010). In actual all the apolipoprotein content of HDL constantly shuttles between circulating lipoprotein particles and HDL particles (Vedhachalam et al., 2010). Hence HDL is considered as a tool for many other functions related to homeostasis along with lipid transport (Eisenberg, 1984).

Both nascent discoidal and spherical HDL particles in circulation have apo $A-I$ as chief protein component (Lund-Katz et al., 2003). Molecules of $apoA-I$ organized in a belt-like manner, surrounding the phospholipid (PL) bilayer at the edge in the discs of reconstituted HDL (rHDL). Two $apoA-I$ molecules collectively form the double belt model which is the most acceptable model representing the discoidal structure of HDL particle (Segrest et al., 1999). Chemical cross linking and mass spectrometery methods have been used to analyze the helix arrangement of molecules. (Davidson & Silva, 2005; Thomas et al., 2006). Both apo $A-I$ molecules in the discs align in anti-parallel manner and stabilized by salt bridges. Indeed, number of apo $A-I$ molecules determine the size of discoidal HDL particle but in case of discrete sized particles the number of apo $A-I$ particles remains constant (Li et al., 2004).

HDL particles also contain apo E Lipoprotein which form complexes with PL arranging it's a helixes in perpendicular fashion to the acyl chains of PL molecules (Narayanaswami et al., 2004; Schneeweis et al., 2005). As compare to apo $A-I$,

apo E form quasi-spherical particles by folding into a hairpin like helix to form complex with PL polar head groups (Hatters et al., 2009; Peters-Libeu et al., 2006, 2007). It has been cleared in an in- vivo study that various factors are involved in remodeling, maturation and release of spherical HDL particles in circulation which were formed by the interaction of discoidal nascent HDL particles with $ABCA-I$ (Lund-Katz et al., 2003).

Spherical HDL particles are composed of a neutral lipid core which is formed by cholesterol esters (CE) and Triacylglycerols (TAG) covered by a single layer of PL and cholesterol molecules and, as compare to discoid particle, lack a specific edge to constrain the apo lipoproteins, however, protein-protein interactions in both spherical HDL and double-belt model of discoidal HDL particles are exactly alike (Silva et al., 2008). Three apo $A-I$ molecules are arranged to form trefoil arrangement having intermolecular salt bridges and conformations exactly same as that of the double$-$*belt* model of discoidal particle.

2.5.3 Proteome of HDL

Unlike other lipoprotein classes, HDL contains huge number of different memberane bound and plasma proteins (Zannis et al., 2006). Proteome component of HDL is composed of apolipo-proteins, enzymes, lipid transfer proteins (LTP), acute-phase response proteins, proteinase inhibitors and various other protein components.

Major functional HDL components are apolipoprotein and enzymes while the role of other minor protein components (complement regulation, acute-phase response and protection against infections) was came into focus as a result of advancement in proteomic technologies (Heinecke, 2009; Shah et al., 2013). More than 80 proteins were identified in human HDL by these proteomic studies. (Hoofnagle & Heinecke 2009; Shah et al 2013).Approximately 70% of total HDL proteins content is formed by apolipo protein A-I which is major component of almost all HDL particles (Asztalos & scheafer, 2003 a, b Shceafer et al., 2010).

Activiation of lecithine-cholesterol acyltrasferase (LCAT), preparing HDL for multiple anti-atheroganic function and interactions with cellular receptors are the major functions performed by apo $A-I$. It is an important constituent of HDL particle as it forms about 70% of the particle (Asztalos & scheafer, 2003 a, b; Shceafer et al., 2010). Like other many Apo lipoproteins, apo $A-I$ is also synthesized and released by liver and intestine (Zannis et al., 1985) and possesses binding ability for lipids, act as detergent, shuttle between lipoprotein particles, present in very low density lipoproteins and chylomicrons.

$Apo-AII$ comprises almost $15-20\%$ of total HDL protein content and is second major apoliportien in HDL particle. Approximately half of HDL particles may have apo $A-II$ (Duriez & Fruchart, 1999).As compare to apo $A-I$ it is more hydrophobic and mainly consists of two polypeptides having same amino acid sequence (Puppione et al., 2009; Shimano, 2009). It is also produced in liver and intestine.

Apo $A-IV$ is another component of HDL particle (Duka et al., 2013) prepared in intestine and released into circulation with chylomicrons and is most hydrophilic apolipoprotein circulates in free form as well as exchanges between lipoprotein.

Apo Cs is a family of apolipoproteins mainly synthesized in the liver (Albers et al., 1979). All of the members of this family readily exchange between lipoproteins (Jong et al.,1999). Apo $C-I$ is the smallest apolipoprotein having strong positive charge, associate with HDL and VLDL and act as modulator of several proteins (Shachter, 2001). It plays a major role as LCAT activator and inhibitor of cholesteryl ester transfer protein (CETP) as well as of hepatic lipase (Soutar et al., 1975).

Apo $C-II$ is also associated with HDL and VLDL and help to activate various triacyl glycerol lipases. Apo CIII is also a component of HDL and VLDL, act as inhibitor of lipoprotein lipase (LPL) and hepatic lipase (Jong et al.,1999). Its over expression in mice induces hypertriglyceridemia (Kim et al., 2008).

Apo D is HDL associated glycoprotein (Mc Conethy & Alaupovic, 1973) and belongs to lipocalin family. Its expression has been reported in liver, intestine and

many other tissues where it transports small hydrophobic ligands and specially arachidonic acid (Rassart et al., 2000).

Apo E is one of the major structural and functional glycoprotein associated with HDL but present in very less amount as compare to $Apo-A-I$ (Utermann, 1975). It is expressed in many tissues such as of liver, ductless glands, CNS and tissue macrophages. It presents detergent like properties specially for PL and plays same role as that of Apo $A-I$ and $ApoA-II$ (Lund-Katz & Philips, 2010).

APO F is called lipid transfer inhibitor protein (LTIP) due to its specificity for CETP inhibition. It is synthesized in liver and made protein highly acidic due to its heavy glycosylation with both $O-$and $N-$Linked sugar groups (Lagor et al., 2009).

Apo J is composed of two sub units, alpha and beta and also known as clusterin (De Silva et al., 1990 a, b). It involves in binding of hydrophobic molecules as well as of specific cell surface receptors.

Apo $L-I$ expression is reported in pancrease, lungs, prostate, liver, placeta and spleen and show high affinity to phosphatidic acid (Zhaorigetu et al., 2008). It is structurally and functionally similar to proteins belong to the $Bcl-2$ family which are involved in regulation of intra cellular apoptosis.

Apo M is HDL associated protein (Axler et al., 2007) involves in small hydrophobic molecular binding, especially sphingosine-1 phosphate (SIP) (Christoffersen et al., 2011; Ahnstrom et al., 2007). It is produced in liver and kidney.

Apo O is a minute fractioned HDL associated apolipoprotein manifested in many tissues in man (Lamant et al., 2006). It is also found in HDL and VLDL and is related to proteoglycan family. Its physiological function is still not known (Nijstad et al., 2011).

2.5.4 Enzymes

LCAT is responsible for cholesterol esterification to cholesteryl esters in plasma lipoproteins, not only in HDL but also in other particles containing apo-B. Due

to its co-purification, it is believed that LCAT is closely linked with Apo D (Holmquist, 2002). It manifests itself in the liver and to some extent in testes and in the brain. Most of its activity (about 75%) is associated with HDL which is due to its tertiary structure maintained by two disulfide bridges along with a covered active site. (Rousset et al., 2009).

Human paraoxanases (PON) is the group of enzymes including PON 1, PON 2 and PON 3 and are calcium dependent lactonases. (Goswami et al., 2009) PON 1 is completely associated with HDL and synthesized in liver, kidney and parts of large intestine (Mackness et al., 2010).

Platelet activating factor acetyl hydrolase ($PSF - AH$) is calcium independent enzyme responsible for degradation of PAF into biologically quiscent lyso-PAF (Mallat et al., 2010). It is produced in the brain, placenta and white adipose tissue while the major source of the enzyme in circulation are macrophages. (Mclntyre et al., 2009).

2.5.5 Acute Phase Response Proteins

A huge family of positive acute-phase response proteins is also associated with HDL which shows increased concentration during acute inflammation (Vaisar et al., 2007; Heinecke, 2009). As compared to aolipoproteins, concentration of these proteins is much less in HDL during normal conditions, whereas concentration of HDL associated apolipoprotiens in plasma is reduced in acute inflammation such as of apo $A - I$ and apo $A - IV$ (Navab et al., 2004) that is why these proteins are known as negative acute–phase response proteins.

Serum amyloid A(SAA) proteins represented by three isoforms SAA1, SAA2 and SAA4 are synthesized in liver of humans and are released during acute phase of the inflammatory response as major reactants (Khovidhunkit et al., 2004).

Fibrinogen is another acute phase protein synthesized in the liver, involved in platelet aggregation and by the activity of thrombin transformed into fibrin during blood clotting.

Lipopolysaccharide binding protein (LBP) is also an acute phase response glycoprotein responsible for lipopolysaccharide (LPS) diffusion with the help of attachment of the lipid A fraction of lipopolysaccharide of Gram-negative bacteria(Wurfel et al., 1994).

2.5.6 Complement Components

A large family of complement components such as C3, C4, $C4b-$bidnig proteins and C9 is associated with HDL and involve in the activation of complement system through different pathways. Vitronectin is major example of complement regulation protein associated with HDL (Heinecke, 2009).

2.5.7 Lipid Transfer Proteins

Major examples of this group are phospholipid transfer proteins (PLTP) and cholestryl ester transfer protein (CETP). Among them PLTP is synthesized in liver, pancreas, kidney lungs and placenta and primarily involves in extracellular phospholipid transport and play a key role in the innate immune system (Albers et al., 1996). CETP is synthesized in liver and adipose tissue and physiologically functions as it commutes between HDL and apo B holding lipoproteins (Quinet et al., 1991) and involves in shuttling of triglycerides and cholesteryl esters between them. Its molecular structure is explained by Qui et al. (Qui et al., 2007).

