ASSOCIATION OF HAPTOGLOBIN GENE POLYMORPHISM IN DIABETES AND CARDIOVASCULAR DISEASE IN NORTH INDIAN POPULATION

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Biotechnology

By

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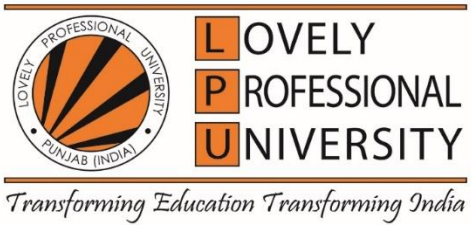

LOVELY PROFESSIONAL UNIVERSITY, PUNJAB

2024

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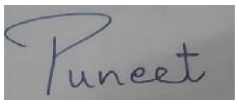
ABBREVIATIONS

T1DM	Type1 Diabetic mellitus
T2DM	Type2 Diabetic mellitus
CVD	Cardiovascular Disease
AS-PCR	Allele Specific-Polymerase Chain Reaction
HP	Haptoglobin
HPr	Haptoglobin related gene
BMI	Body Mass Index
WC	Waist Circumference
HC	Hip Circumference
WHR	Waist-Hip ratio
TC	Total Cholesterol
TG	Triglyceride
HDL	High Density lipoprotein
LDL	Low Density lipoprotein
VLDL	Very low density lipoprotein
APOA1	Apolipoprotein A1
AIP	Atherogenic index plasma
SNP	Single nucleotide polymorphism
DNA	Deoxyribonucleic acid
BP	Blood pressure
DBP	Diastolic Blood pressure
SBP	Systolic Blood pressure
MAP	Mean Arterial pressure
PP	Pulse pressure
CNV	Copy number variant
HbA1C	Glycosylated haemoglobin
FPG	Fasting plasma glucose

DECLARATION

I, hereby declared that the presented work in the thesis entitled "Association of Haptoglobin gene polymorphism in Diabetes and Cardiovascular disease in North Indian population" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision Dr. Rattandeep Singh, working as Professor, in the Bioengineering and Biosciences of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



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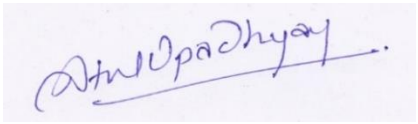
CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Association of Haptoglobin gene polymorphism in Diabetes and Cardiovascular disease in North Indian population" submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the Bioengineering and Biosciences, is a research work carried out by Puneet (Registration No. 11512761), is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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ABSTRACT

Diabetes is a non-communicable disease that is ravaging both industrialised and developing nations. In 2011, 366 million individuals were impacted globally, and this number is expected to climb to almost 552 million by 2030. Diabetes is a chronic, and life-threatening metabolic illness brought on by a high blood glucose level in the body. Type 2 diabetes mellitus (T2DM) progresses slowly and is difficult to detect in its early stages. One of the most serious outcomes of T2DM is cardiovascular disease (CVD).

Similarly, throughout the last two to three decades, a CVD epidemic has emerged throughout the world. In 1990, it was estimated that 5.3 million fatalities were caused by CVD in industrialized nations, while the comparable numbers for underdeveloped nations were between 8 and 9 million (i.e. a relative excess of 70%). More over half of those with T2DM experience a coronary artery disease, a stroke, or a heart attack.

Obesity is becoming a global epidemic in both children and adults. It is associated with numerous co morbidities such as cardiovascular disease (CVD), type 2 diabetes, hypertension, certain cancers and sleep disorder. Increase in adipose mass results in obesity and modulation of several factors in white adipose tissue. Haptoglobin (Hp) protein is up regulated in adipose tissue in obesity.

Hp is a glycoprotein produced in the liver in response to cytokines including IL-1, IL-6 (interleukin), and TNF (Tumour Necrosis Factor). Hp is a protein that looks like an immunoglobulin in which disulfide bridges connect two alpha (light) and two beta (heavy) chains (S-S). The HP gene is found on chromosome 16q22.1, which is the long arm of the chromosome. The HP1 and HP2 are the two autosomal co-dominant alleles. HP1 has five exons, and the HP2 allele was created by the duplication or multimerization of exons 3 and 4 of HP 1, in total there are seven exons in this gene.