2.5.8 Lipidome

Wiesner & colleagues are the pioneers who gave reference values for HDL lipidome (Wiesner et al., 2009). Furthermore, phospho and sphingo-lipidome of five major subpopulations of HDL have been reported which helped in further characterization of HDL composition and inherent heterogeneity (Camont et al., 2013).

2.5.9 Phospholipids

About 32−35 mol of total lipid content in HDL is composed of phosphatidyl choline which is major plasma phospholipid (Wiesner et al. 2009). It is a consistent part of the structure of all subpopulations of HDL. Lyso PC (Camont et al. 2013) phosphtidylethanol amine (PE) (Wiesner et al. 2009), plasmalogens (Stahlman et al. 2013) and negatively charged phospholipids such as phosphatidylinositol (PI), Phosphatidylglycerol (PG), Phosphatidic acid (PA), Phosphatidylserine (PS) and cardiolipin (Rosenson et al.) are other major subclasses of phospholipids present in HDL lipidome.

2.5.10 Sphingolipids

Major sphingolipid in circulating HDL is sphingmyelin, a well known structural lipid responsible for increasing surface lipid rigidity and is $5.6 - 6.6 mol\%$ of total HDL lipid content (Wiesner et al. 2009, Stahlman et al. 2013).

Sphingosine −1 phosphate (S1P) is more important among lysosphingolipids (Kontush & Chapman, 2012) performs important function in vascular biology as a biologically active lipid (Lucke & Levkau, 2010). Lysosphingomylin and lysosulfatide are other examples of bioactive lysosphingolipids associated with HDL (Lhomme et al., 2012).

2.5.11 Neutral Lipids

Surface lipid layer of HDL particle is composed of many unesterified (free) sterols which regulate its fluidity. Cholesterol is a principle sterole among them. A variety of other sterols such as lathosterol, ergosterol, phytosterols, oxysterols and estrogens are minor components of HDL particle (Kontush & Chapman, 2012).

Cholesteryl esters (CE) are formed by trans-esterification of PL and cholesterol catalyzed by LCAT and about 80% of CE are formed in plasma HDL. They form about $36 mol\%$ of total HDL lipid and as being highly hydrophobic, act as major component of lipid core (Camont et al. 2013 , Stahlman et al., 2013).

Most important HDL CE is cholesteryl inoleate. Oleic acid, palmitic acid and linoleic acid moieties containing triacylglycerid species are also associate with HDL (Lhomme et al., 2012) and form about $3mol\%$ of total lipid of HDL (Kontush et al., 2007; Weisner et al., 2009; Stahlman et al., 2013).

2.5.12 Subclasses

Human plasma HDLs family includes highly heterogenous group of lipoproteins and have been classified into various subgroups on the basis of difference in size, shape, density and chemical composition of proteins and lipids. The two principle HDL subclasses were isolated by different ultra-centrifugation methods. For example rate-zonal ultracentrifugation (Franceschini et al., 1985) or single vertical spin ultra-centrifugation (Kulkarni et al., 1997).

These two identified subclasses are HDL2 and HDL3. Among them HDL2 was less dense (1.063−1.125 g/ml) and relatively lipid-rich form, while HDL3 was more dense (1.125−1.21g/ml) (Havel et al., 1955) and relatively protein-rich form.

Furthermore two subdivisions of HDL2 and three subdivisions of HDL3 have been isolated by non-denaturing polyacrylamide gradient gel electrophoresis (Nichols et al., 1986) and by density gradient ultra centrifugation (Kontush et al., 2003). Another analytical way to separate HDL particles is agarose gel electrophoresis in which HDL particles can be separated due to thier surface charge and shape into α-moving particles which include circulating HDL, nascent discoidal and poorly lipidate HDL. These two subclasses can be isolated by using coomasive blue stain or anti-apolipoprotein $A-I$ antibodies (Favari et al., 2004). Concentration of Plasma pre-β HDL can also be measured by sandwich enzyme immunoassay (Miida et al., 2003). 2−dimentional (2D) electrophoresis method is the combination of agarose gel and the GGE, by which one can separate HDL on the basis of charge in the first run and on the basis of size in the second run. Isolation of distinct HDL subclasses can be achieved by using typical anti-apo $A-I$ antibodies. (Asztalos et al., 2007). About 12 different apo $A-I$ containing HDL subclasses can be isolated by this method such as pre β(Pre- β_1 and Pre β_2), $\alpha(pre\alpha_1, pre\alpha_2, pre\alpha_3)$

on the basis of mobility and size of the particles (Asztalos and Schaefer, 2003 a, b). HDL can also separated, on the basis of protein component, into apo A-I containing particles with (LpA−I and LpA−II) and without apo A−II (LpA−I) by an electroimmuno-diffusion technique in agarose gel (Franceschini et. al., 2007).

Various HDL subclasses have also been isolated by nuclear magnetic resonance (NMR) method on the basis of characteristic lipid methyl group, NMR signal of each lipoprotein particles of a specific size (Otvos et al., 1992). Three HDL subclasses can be isolated by NMR signals are: large HDL with $8.8 - 13.0$ nm diameter, medium HDL with $8.2 - 8.8$ nm diameter and small HDL with $7.3 - 8.2$ nm diameter. All these techniques (NMR, GGE and 2D-gel electrophoresis) are being used in number of laboratory tests to determine HDL subclasses (Mora, 2009). But number and codification of HDL subdivisions is not consistent among different techniques. Further more if one technique is used for the measurement of concentration of HDL subclasses, the other method is being used to subclasses in relation to the total content, as a result, on the basis of physical properties of particles, HDL have been classified by a panel of experts and five HDL subclasses were described on the basis of integration of terminology from several methods. These subclasses includes small, very small, medium, large and very large HDLs (Rosenson et al., 2011). This classification may help to determine the co-relation between HDL subdivisions and cardiovascular risk along with the efficacy of HDL modifying drugs in near future.

2.5.13 HDL and Reverse Cholesterol Transport (RCT) Pathway

Athero-protective role of HDL against cardiovascular disease (CVD) is regulated by cholesterol efflux from tissues and by modulating inflammation. Movement of cholesterol from peripheral cells to the liver for removal from the body is facilitated by HDL (Oram & Heinecke, 2005). Reverse cholesterol transport (RCT) is the phenomena in which cholesterol is vanished from macrophages present in the

walls of blood vessels and this pathway is critically involved in the prevention of development of atherosclerotic plaque (Cuchel & Rader, 2006).

First of all liver secretes the apo $A-I$ to begins the life cycle of HDL and nascent discoidal lipid poor HDL particles are formed when this apo $A-I$ attaches with circulating cholesterol and phospholipids. In the sub endothelial macrophages and fibroblasts, cholesterol efflux is triggered by these immature HDL particles and through the interaction with $ATP-$ binding cassette transporter $A1(ABCA1)$ (Duong et al., 2006 & 2008). These HDL particles help in the storage of cholesterol in their core by esterification through lecithin-cholesterol acyltransferase (LCAT). Now two major types of spherical, mature HDL particles (HDL2 and HDL3) are formed which drop their cholesterol in the liver directly through scavenger receptor B-I (SR-BI) (Adorni et al., 2007; Zannis et al., 2006) in one way or indirectly deliver cholesterol to LDL or VLDL particles on the other way and liver takes up this cholesterol through LDL receptors (Mahley, 1988; Mahley et al., 2006). A specific HDL associated protein, cholesterol ester transfer protein (CETP) facilitates this shifting.

Finally cholesterol is removed from body through feces in the form of neutral steroids or bile acids and this phenomena of cholesterol removal was named as RCT (Lewis & Rader 2005; Azzam & Fessler, 2012). More ways of participation of apo A-I loaded HDL particles in the RCT and shifting of cholesterol from peripheral cells to the liver are introduced (Lund-Katz & Phillips, 2010) and a significant breakthrough is underway to understand the antioxidant and vasoprotective effects of these particles.

2.5.14 Anti-Inflammatory Effects of HDL

Atherosclerosis is an inflammatory disorder in which various immune cells and their products such as cytokines and chemokines continuously migrate and adhere into the walls of blood vessels (Ross, 1999). Inhibition of monocyte chemoattractant protein$-1(MCP-1)$ production and anti-inflammatory role of HDL have

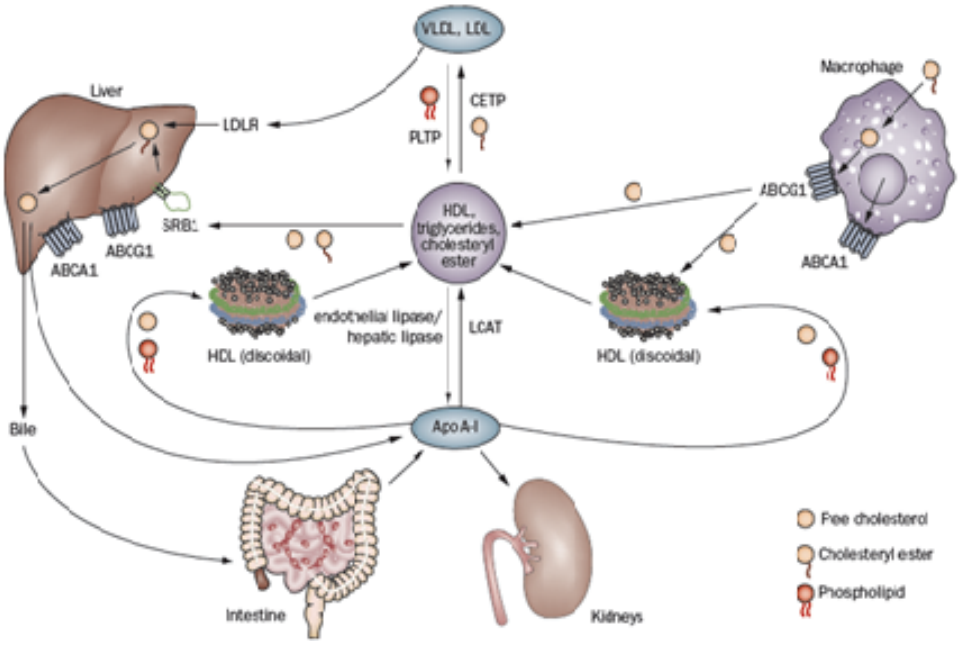

FIGURE 2.2: Lipid biosynthesis, storage and elimination (Navab et al., 2011).

been reported in murine vascular smooth muscles of aorta and human endothelial cells (Mackness et al., 2004, Tolle et al., 2008). Inhibition of expression of endothelial adhesion molecules i.e. vascular cells adhesion molecule-1 (VCAM-1), E-selectin & intercellular adhesion molecule$-1(ICAM-1)$ by HDL has also been demonstrated in an in$-$vitro study (Cockerill et al., 1995 & 1999; Baker et al., 1999) but in case of human aortic and coronary artery, these observations could not be repeated (Stannard et al., 2001; Zhang et al., 2002). However, some of these in-vitro results could be confirmed in some in-vivo studies (cockerill et al., 2001). As in a demonstration, infusion of reconstituted HDL (rHDL) and insertion of nonocclusive collar around carotid arteries in rabbits, inhibition of VCAM-1, ICAM-1 and MCP-1 produciton and leukocyte infilteration as well as stoppage of reactive oxygen species creation in the walls of artery is reported by Nicholls et al. (Nicholls et al., 2005).

2.5.15 Antioxidant Effects of HDL

Development of atherosclerotic lesions involves oxidation of LDL in the walls of arteries (Ross, 1999). HDL involves in the inhibition of mild oxidized LDL (ox LDL) in healthy persons because various antioxidant enzymes i.e LCAT, PON-I

and PAF-AH are associated with HDL. HDL is involved in detoxification of ox LDL (Garner et al., 1998) due to the activity of CETP which is involved in transference of hydroperoxides from oxLDL to HDL and thus oxidized HDL may remove from circulation. On the other hand, in subjects with CAD, HDL may lose this ability (Navab et al., 2000 & 2001).

2.5.16 Anti-apoptotic Effects of HDL

ox LDL, proinflammatory cytokines and unavailability of growth factors play triggering role in atherosclerosis represented by cell death as a result of endothelial injury. Suc et al observed that HDL, specifically its apo−AI content is involved in blocking of intracellular signaling in apoptosis and prevention of oxLDL induced apoptosis (Suc et al., 1997). It has been studied in demonstrations on endothelial cells incubation with HDL, that apoptosis induced by TFA, and by deficiency of growth factors, could be avoided (Nofer et al., 2001; Sugano et al., 2000).

2.5.17 Antithrombotic Effect of HDL

HDL also play a principle role in homeostasis in such a way that it is involved in regulation of platelet adherence by blocking platelet movement and assemblage (Cockerill et al., 1999; Nofer et al., 2010). In another way, it has been reported that expression of thrombin-induced tissue factor in human endothelial cells is inhibited by HDL (Viswam bharan et al., 2004) and two important anticoagulants such as protein C and protein S show their enhanced activities and activation of factor X is inhibited in the presence of HDL so that plasmatic coagulation is also influenced by HDL (Griffin et al., 1999).

2.6 Total Cholesterol (TC)

Total cholesterol is the total amount of cholesterol in the blood which includes low-density lipoprotein (LDL) and high-density lipoproteins (HDL).

2.6.1 Cholesterol and its Significance

Cholesterol is a fat like substance, found in the blood stream. It is major structural component of cellular membranes, myelin of neurons, steroid hormones and bile acids and play important role in physiological function of Hedgehog protein (Auderset et al., 2016). Cholesterol is the principle sterol in the living body which is needed to synthesize cell membranes and to regulate the fluidity of membranes across a range of temperatures (Lohe et al., 2010). It is also an important component of human brain as an adult human brain contains about 35 grams of cholesterol (Dietschy & Turley, 2001). So the human brain is considered as most cholesterol rich organ (Björkhem, & Meaney, 2004). Abnormalities related to cholesterol metabolism can cause structural and physiological abnormalities of central nervous system i.e Smith−Lemli−Opitz syndrome (DeBarber et al., 2011), Niemann−Pick type C disease (Madra & Sturley, 2010), Huntington disease (Block et al., 2010) & Alzheimer disease (Di Paolo & Kim, 2011). All these metabolic abnormalities in turn can lead to cause disturbances in other metabolic processes like cholesterol synthesis pathway, membrane receptors associated lipid transport in cells, lipoprotein accumulation and signaling molecules (Herz, 2009).

The normal mean serum cholesterol should be $118.4 \pm 32\ mg/dL$ but cholesterol level of individuals can be affected by more saturated fat content in diet, genetic inheritance and variety of metabolic defects i.e type II diabetes mellitus. Above or below the normal range of serum cholesterol level is critical. Changes like increase or decrease in risk factors such as of cholesterol level over time, have been reported in various demonstrations related to different population living in different regions (Capewell et al., 2000; Evans et al., 2001; Suh, 2001; Vartiainen et al., 2000).

Various disorders are linked with non-optimal cholesterol level (some are described above) especially elevated cholesterol level in the blood is more critical as being an accelerator or amplifier in case of atherosclerosis which leads into peripheral vascular disease, stroke and heart attack (Nseka 2005). Elevated cholesterol level has been proven as risk factor of CHD/CVD by various epidemiological studies and an inverse relationship between cholesterol level and CVD risk has been reported

in different populations in different regions of the world (Law & Wald, 1994; Law et al., 1994; Law et al., 2003).

2.6.2 Cholesterol as a Risk Factor of ASCVD

Increased prevalence of atherosclerosis and related cardiovascular diseases (CVD) have compelled the researchers to study causes and risk factors of their development. Atherosclerotic cardiovascular disease (ASCVD) and its pathophysiological representations like myocardial infarction and ischaemic stroke have been declared as the major cause of mortality throughout in all the populations. Multiple studies have been conducted to demonstrate the risk factor of increased cardiovascular events (Yusuf et al., 2004). Subendothelial cells play a vital role in the pickup of cholesterol from blood stream and development of atherosclerotic plaque. Cholesterol especially LDL cholesterol, as being approximately 60% of the total cholesterol content in the blood stream, is consumed by macrophages. In the case of elevated blood cholesterol level, macrophages take up cholesterol more than their ability to metabolize it and develop into foam cells which play important role in the atherosclerotic plaque development (Bronner et al., 1995; Gorelick et al., 1997; Warlow, 1998). Hypercholesterolemia have been declared as a leading cause of atherosclerosis and clinicians are commonly used cholesterol-lowering therapy to treat CVD (Lewington et al., 2008; Mills et al., 2008) but in most clinical demonstrations only about 30% reduction of risk related to CVD is achieved which enforces the researchers to take other risk factors into account (Baigent, 2010; Afilalo et al., 2007; Hirayama & Miida, 2012). Multiple lines of evidence have established that atherosclerotic plaque formation is not only depends upon the quantity but also the quality and characteristics of the lipoproteins in blood stream.(Rizzo & Berneis, 2006; Packard, 2006). A significant collection of evidences indicate that the ASCVD development and progression involves the activity of cholesterol enriched LDL and other lipoproteins having apolipoprotein B such as very low density lipoproteins (VLDL) along with remnants, intermediate density lipoproteins (IDL) and lipoprotein (a) Lp (a). (Goldstein & Brown, 2015). Most of these demonstrations have revealed that LDL is the principle cause of

atherosclerotic plaque formation while an inverse reciprocal relationship is found between HDL and atherosclerotic CVD risk (Krauss, 2010).

2.6.3 Metabolic Regulation and Excretion of Cholesterol

Most of the cholesterol content in the body is synthesized in the liver from various types of food, principally from saturated fats got from animal products and almost 25% of the total cholesterol formed in intestine. It may also be absorbed from food or reabsorbed from bile. Large lipid rich, apolipoprotein B 48 containing lipoprotein particles, the chylomicrons are responsible for the transportation of cholesterol from intestine to the liver. These intestinally derived chylomicrons take another apolipoprotein apo E from HDL in blood stream and partially dilapidated by the activity of lipoprotein lipase before entering into the liver by B/E receptors (Goldstein & Brown, 2009). Now released cholesterol along with de novo synthesized and hepatically derived cholesterol, is repackaged and take up triglycerides and phospholipids, become soluble in apo B 100 and released into the blood stream as VLDL. Lipoprotein lipase present in the walls of blood vessles remove its triglycerides and it becomes VLDL remnants after delipidation which in turn deformed into LDL. Apo E is responsible for the removal of most of delipidated VLDL through LDL rceptors present in the liver and shuttle back to HDL. Usually LDL receptors, found in almost all types of cells in the body take up LDL particles for their catabolism (Goldstein & Brown, 2009). Hence LDL act as vehicle for cholesterol transport throughout the body to synthesize cellular membranes and steroid hormones while HDL plays its role in reverse cholesterol transport and inhibition of LDL oxidation (Khera et al., 2011). Cholesterol is completely inert molecule and whether it is dietary in origin or synthesized de novo by liver or intestine, it can be eliminated from the body by three ways.

1. Liver convert the cholesterol into bile acid, which, along with phospholipids is removed into the bile and bile further reabsorbed or excreted with feces.(Fagerholm, 2008).