In India, there is no work on the molecular level related to Hp gene. This is the first extensive genotypic analysis in northern India. Some studies have shown that the Hp 2-2 genotype raises the possibility of coronary diseases. The impact of Hp 2-2 protein in both cardiovascular and diabetes diseases was being investigated for the first time in this work.

In India, there is no work on the relationship of Hp with anthropometric, lipid profile and BMI. This study has never before conducted a genotypic investigation in northern India. It was determined in 123 type2 diabetes, 150 cardiovascular, 64 cardiovascular with type2 diabetes

and 130 normal individuals. The identification of Hp2-2 genotype was determined by allele-specific PCR. The relationship of Hp2-2 with obesity, lipid profile and blood pressure and arterial pressure were determined by student t-test and chi square test. The association of haptoglobin2-2 was found to be statistically significant with total cholesterol, high density lipoprotein, triglycerides, very low density cholesterol but not in low density lipoprotein cholesterol in case of diabetics and it was statistical significant in cardiovascular as well as cardiovascular with diabetics ($p<0.05$). Apo lipoprotein A1, HbA1c, systolic, diastolic blood pressure, mean arterial pressure and pulse pressure, alcoholic, physical activity, smokers, body mass index were found to be statistical significant with haptoglobin2-2 genotype in case of cardiovascular and cardiovascular with type2 diabetic mellitus participants. It concluded that Hp2-2 may be a marker of baseline characteristics and serum levels in individuals having diabetics, cardiovascular disease and cardiovascular with diabetics.

Haptoglobin (Hp) is an acute phase protein and plasma inflammatory marker. It is important in both infectious and non-infectious illnesses. Inflammatory illness disorders have been linked to a number of single nucleotide polymorphisms (SNP) of Hp. By understanding the structural and functional effects of several genetic determinants of Hp, the genetics underlying these disorders may be more clearly understood. A total of 57 SNPs, including 21 missense variations, three inframe deletions, 18 intron variants, eight non-coding transcript exon variants, five 5'UTR variants, one 3'UTR variant, and one intergenic variants, were randomly selected from the dbSNP, NCBI, and SNPedia databases. This is the first report that thoroughly examines bioinformatics of non-synonymous (nsSNP) and non-coding SNPs in the Hp gene. By using SNAP2 server, the structural predicted impact score of SNP was examined. SIFT, polyphen2 and the mutation accessor database were used to analyse the detrimental, tolerable and most likely harmful effects of nonsynonymous SNP. The FuncPred and Regulomedb databases were used to analyze the functional and structural effect score of synonymous SNPs. The haptoglobin-haemoglobin interaction was analyzed by PP check tool. DNA sequencing of haptoglobin 2-2 genotype were examined in human samples and showed A>C mutations and two SNP's rs3852780 and rs5471 were associated with transcription factor binding site and the variation is more likely to be a regulatory variable that affects gene expression. Hence, these mutations are linked to illnesses.

Keywords: Lipid profile, Obesity, HbA1c, haptoglobin2-2, ApoA1, systolic, diastolic blood pressure. Databases, genetics variants, Bioinformatics, nsSNP's.

Graphical Abstract

Title of the thesis: Association of Haptoglobin gene polymorphism in Diabetes and Cardiovascular disease in North Indian population