2. Very small amount of cholesterol is consumed in the synthesis of steroid hormones(Praseetha & Thampan, 2009).

3. It can also be disposed off via shedding of skin cells as well as of intestine (Simons & Gerl, 2010).

Regulation of cholesterol level in the cells is due to cholesterol synthesis pathway and LDL-receptor pathway because both these pathways are most sensitive to the presence of cholesterol. So free cholesterol level must be maintained in narrow range because its increased amount is dangerous for normal membrane working (Steck & Lange, 2010). Excessive amount of free cholesterol is esterified and accumulated as lipid droplets to stop its storage in cell membranes (Steck & Lange, 2010). Increased cholesterol efflux from peripheral tissue and transfer of cholesterol to the liver is the responsibility of HDL and lipid homeostasis can be adjusted by maintaining the cholesterol synthesis, ingestion, esterification and egestion through various downstream feedback mechanisms(Steck & Lange, 2010).

2.6.4 Cholesterol Biosynthesis Pathway

Cholesterol is well known sterol, having complex four ring structure and is used as precursor for steroid hormones and bile acids. Much of plasma cholesterol is in esterified form. In mammals, cells take cholesterol principally either by new synthesis from aetyl-CoA or through LDL-receptor mediated intake from outside of the cells (Brown & Goldstein, 1986). Most of the tissues, other than of liver, get cholesterol from de novo synthesis (Dietschy & Turley, 2001). Cholesterol biosynthetic pathway is strictly regulated by various feedback responses to maintain intracellular state (Goldstein & Brown, 1990) and this complex feedback mechanism is regulated by recently identified sterol sensors (Goldstein et al., 2006). Synthesis of cholesterol takes place through isoprenoid biosynthetic pathway (Waterham, 2006) which is started by acetyl (coA precursor. This precursor coenzyme is passed through six enzymatically controlled steps and finally produced isopentenyl- pyrophosphate, which is the basic isoprene unit of all isoprenoids. There are almost twenty enzymes are used to synthesize cholesterol (Waterham, 2006).

Formation of isopentenyle-pyrophosphate from acetyle CoA is the first step in the cholesterol biosynthetic pathway. Isopentenyl pyrophosphate formation is a complex set of reactions initiated with the formation of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) from acetyl-CoA. HMG-CoA is reduced, by the catalytic activity of $HMG-CoA$ reductase (HMGR) by using two molecules of NADPH, into mevalonate.

Cholesterol and isoprenoids formed through this most committed step of mevalonate pathway are used to produce dolicol, tRNA, heme A, ubiquinone and some other proteins like Ras and Rabs (Goldstein & Borown 1990). Molecular analysis of HMG-CoA reductase showed that the crystalline catalytic site containing domain of the enzyme forms tight tetramers (Istwan and Deisenhofer, 2000). Mevalonate is transformed into 3-isopentenyl pyrophosphate which is decarboxylated into isopentynyl pyrophosphate, an activated isoprene unit.

In the next step squalene is synthesized by the isomerization and condensation of six molecules of isopentynyl pyrophosphate through various intermediate steps. This synthesis is mediated by squalene-synthase enzyme which is mostly regulated by the concentration of cholesterol in the cell and hence plays a determinative role in directing the farnesyl-pyrophosphate to enter into the sterol or non-sterol directions of the pathway (Tansey & Shechter, 2000).

Squalence in converted into cyclized structure lanosterol which is subsequently transformed into cholesterol in the membrane of endophasmic reticulum. Although all the metabolic intermediates and enzymatically controlled steps of the reaction are well known but still need to elaborate the sequence of reactions between lanosterol and cholesterol (Waterham & Wanders, 2000).

2.7 Triglycerides (TG)

Triglycerides (TG) also called Triacylglycerol's or Triacylglcerides (TAG), are triesters composed of glycerol (alcohol) and three fatty acids (Svennerholm, 1977). In triglycerides carboxyl ($-COOH$) groups of fatty acids are joined with the hydroxyl ($HO-$) groups of glycerol to from ester linkages.

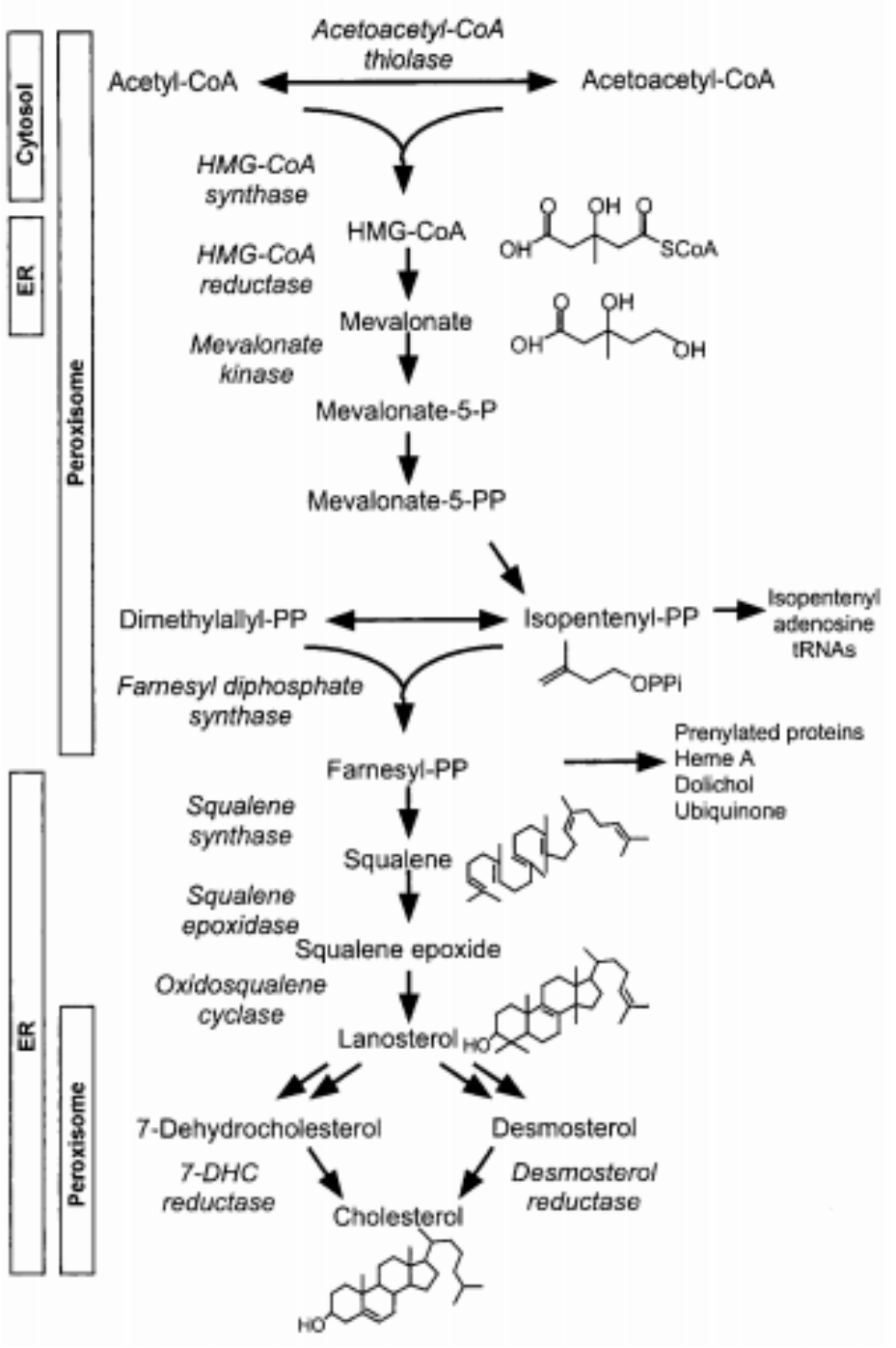

FIGURE 2.3: Cholesterol biosynthetic pathway (Olivier & Krisans, 2000).

Serum triglycerides (TGs) collectively form a most variable component among all the lipids which are being measured in routine clinical practice. Normally fasting triglycerides should be less than 1.37 mmol/L but it can be reached up to 100 or more than $100 mmol/L$ in some cases. Elevated triglycerides level is known as hypertriglyceridemia. Although the hypertriglyceridemia can be classified in multiple ways but more common and useful classification scheme includes: (1)-mild to moderate hypertriglyceridemia with >1.7−<10.0 mmol/L (TGs) and (2)–Sever hypertriglyceridemia with >10.0 mmol/ L TGs (Hegele et al., 2014).

2.7.1 Causes of Hyper Triglyceridemia

Most cases of hypertiglyceridemia are complex multifactorial because of combined effect of polygenes and environmental factors. But relative effect of polygenic aetiology and environmental factors can vary person to person such as in some individuals effect of environment may dominate and in others genic role can be major contributor. So effect of multiple genes is the major barrier for genetic investigation of polygenic hypertriglyceridemia. Mild-moderately high hypertriglyceridemia is a typical multifactorial disorder and is the consequence of combined effect of more than 30 gene variants along with life style factors such as obesity (Hegele et al., 2014), more alcohol consumption and unmanaged diabetes, effect of drugs etc. More important, single variation among variants of these genes has no significant effect on triglycerides level but cumulative burden of these variants can cause hypertriglyceridemia (Hegele et al., 2014). As compare to normal controls, in individuals having hypertriglyceridemia, a score of these variants at 32 gene loci has been constructed (Johansen et al., 2012).

Monogenic hypertriglyceridemia is a rare, autosomal, recessive disorder with severe clinical manifestations in early childhood caused by mutations in six different genes such as LPL, APOC2, APO45, LMF1, GPIHBP1 and GPD1. Most common forms of monogenic hypertriglyceridemia are caused by biallelic mutations of LPL gene. However, it can also be occasionally caused by mutations in apo E gene.

2.7.2 Remnant Cholesterol

Remnant cholesterol is the fraction of cholesterol formed by triglyceride-rich lipoproteins. Multiple techniques are employed to detect the remnants and remnant cholesterol content can be measured as:

$$Remant\ cholesterol = Total\ cholesterol - HDL\ cholesterol - LDL\ cholesterol$$

(Varbo et al., 2013 a; Varbo et al., 2013b; Jørgensen et al., 2012; Nordestgaard et al., 2007; Nordestgaard, 2016).

This measurement method has an advantage over other's because it can be calculated with the help of results obtained by non-fasting lipid profiling without any additional expenditure and without more ultracentrifugation methods application. Elevated remnant cholesterol is more likely to be an increased risk factor of CVD (Varbo et al., 2014; Varbo et al., 2013 a; Varbo et al., 2013 b; Jørgensen et al., 2012).

2.7.3 Clinical Effects of Hypertriglyceridemia

Severely elevated level of triglycerides can be a cause of actue pancreatits (Whitcomb, 2006). Although the mechanisms involved behind the association of elevated triglyceride level and pancreatitis are not clear, however, it may cause disruption of capillary network in pancreas or tissue necrosis in pancreas which may leads into disruption of pancreatice enzymes activation and secretion.

Mild-moderately high hypertriglyceridemia is considered as a risk factor for cardiovascular disease (CVD). (Varbo et al., 2013 a; Varbo et al., 2014). Although, the exact mechanism lies behind the association of elevated triglyceride rich lipoprotein level and atherosclerosis is still debatable but these cholesterol containing TGRLs are more likely to be a principle cause of atherosclerosis (Varbo et al., 2013 a; Varbo et al., 2013b; Varbo et al., 2014; Jørgensen et al., 2012).The relative risk of CVD, although depends upon sex and race, may increase with 1.0 $mmol/L$ increase of triglycerides, between the range of 1.14 to 1.80 along with the adjustment of factors like HDL-c (Chapman et al., 2011).

2.7.4 Triglycerides and Cardiovascular Diseases (CVD) Epidemiology

Elevated levels of plasma triglycerides, more accurately called triglyceride-rich lipoproteins, as a cardiovascular risk factor have long been a focus of discussion. Many individuals having chylomicronaemia syndrome and elevated triglyceride level do not have atherosclerosis and (CVD) (Brunzell, 2001) as compare to those having distinctly elevated cholesterol level as the cases of hypercholesterolemia (Chapman et al., 2011). This observation initially generated a controversy about the association of triglycerides and CVD, However, the possible reason behind this observation is the fact that in case of severe hypertriglyceridemia with triglycerides level of $> 50\ mmol/L$, the TGLs become much larger and cannot enter into intima of blood vessels, so cannot be a cause of atherosclerotic development and progression (Nordestgaard et al., 1988; Nordestgaard & Zilversmit, 1988), while in the case of mild-moderately high level of triglycerides with $2 - 10\ mmol/L$, the lipoproteins are of much smaller size and can easily be entered into intima of blood vessel can be potential cause of atherosclerotic development (Shaikh et al., 1991; Nordestgaard et al., 1995).

Triglycerides have the ability to be broken down in most of the tissues, but cholesterol cannot be degenerated in most cells, So elevated triglyceride level is marker for increased remnants loaded with cholesterol, which after get entry into the wall of vessel, cause low-grade inflammation, development of atherosclerotic plaques and consequently progression of CVD. Different meta- analyses conducted in 199Os concluded that elevated fasting and non-fasting triglyceride level were linked with increased risk of coronary heart disease (CHD), although HDL-c level had already been adjusted (Hokanson & Austin, 1996). Studies based upon Copenhagen City Heart Study and the Women's Health Study proposed that elevated non-fasting triglycerides levels were tightly linked with increased risk of myocardial infarction, ischaemic heart disease, ischaemic stroke and mortality due to coronary events (Nordestgaard et al., 2007; Bansal et al., 2007; Freiberg et al., 2008). Data recorded by Emerging Risk Factor Collaboration in 2009 (Sarwar et al., 2009),

based on 68 long term, prospective studies including 302430 individuals and 12785 coronary incidents, concluded that elevated fasting and non-fasting triglycerides were the major cause of increased risk of CHD.

Genome-wide association studies (GWAS) have also proposed that elevated triglycerides are associated with CVD (Teslovich et al., 2010; Do R et al., 2013). It has been recommended in another study that variants linked with triglyceride elevation were the cause of CVD, even maintaining low HDL level (Do R, et al., 2013), which also proved that there is no association between genetically low HDL-c and CVD.

So being a part of triglyceride-rich lipoprotein, it can be a potent cause of atherosclerotic development and CVD instead of elevated triglycerides. Unlike triglycerides, the cholesterol deposits in the macrophage foam cells in arterial intima which form atherosclerotic plaques and remnant lipoproteins just as LDL have potential to enter into the intima of blood vessels (Shaikh et al., 1991; Nordestgaard et al.,1992) while chylomicrons does not enter into arterial intima because of having large size (Nordestgaard et al., 1988; Nordestgaard & Zilversmit, 1988). These remnants, being having larger size, can be entangled by LDL in arterial intima or may attach to proteoglycanes outside the cells (Goldberg et al., 2011; Shaikh et al., 1991; Nordestgaard et al., 1995; Proctor et al., 2002). Free fatty acids and monoacylglycerols like substances are liberated when lipoprotein lipase (LPL) attacks on the surface of remnants present in arterial intima or at the vascular endothelium (Goldberg et al., 2011), which consequently ends into local injury and inflammation (Goldberg et al., 2011; Saraswathi & Hasty, 2006; Rutledge et al., 2000). Unlike LDL, these remnants also involve in foam cell formation by interacting with macrophages (Goldberg et al., 2011).

Chapter 3

Material and Method

3.1 Study Design

A cross-sectional study was conducted including 250 subjects ranging from 23 to 88 years age in span of three months from start of the study which consisted of 133 males and 117 females. All patients were residents of Mirpur Azad Kashmir and all the Subjects were informed about procedure along with risk and benefit of study and were included in the study only if voluntarily consented to participate in the study after due explanation of the purpose of the study was done.

3.2 Sample Size Selection

Sample was selected through simple random sampling technique and on the basis of previous work and literature review.

3.3 Inclusion and Exclusion Criteria

Those subjects were included in this study which were not using any type of lipid lowering drug like statin etc. and were not the patients of CVD and thyroid disorder move. Moreover participants had no pregnancy no smoking and diabetic history. Although some of them were the patients of high blood pressure.

3.4 Blood Specimen Collection

5ml of blood samples were collected from patients, 1 ml blood was immediately put into EDTA bottle and the remaining 4 ml of blood was put in a plain bottle and both were labeled, capped and the EDTA sample was gently mixed with the anticoagulant and used for the blood groups determination while samples in the plain bottles were allowed to clot before centrifuging for five minutes at 3400 revolutions/minute and the serum were separated immediately into plain sterile sample bottles with Pasteur pipette and used for Lipid profile analyses. We collected demographic and clinical data including age, sex, ABO blood type, Rh factor, serum levels of TC, LDL-c, high-density lipoprotein cholesterol (HDL-c) and triglycerides. Blood samples for cholesterol and triglyceride measurements were collected after overnight fasting.

3.5 Biochemical Determinations

3.5.1 Assessment of Serum Total Cholesterol by CHOD-PAP Enzymatic Photometric Method

The enzymatic procedure used for total cholesterol determination in serum based upon the enzymatic endpoint method published by Allain (Allain et al. 1974) and Meiattini et al. in 1978).

Commercial kit RX MONZA by Randox Laboratories(U.K) was used for total cholesterol determination.The assay was performed using a Clinical chemistry Analyzer model: Spinlab, GMDN code (56679) for analysis.

Cholesterol is determined enzymatically in serum in a series of interdependent reactions that hydrolyze cholesterol esters and oxidize the $3-OH$ group of cholesterol. Quantity of reaction byproducts, H_2O_2 is calculated in a peroxidase catalyzed reaction that produces a color. The indicator quinoneimine is formed from

hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. Absorbance is measured at 546 nm. The color intensity is proportional to cholesterol concentration. The reaction sequence is as follows:

Cholesterol ester $+H_2O$ $\underline{cholesterol\ esterase}$ cholesterol + fatty acid,

Cholesterol + O_2 $\underline{cholesterol\ oxidase}$ cholesterol-3-one $+H_2O_2$,

$2H_2O_2$ + 4-aminoantipyrine $+phenol$ $\underline{Peroxidase}$ $quinoneimine + 4H_2O$.

3.5.2 Triglycerides Quantitative Determination by Enzymatic Glycerol Phosphate Oxidase/Peroxidase Method

Triglycerides were measured enzymatically (Bucolo and David, 1973; Fossati and Prencipe, 1982; Grossman, 1976) in serum by employing a series of interdependent reactions in which triglycerides are hydrolyzed to generate glycerol. Glycerol is then oxidized by the action of glycerol oxidase and H_2O_2, one of the reaction products, is calculated as described above for cholesterol. The indicator quinone is produced from hydrogen peroxide and 4-aminophenazone in the presence of p-chlorophenol and peroxidase. Absorbance is measured at 546 nm (Bucolo and David, 1973; Fossati and Prencipe, 1982; Grossman, 1976). The reaction sequence is as follows.

Triglycerides + $3H_2O$ $\underline{lipoprotein - lipase}$ glycerol + fatty acids,

Glycerol + ATP $\underline{glycerokinase}$ glycerol-3-phosphate + ADP,

Glycerol-3-phosphate + O_2 $\underline{glycerophosphate - oxidase}$ dihydroxyacetone phosphate $+H_2O_2$,

H_2O_2 + 4-aminophenazone + p-chlorophenol $\underline{peroxidase}$ $Quinone + 2H_2O$.