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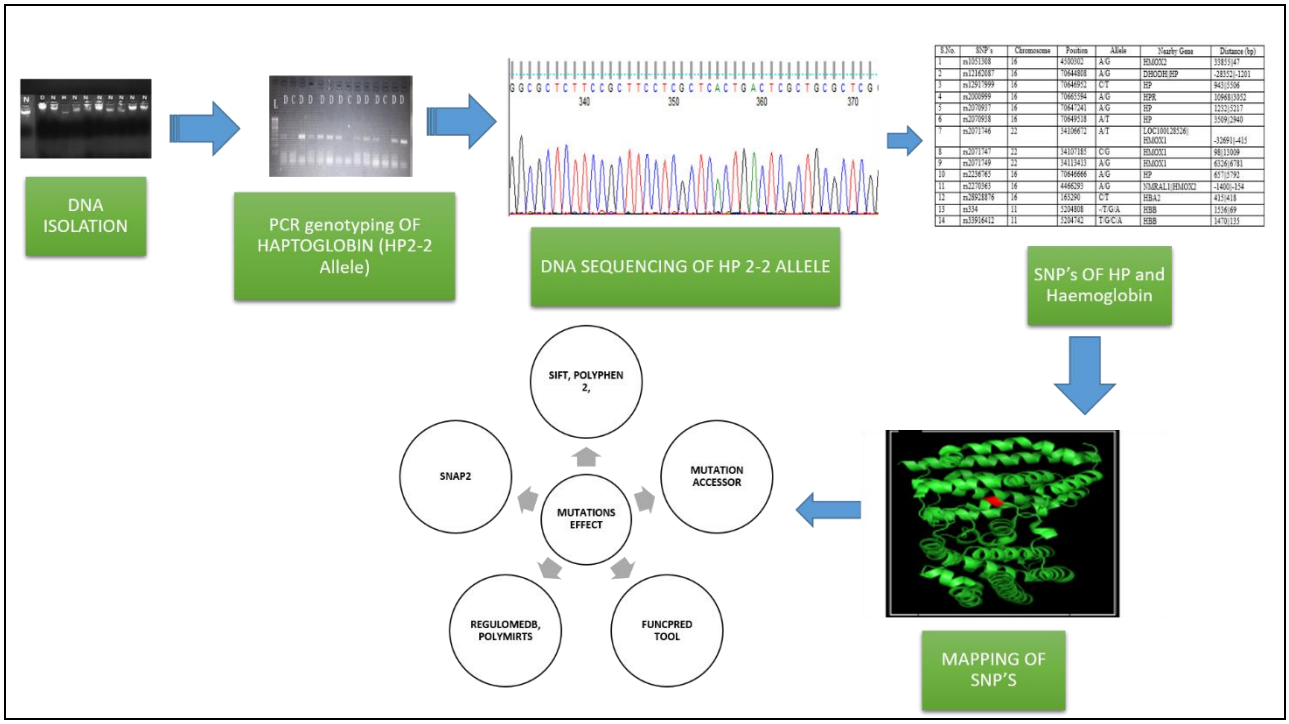
Summary of graphical abstract: Haptoglobin (Hp) is a protein that is mostly synthesized in the liver and lungs. It is currently being researched as a possible biomarker for a number of illnesses, such as different kinds of malignant neoplasms. It possesses diverse physicochemical characteristics that distinguish it from other proteins. Haptoglobin has three genotypes i.e. Hp1-1, Hp2-1 and Hp2-2. These genotypes are influenced by a combination of inherited allelic differences. The extraction of DNA from total 467 human blood samples in which Diabetic mellitus (DM) were 123 patients, Cardiovascular (CVD) were 150 patients, Cardiovascular with Diabetics (CVDWDM) were 64 and control (N) were 130 participants were included. The allelic or genotypic analysis of Hp2-2 genotype were performed by using Allele-specific PCR. The latter is sequencing of these samples to determine the mutations. Further, Identify the SNP's of Hp by using NCBI tool. After the mapping of mutations with their respective structures by using Pymol software. Each mutations effect was checked by different servers or tools (SIFT, Polyphen2, Mutation accessor, FuncPred, SNAP2, Regulomedb and PolymiRTS).

Graphical abstract:



Caption: Genetic polymorphism and SNP analysis of HP gene

Name and signature of all authors: Puneet, Dr. Rattandeep Singh and Dr. Atul kumar Upadhyay

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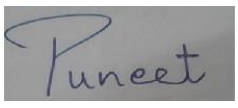


PUNEET

CHAPTER-I

INTRODUCTION

1.1 GENERAL INTRODUCTION

A chronic, non-communicable condition known as diabetes mellitus causes elevated glucose levels. Diabetes is characterized by insufficient pancreatic insulin production or inefficient insulin secretion, both of which can be inherited or acquired. Diabetes comes in a variety of forms, i.e. Type 1 diabetic mellitus (T1DM) or insulin dependent a kind of diabetes and NIDDM (Non-insulin dependent diabetic mellitus), sometimes known as type 2 diabetes, is one of several particular kinds of the disease (others include pancreatic damage from chemicals, infections, etc.) (Mohieldein et al, 2013). Diabetes has spread across the globe, affecting individuals (>170 million). It is the most common causes of death in the United States. The number is expected to reach 366 million by 2030. Type 2 diabetes mellitus (T2DM) is due to the impairment of insulin secretion from pancreatic beta cells as well as resistance of insulin hormone in peripheral tissues, particularly muscle and liver cells. In India, (31.7 million in 2000 and it was estimated that 79.4 million in 2030), In China (20.8 million in 2000 and it was estimated that 42.33 million in 2030), and the United States (17.7 million in 2000 and it was estimated that 30.3 million in 2030) are the top three countries in terms of T2DM individuals with diabetes, according to Wild et al. (2004) (Singh, 2011). Similarly, throughout the last two to three decades, a CVD (cardiovascular disease) epidemic has evolved in emerging nations. In 1990, it was estimated that 5.3 million fatalities were caused by CVD in industrialized nations, whereas the comparable figure for developing countries was between 8 and 9 million (i.e. a relative excess of 70 percent). Developing nations currently provide a larger part of the global burden of CVD than developed countries (Einarson et al, 2018). Obesity in both children and adults is becoming a global epidemic. It's linked to several other co-morbidities, including CVD, type 2 diabetes, hypertension, certain cancers, and insomnia. The deposition of adipose mass in white adipose tissue during high levels of several factors and obesity, haptoglobin is upregulated in adipose tissue (Chiellini et al, 2002).

Haptoglobin (HP) is a sialoglycoprotein. It is secreted by the hepatic cells as well as by other tissues such as the kidney, brain, intestine, arterial vessels, lungs, spleen, and skin. Haptoglobin is found in all mammals, e.g. in the adipose tissue of cattle, also found in zebrafish, as well as in some birds (Galicia and Ceuppens, 2011 and Wan et al, 2021). Two α and two β chains, joined by disulfide bonds in a quaternary structure, make up the hemoglobin-binding plasma protein known as haptoglobin (Dobryszycka,1997 and Tamara et al,2020). Due to the occurrence of several phenotypes that are highly prevalent in the human population, haptoglobin has undergone significant genetic research. While Hp2 has a partial duplication of

the α chain, HpF and HpS vary from one another in the amino acid at position 53 of the α chain. The human Hp2 gene was created by a crossing over between a HplF and a HplS gene, according to a partial sequence of the gene (Bensi et al, 1985). When the intravascular hemolysis occurs, about 10% of the RBC breakdown occurs, i.e., 650mg per day or 27mg per hour. Free Hb (haemoglobin) in the plasma moves via the glomerular filter, leading to renal impairment. Haptoglobin binds with free haemoglobin with the highest affinity, i.e., KD-10^{-15}M, this helps stop it from getting deposited in the glomeruli and nearby tubule cells of the kidney as well as preventing oxidative tissue damage. When the Hb-Hp complex binds with the CD163 scavenging molecule, it starts the secretion of HMOX1 (heme oxygenase) and Interleukin-10 protein in human macrophages cell and in tissue ex-vivo (Galicia and Ceuppens, 2011 and Cooper et al, 2013). CD163 is a Hb-scavenging molecule i.e. connected to the SRCR family class B and it is found on the surface of macrophages. It is upregulated by the anti-inflammatory factors, i.e. glucocorticoids and Interleukin-10, and it is downregulated by proinflammatory cytokines, i.e. lipopolysaccharide, interferon-α, and TNF-α. Hp levels rise and fall during an inflammatory response due to hepatocellular deficiency and in haemolytic conditions (Yang et al, 1993 and Abah et al, 2018). Haptoglobin (Hp) is a plasma protein that helps to eliminate free haemoglobin (Hb) and prevents Hb from oxidising serum or cellular proteins and lipids. It may be important in illnesses like type 2 diabetes that are linked to inflammation and oxidative stress (Bhardwaj et al, 2020). A HP locus can be found on the chromosome 16q22. Hp1 and Hp2 are the two co-dominant alleles in HP, leading in three genotypes: Hp1-Hp1, Hp2-Hp1 and Hp2-Hp2. According to initial reports, Hp2 is the outcome of gene duplication that added two exons to Hp1. However, it has been demonstrated that modification in the structure of Hp gene results from a mixture of old and new deletions (NS Zheng et al,2017). According to recent evidence, old hominins and pre-historic African humans both conveying sequencing concept with the breakpoint sequence present on Hp2 but not on Hp1. The haptoglobin 2-2 phenotype has been connected to oxidative stress and CVD risk in those with glycaemia. It's been claimed that diabetes patients with the HP2-2 genotype have a higher chance of acquiring cardiovascular disease (Szafranek et al, 2002 and Dalan and Ling, 2018). Obesity regulates Hp expression in WAT (White adipose tissue), which could have essential implications for understanding the function of this component, such as its unique function in fat cell differentiation or possible angiogenic ability in an extended adipose mass (Chiellini et al, 2002). However, just a few research has looked at the link between haptoglobin and diabetes. Because mutations in the human haptoglobin gene are linked to an increased risk