3.5.3 Assessment of HDL by Homogenous Enzymatic Direct Assay

3.5.3.1 Assay Procedure

HDL-c was measured according to the CHOD-PAP method by photometric system (Lopes-Virella et al. 1977). 200ul serum was mixed with 500ul of HDL precipitation reagent (Echoline, Merck manufactured by DiaSys GmbH Germany) and kept for 15 minutes at room temperature. Then centrifuged it for 20 minutes at 2500 g. After about an hour 100ul of clear supernatant was blended with 1000ul of cholesterol reagent for cholesterol determination and incubated for 5 minutes at $37c^{\circ}$. Absorbance of the sample against the reagent blank value was measured by Spinlab autoanalyzer after half an hour at 546 nm wavelength with 1 cm optical path at $37c^{\circ}$.

3.5.3.2 REAGENT

Precipitant was ready to use with concentration as follow

Phosphotungstic acid :1.4 mmol/L Magnesium chloride :8.6 mmol/L

3.5.3.3 Principle

The basic methodology is described as follows,

Chylomicrons, VLDL and LDL are precipitated by put in phosphotungstic acid and magnesium chloride ions to the sample. HDL accumulated in supernatant after centrifugation. Their cholesterol content was assessed enzymatically using Echoline cholesterol reagent.

3.5.4 Assessment of LDL by Homogenous Enzymatic Direct Assay

3.5.4.1 Assay Procedure

LDL cholesterol was measured by applying CHOD-PAP method through photometric system (Nakamura, 1997). 100ul serum was mixed with 1000ul of LDL precipitation reagent (Echoline,Merck manufactured by DiaSys GmbH Germany) and kept at room temperature for 15 minutes. Then centrifuged it for 20 minutes at 2500 g. After about an hour 100ul of clear supernatant was blended with 1000ul of cholesterol reagent for cholesterol determination and kept for 5 minutes at $37c^{\circ}$. Absorbance of the sample against the reagent blank value was assessed by Spinlab autoanalyzer after half an hour at 546 nm wavelength with 1 cm optical path at $37c^{\circ}$.

3.5.4.2 REAGENT

Precipitant was ready to use with concentration as follow:

Heparin :100000 ul Sodium citrate : 64 mmol/L

3.5.4.3 Principle

The basic methodology is described as follows, Low density lipoproteins (LDL) are precipitated by adding heparin. High density lipoproteins (HDL) and very low density lipoproteins (VLDL) are confined in the supernatant after centrifugation and assessed enzymatically by the CHOD-PAP method. The LDL concentration is determined as the difference of total cholesterol and cholesterol in the supernatant.

LDL-c = Total Cholesterol - Cholesterol in supernatant

3.6 ABO Blood Grouping

ABO and Rh blood status was assessed by using standard tile techniques (Mannessier, 1992 and Betremieux et al. 1994) with appropriate negative and positive

controls using one drop of whole blood mixed with one drop of appropriate antisera and rocked gently for agglutination. Quality monoclonal blood grouping reagents manufactured by "Rapid Labs Limited" (United Kingdom) were used.

3.7 Statistical Analysis

The data produced at the end of this study was analysed by using Excel and statistical package for the social sciences (SPSS) version 20 to carried out mean values and standard deviation. While $\alpha = 0.05$ was considered as significance level and value of p less than 0.05 ($p < 0.05$). The significance difference among various blood phenotypes was measured by two way Analysis of Variance (ANOVA) and where required by student "t" test.

Chapter 4

Results and Discussion

4.1 Sample Collection

In this study, the blood samples are collected from 250 randomly selected subjects of lipids homeostasis problem who have visited District Headquarter Hospital Mirpur Azad Kashmir during June 2018 to Aug 2018. Among these patients, 117(46.80) were Female and 133(53.20) were male whereas the mean age of the patients was found 50.90 ± 13.09 years (Figure 4.1).

4.2 Blood Group Antigens

The blood samples of 250 patients were processed for identification of Antigens. The standard tile technique method was used for this purpose. As a result of ABO blood grouping method, patients were found to belong all four categories i.e. blood group A ($n = 62$), blood group B ($n = 78$), blood group AB ($n = 26$) and blood group O ($n = 84$). Therefore, the ratio of blood group O was found highest and the ratio of blood group AB is found lowest among the 250 patients (Fig. 4.2).

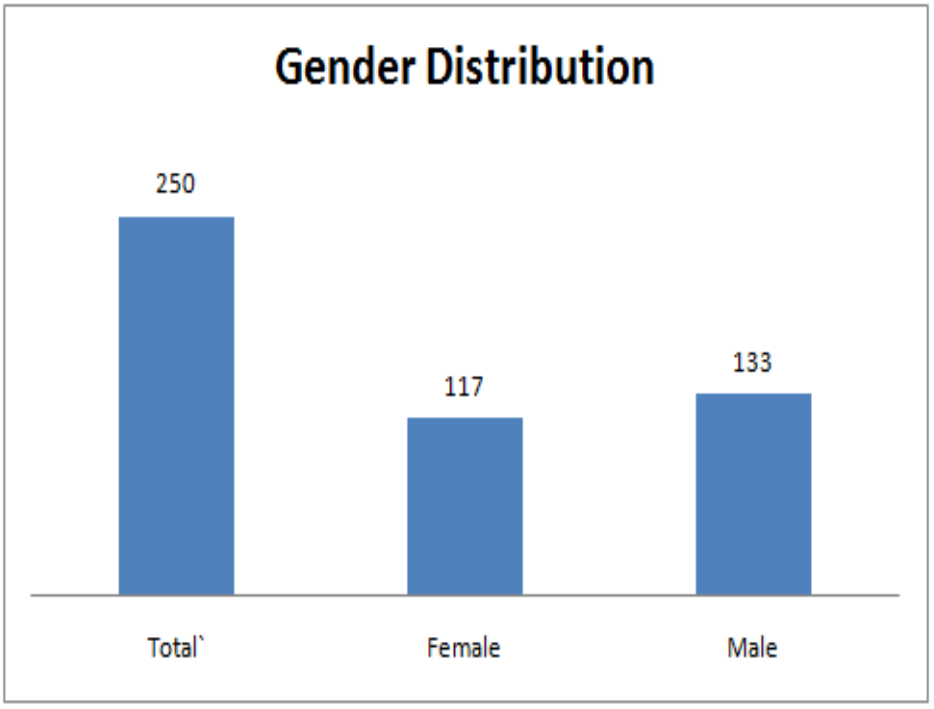

FIGURE 4.1: Gender Distribution of Patients

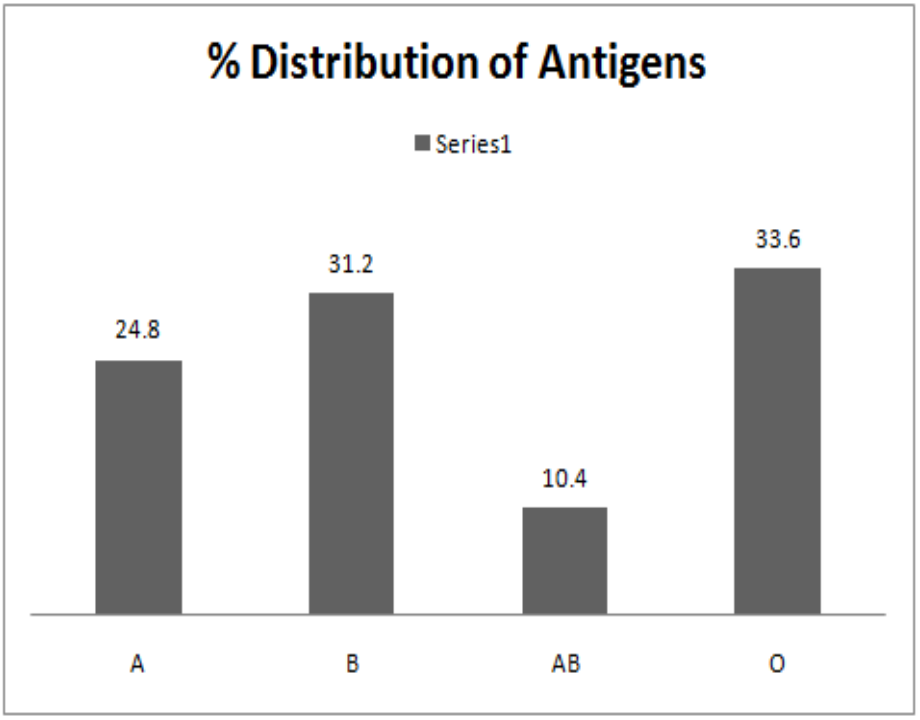

FIGURE 4.2: Distribution of ABO Blood Group Antigens

ABO blood grouping test showed that the all four type of blood groups were distributed among male and female both type of patients. The distribution of blood group antigens along with gender distribution is given in **Table: 4.1**.

TABLE 4.1: Demographic Distribution of Blood Group Antigens in the Population.

SEX	Blood group A	Blood group B	Blood group AB	Blood Group O
Male	34(24.82)%	43(31.39)%	15(10.95)%	45(32.85)%
Female	28(24.78)%	35(30.97)%	11(9.73)%	3(34.5)%
TOTAL	62(24.8)%	78(31.20)%	26(10.40)%	84(33.60)%

4.3 Lipid Profile of Male Subjects

The collected blood samples of 133 male patients were processed for lipid profile by using standard protocols. Total cholesterol (TC), Low density lipoproteins (LDL), High density lipoproteins (HDL) and Triglycerides (TG) were measured and recorded for data analysis.

4.3.1 Lipid Profile of Blood group A (Male)

Among total 62 patients having blood group A, 34 patients were male. The mean age of male patients having blood group A was found 49.93 ± 13.93 and mean TC level was observed 191.74 ± 52.83 in **Table: 4.2**.

TABLE 4.2: Summary statistics of Lipid profile blood group A (Male).

	Age	TC	HDL	LDL	TG
Mean	49.94	191.74	41.59	91.85	228.76
Standard Error	2.39	9.06	0.51	3.42	40.0
Standard Deviation	13.93	52.83	2.95	19.96	237.81
Sample Variance	194.12	2791.23	8.67	398.37	56555.46
Kurtosis	0.25	1.79	-0.64	-0.86	18.87
Skewness	0.62	-0.79	-0.32	-0.13	3.99
Count	34	34	34	34	34
Confidence Level(95.0%)	4.86	18.43	1.03	6.96	82.98

4.3.2 Lipid Profile of Blood Group B (Male)

In this study, 43 patients were found belonging to blood groups B, among total 78 male patients with blood group B. The mean age of male patients having blood group B was found 53.33 ± 15.40 and mean TC level was observed 199.95 ± 46.73 in **Table: 4.3**.

TABLE 4.3: Summary statistics of Lipid profile Blood Group B (Male).

	Age	TC	HDL	LDL	TG
Mean	53.33	199.95	41.88	88.44	181.42
Standard Error	2.35	7.13	0.47	2.59	13.58
Standard Deviation	15.40	46.73	3.11	16.95	89.07
Sample Variance	237.03	2183.95	9.68	287.35	7933.01
Kurtosis	-0.83	1.83	-1.12	-0.87	0.27
Skewness	0.23	0.99	0.00	0.26	0.81
Count	43	43	43	43	43
Confidence Level (95.0%)	4.74	14.38	0.96	5.22	27.41

4.3.3 Lipid Profile of Blood Group AB (Male)

ABO blood grouping test revealed 15 male patients having blood group AB. The mean age of male patients having blood group AB was found 51.27 ± 10.42 and mean TC level was observed 184.67 ± 31.01 whereas Triglycerides TG was observed 162.20 ± 90.06 in **Table: 4.4**.

TABLE 4.4: Summary statistics of Lipid profile blood group AB (Male).

	Age	TC	HDL	LDL	TG
Mean	51.27	184.67	41.47	84.13	162.20
Standard Error	2.69	8.01	0.79	3.41	23.25
Standard Deviation	10.42	31.01	3.04	13.22	90.06
Sample Variance	108.64	961.38	9.27	174.84	8110.31
Kurtosis	1.40	-0.31	-1.34	4.74	7.91
Skewness	-0.17	-0.33	-0.02	1.45	2.56
Count	15	15	15	15	15
Confidence Level(95.0%)	5.77	17.17	1.69	7.32	49.87

4.3.4 Lipid Profile of Blood Group O (Male)

The ratio of blood group O was observed higher in male patients. 45 male patients have been found with blood group O. The mean age of male patients having blood group O was found 47.91 ± 12.16 and mean TC level was observed 187.33 ± 37.10 in **Table: 4.5**.

TABLE 4.5: Summary statistics of Lipid profile blood group O (Male).

	Age	TC	HDL	LDL	TG
Mean	47.91	187.33	41.78	86.67	188.62
Standard Error	1.81	5.53	0.40	2.49	14.73
Standard Deviation	12.16	37.10	2.68	16.68	98.79
Sample Variance	147.95	1376.32	7.18	278.32	9759.51
Kurtosis	0.31	-0.87	-1.07	-1.27	0.06
Skewness	0.41	-0.39	-0.05	-0.03	1.01
Count	45	45	45	45	45
Confidence Level(95.0%)	3.65	11.15	0.80	5.01	29.68

4.4 Lipid Profile of Female Subjects

Among 250 patients, 117 were female and their blood samples were processed through standard protocols to obtain their lipid profile details. These 117 female patients were categorized into four groups according to their respective blood group.

4.4.1 Lipid Profile of Blood Group A (Female)

In this study, 28 female patients were found to have blood group A (Appendix-V). The mean age of female patients with blood group A was 52.50 ± 11.3. The mean value of TC, HDL, LDL and TG was observed as 206.61 ± 64.35, 40.93 ± 2.81, 93.82 ± 28.44 and 212.71 ± 229.20 respectively in **Table: 4.6**.

TABLE 4.6: Summary statistics of Lipid profile blood group A (Female).

	Age	TC	HDL	LDL	TG
Mean	52.5	206.61	40.93	93.82	212.71
Standard Error	2.14	12.16	0.53	5.37	43.31
Standard Deviation	11.31	64.35	2.81	28.44	229.20
Sample Variance	127.89	4141.43	7.92	808.82	52532.73
Kurtosis	-0.18	6.73	-1.19	5.22	18.84
Skewness	-0.20	1.77	0.19	1.38	4.12
Count	28	28	28	28	28
Confidence Level(95.0%)	4.39	24.95	1.09	11.03	88.87

4.4.2 Lipid Profile of Blood Group B (Female)

There were 35 female patients having blood group B. The mean age of these patients was observed as 51.29 ± 13.09. The mean value of TC, HDL, LDL and TG was found as 193.17 ± 47.41,41.31 ± 2.63, 85.66 ± 20.19 and 183.51 ± 132.11 respectively in **Table: 4.7**.

TABLE 4.7: Summary statistics of Lipid profile blood group B (Female).

	Age	TC	HDL	LDL	TG
Mean	51.29	193.17	41.31	85.66	183.51
Standard Error	2.21	8.01	0.44	3.41	22.33
Standard Deviation	13.09	47.41	2.63	20.19	132.11
Sample Variance	171.45	2247.85	6.93	407.64	17452.32
Kurtosis	-0.14	-0.38	-0.68	-0.42	7.03
Skewness	-0.49	0.53	0.24	0.14	2.42
Count	35	35	35	35	35
Confidence Level(95.0%)	4.50	16.29	0.90	6.94	45.38

4.4.3 Lipid Profile of Blood Group AB (Female)

In this study, the ratio of female patients with blood group AB was lower as compared to other blood groups in female patients. The mean age of the patients was observed as 51.45 ± 15.06. The mean value of TC, HDL, LDL and TG was found as210.0 ± 36.13, 42.18 ± 2.68, 94.73 ± 15.07 and 212.64 ± 113.24 respectively in **Table: 4.8**.

TABLE 4.8: Summary statistics of Lipid profile blood group AB (Female).

	Age	TC	HDL	LDL	TG
Mean	51.45	210	42.18	94.73	212.64
Standard Error	4.54	10.89	0.81	4.54	34.14
Standard Deviation	15.06	36.13	2.68	15.07	113.24
Sample Variance	226.87	1305.40	7.16	227.02	12824.25
Kurtosis	1.21	-0.30	-0.18	0.91	3.72
Skewness	1.04	0.56	-0.01	0.54	1.72
Count	11	11	11	11	11
Confidence Level(95.0%)	10.12	24.27	1.80	10.12	76.08

4.4.4 Lipid Profile of Blood Group O (Female)

The ratio of female patients with blood group AB was highest among female patients of all types of blood groups. The mean age of the female patients with blood group O was observed as 50.77 ± 12.57 whereas the mean value of TC, HDL, LDL and TG was found as 201.28 ± 44.74, 43.0 ± 7.03, 86.23 ± 14.82 and177.0 ± 162.30 respectively in **Table: 4.9**.

TABLE 4.9: Summary statistics of Lipid profile blood group O (Female).

	Age	TC	HDL	LDL	TG
Mean	50.77	201.28	43	86.23	177
Standard Error	2.01	7.16	1.13	2.37	25.99
Standard Deviation	12.57	44.74	7.03	14.82	162.30
Sample Variance	158.08	2001.63	49.37	219.66	26342.74
Kurtosis	-0.32	0.08	29.19	-0.97	25.62
Skewness	0.37	0.36	5.05	0.12	4.70
Count	39	39	39	39	39
Confidence Level (95.0%)	4.08	14.50	2.28	4.80	52.61

4.5 Comparison Between Male and Female Lipid Profile

Below Table (4.10) represents the mean values of Lipid profile among different ABO blood phenotypes of unselected subjects in Mirpur Azad Kashmir. The mean values of age these persons belong to four different blood phenotypes were lying

between 49.24 ± 12.36 years and 52.41 ± 14.35 years. There was no significant difference ($F = 0.809, p = 0.489, p > 0.05$) among mean ages of these blood phenotypes. Mean value of serum total cholesterol (TC) was reported higher in A blood type ($198.45 \pm 58.299\ mg/dl$) and lower in O blood types ($193.81 \pm 41.17\ mg/dl$)as compared to other blood phenotypes but the difference with the other blood types was not significant ($F = 0.12, p = 0.944, p > 0.05$). Mean value of HDL-cholestrol in serum was higher in O blood phenotype ($42.35 \pm 5.17\ mg/dl$) and lower in blood type A ($41.29 \pm 2.88\ mg/dl$)as compared to other blood types but there was no significant difference among values of studied blood types ($F = 0.993; p = 0.396; p > 0.05$). Mean values of LDL-cholesterol in serum was once again higher in blood type A ($198.45 \pm 58.299\ mg/dl$) and power in O blood type ($86.46 \pm 15.75 mg/dl$) as compared to other blood phenotypes but the difference was insignificant ($F = 1.502, p = 0.214, p > 0.05$). The mean value of serum triglyceride level was also higher in A blood type ($221.52 \pm 232.19\ mg/dl$) and lower in B blood type ($182.36 \pm 109.70\ mg/dl$) with no significant difference with other blood groups ($F = 0.965, p = 0.409, p > 0.05$).

TABLE 4.10: Lipid profile among different ABO blood groups.

Parameters	Blood Group A	Blood Group B	Blood group AB	Blood group O	F-value	P-value
AGE	51.10 ± 12.777	52.41 ± 14.35	51.35 ± 12.312	49.24 ± 12.36	0.8093	0.4898
TC	198.45 ± 58.299	196.91 ± 46.854	195.38 ± 34.977	193.81 ± 41.17	0.127	0.944
HDL	41.29 ± 2.882	41.63 ± 2.901	41.77 ± 2.860	42.35 ± 5.175	0.9935	0.3965
LDL	92.74 ± 23.968	87.19 ± 18.403	88.62 ± 14.737	86.46 ± 15.752	1.5029	0.2144
TG	221.52 ± 232.190	182.36 ± 109.70	183.54 ± 101.573	183.23 ± 131.408	0.9653	0.409

Table **Table: 4.11** shows the mean serum lipid profile of blood group **A** subjects between male and female. The male blood group **A** subjects had a non significant ($p > 0.05$) lower mean total cholesterol (191.7 ± 52.8 vs 206.6 ± 64.3) and low density lipoproteins (91.85 ± 19.95 vs 93.8 ± 28.4) levels as compare to female blood group **A** subjects while non-significant ($p > 0.05$) higher levels of high density lipoproteins (41.5 ± 2.9 vs 40.9 ± 2.8)and serum triglycerides (228.76 ± 237.8 vs 212.7 ± 229.2) in males as compared to female **A** blood group subjects were observed.

TABLE 4.11: Lipid Profile of Male and Female blood group A subject.

Parameters	Male(n=34)	Female(n=28)	t-value	p-value
TC (mg/dl)	191.7 ± 52.8	206.6 ± 64.3	0.165	0.869
HDL (mg/dl)	41.5 ± 2.9	40.9 ± 2.8	0.186	0.853
LDL (mg/dl)	91.85 ± 19.95	93.8 ± 28.4	0.379	0.706
TG (mg/dl)	228.76 ± 237.8	212.7 ± 229.2	0.394	0.694

Table: 4.12 shows the mean serum lipid profile of blood group **B** subjects between male and female. Male blood group **B** subjects had non significant ($p > 0.05$) higher mean total cholesterol (199.9±46.7 vs 193.1±47.4), high-density lipoprotein (41.8 ± 3.11 vs 41.3 ± 2.6) and low-density lipoprotein (88.4 ± 16.9 vs 85.6 ± 20.1) levels as compared to female subjects of **B** blood group while non significant ($p > 0.05$) low level of serum triglycerides was observed in males (181.4 ± 89.0 vs 183 ± 132.1) as compare to female **B** blood group subjects.

TABLE 4.12: Lipid Profile of Male and Female Blood Group B Subject

Parameters	Male($n = 43$)	Female($n = 35$)	t-value	p-value
TC(mg/dl)	199.9 ± 46.7	193.1 ± 47.4	0.264	0.792
HDL(mg/dl)	41.8 ± 3.11	41.3 ± 2.6	0.192	0.848
LDL(mg/dl)	88.4 ± 16.9	85.6 ± 20.1	0.258	0.769
TG(mg/dl)	181.4 ± 89.0	183.5 ± 132.1	0.468	0.641

Table: 4.13 shows that the mean serum lipid profile of blood group **AB** subjects between male and female. Male blood group AB subjects had non-significant lower ($p > 0.05$) mean total cholesterol (184.6 ± 31 vs 210 ± 36.1), high density lipoprotein (41.46 ± 3.04 vs 42.18 ± 2.67),low density lipoproteins (84.13 ± 13.22 vs 94.72 ± 15.06) and serum triglycerides (162.2 ± 90.05 vs 212.63 ± 113.24) levels as compared to female blood group **AB** subjects.

TABLE 4.13: Lipid Profile of Male and Female blood group AB subject.

Parameters	Male($n = 15$)	Female($n = 11$)	t-value	p-value
TC(mg/dl)	184.6 ± 31	210 ± 36.1	0.037	0.970
HDL(mg/dl)	41.46 ± 3.04	42.18 ± 2.67	0.265	0.793
LDL(mg/dl)	84.13 ± 13.22	94.72 ± 15.06	0.038	0.970
TG(mg/dl)	162.2 ± 90.05	212.63 ± 113.24	0.118	0.907

Table: 4.14 shows that mean serum lipid profile of blood group **O** subjects between male and female. Male blood group **O** subjects had non-significant lower($p > 0.05$) mean total cholesterol (187.33 ± 37.0 vs 201.28 ± 44.73) and high-density lipoproteins (41.77 ± 2.67 vs 43.0 ± 7.024) levels as compared to female blood group **O** subjects while non-significant higher levels of low density lipoproteins (86.66 ± 16.68 vs 86.23 ± 14.82) and serum triglycerides (188.62 ± 98.79 vs 177.0 ± 162) were observed in males as compare to female blood group **O** subjects.

TABLE 4.14: Lipid Profile of Male and Female blood group O subject.

Parameters	Male($n = 45$)	Female($n = 39$)	t-value	p-value
TC(mg/dl)	187.33 ± 37.0	201.28 ± 44.73	0.063	0.949
HDL(mg/dl)	41.77 ± 2.67	43.0 ± 7.02	0.155	0.877
LDL(mg/dl)	86.66 ± 16.68	86.23 ± 14.82	0.449	0.654
TG(mg/dl)	188.62 ± 98.79	177.0 ± 162	0.349	0.727

4.6 Discussion

Cardiovascular diseases include coronary artery disease CAD and other heart diseases have been proved as the major cause of mortality all over the world (Mendis et al., 2011) and lipids and lipoproteins which play significant role in metabolism have seek attention in case of association with coronary heart disease (Omorogiuwa and Ozor, 2015). Dyslipidemia have been accepted as a major risk factor of cardiovascular diseases, moreover, cardiovascular disease risk factors are increased with the increase in total serum cholesterol (TC), low-density lipoprotein cholesterol (LDL-c) and serum triglycerides (TGs) but these risk factors are inversely related to serum high-density lipoprotein cholesterol (HDL-c) thus it has been considered that HDL-c plays a protective role against heart diseases and stroke as being involved in reverse cholesterol transport (Hatmi et al., 2007; Klop et al., 2013).This study was planned specifically to measure the serum lipid profile and its association with different ABO blood phenotypes of randomly selected subjects in Mirpur Azad Kashmir and to find out which blood group phenotype is more likely to be vulnerable to cardiovascular diseases. Results of this research work

represented that there was no significant relationship of the levels of serum total cholesterol (TC), triglycerides (TG), and lipoproteins with different ABO blood phenotypes. These findings bear out the observations of Amirzadegan et al., which proved no significant relationship between ABO blood phenotypes and lipid profile levels along with prevalence of cardiovascular risk factors among the patients with CAD (Amirzadegan et al., 2006). Lack of positive correlation between various parameters of lipid profile and ABO blood types in Saudi Arabian population was also observed by Anjum et al. and the same sorts of findings were got by the work of Ghazaee et al. on Iranian population (Anjum et al., 2017; Ghazaee et al., 2014).

Several demonstrations have been conducted to prove that ABO blood types, especially O-phenotype having individuals have higher chances of cardiovascular incidents due to more association with cardiovascular risk factors (Ketch, 2008; Fang et al., 2006; Nixon , 2004; Kaur, 2012). Another investigative study by Contiero et al. on Italian population showed no significant relationship of high density lipoproteins and low density lipoproteins with ABO blood types whereas levels of serum triglycerides were found higher in patients having B antigen (B and AB) as compare to those which did not posses this antigen (Contiero et al.,1994). Although no significant relationship was found in the present study, however levels of serum total cholesterol, triglycerides, and low density lipoproteins were reported higher than the required levels of these parameters and high density lipoproteins level was lower than the required value of HDL-c in blood type A as compare to other blood types which suggests that blood phenotype A holder subjects are more likely to be genetically predisposed to cardiovascular disease risk factors while levels of serum total cholesterol and low density lipoproteins were reported lower and of high density lipoproteins was higher in blood type O which suggests that O blood type is more likely to be protective against atherogenic factors. It may be due to genetic predisposition or may be the result of immunological interaction in different ABO blood phenotypes against different diseases. This investigation contradicts the previous findings of Ketch, Fang et al., Kaur and Contiero et al. (Ketch, 2008; Fang et al., 2006; Kaur, 2012; Contiero et al.,1994). This work also disagree with that of Girgla et al. and Airhomwanbor et al. (Girgla et al., 2011;

Airhomwanbor et al.,2018) in which they observed a positive correlation between AB blood group and various parameters of lipid profile in north Indian population and population of Ekpoma, Nigeria respectively. Besides it, this study vindicates the work of Iheanacho et al. (Iheanacho et al., 2018) at Aba Metropolitan which revealed that individuals possessing blood type A have more chances to acquire CVD than blood type O possessing persons. Present study also certify the results of the work performed by Stakisaitis et al. (Stakisaitis et al., 2002) in which It has also been found that A blood group phenotype have more affinity for developing cardiovascular diseases and O phenotype is more protective against CVD risk factors. In fact, higher levels of serum total cholesterol, triglycerides and low density lipoproteins as well as low level of high density lipoproteins have strongly been considered as the key risk factors for the development of atherosclerosis and CVD so, results of present study reveal that individuals having blood phenotype A may be at risk of development and progression of atherosclerosis and CVD.

Chapter 5

Conclusion and Future Work

In conclusion, no significant relationship was reported between various Lipid profile parameters and ABO blood group phenotypes of the population under study in this investigation. Since influence of ABO blood types on the risk factors of CVD has long been considered by researchers, however, mechanistic basis underlying this correlation is still unclear. Present study reveals that individuals with A blood type are more likely to be at risk of development and progression of CVD. While O blood type having individuals are more likely to be protective against these risk factors. Correlation between ABO blood types and ASCVD risk factor are remained unclear in the residents of Mirpur Azad Kashmir region. The reason behind it is that the family history and genetic predisposition are strongly associated to Lipid profile so may play influential role in the pathogensis of these disorders. In our opinion many other factors such as environmental factors may play role as predisposing force in the prevelance of CVD.

It is suggested that more precise and comprehensive study with larger sample size can be helpful to measure and quantify the possibilities of cardia problems in specific blood phenotype as compared to other blood types. Also there is a need to associate Rh status of population of this region with Lipid profile and other possible influential factors. Also there might be few genes up regulated for certain blood groups and relationship with cholesterol level in the population of the area under study.

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