of diabetic complications, haptoglobin might be used as a prognostic biomarker for viral induced autoimmune T1DM and T2DM (Jelena et al, 2013).

To investigate association of haptoglobin gene polymorphisms in T2DM and CVD in north Indian cohorts, the study was designed based on this principle.

1.2 ANTHROPOMETRIC PARAMETERS

Obesity is often assessed based on physical characteristics. BMI, hip circumference, Waist circumference, hip ratio, and Waist-hip ratio are some of the physical indicators of obesity. Despite the availability of more advanced methodologies, anthropometric measures such as height, weight, waist-hip ratio (WHR), and Body Mass Index (BMI) have long been used to research genetic structure and forecast risk factors for a variety of complex disorders that affect human health.

- BMI

The Body Mass Index (BMI) is a measurement of someone's weight in relation to their height. It's a good indicator of obesity and overweight. Our height and weight are used to compute it. It's a way to evaluate body fat and an excellent predictor of illness risk. BMI rises with age until it reaches a certain point.

Weight category	**BMI ($kg/m^{2)}$**
Underweight	Less than 18.5
Norm`1al range	18.5-24.9
Overweight	25.0-29.9
Obese Class I Class II Class III	Greater than and equals to 30 30.0-34.9 35.0-39.9 Greater than and equals to 40

Table 1. BMI categories

The higher our BMI, the risk of certain diseases will be higher i.e. Cancer, Heart diseases, Breathing problems, T2DM, CVD (Singh and singla,2020).

- Waist circumference (WC)

A person's waist circumference is a measurement of their abdomen. The narrowest point between the rib cage and hips should be used to estimate a person's waist circumference (Singh and singla,2020).

- Hip circumference (HC)

The hip circumference of a person is measured at the greatest posterior stretch of the buttocks. The measurement technique mentioned under Data Collection Methods should be followed to maintain uniformity in measurement (Singh and singla,2020).

- WAIST-HIP RATIO (WHR)

It is determined by a person's waist and hip circumferences. It identifies abdominal fat and is a more accurate gauge of cardiovascular danger compared to BMI. The waist-hip ratio in men is 0.90, whereas in women it is 0.85 (Singh and singla,2020).

Health risk	**Women**	**Men**
Less	less than and equals to 0.80	less than and equals to 0.95
Intermediate	0.81-0.85	0.96-1.0
More	Greater than and equals to 0.86	Greater than and equals to 1.0

Table 2. Waist-hip ratio in Men and Women

1.3 BIOCHEMICAL PARAMETERS

The Biochemical parameters include Lipid profile, APOA1 and HbA1c tests.

1.3.1 LIPID PROFILE

It consists the cholesterol, triglycerides, high density lipoprotein, very low density lipoprotein and low density lipoprotein.

- **TOTAL CHOLESTEROL**

Cholesterol is a waxy fat-like molecule produced by the liver and other cells and contained in a variety of meals, including dairy products, eggs, and meat. Cholesterol is required for normal physiological function. Its cell walls, or membranes, require cholesterol to create hormones, vitamin D, and bile acids, which aid fat digestion. However, the body only requires a certain quantity of cholesterol to satisfy its requirements. When there is too much, health concerns such as heart disease might arise. It can be used to assess the risk of a coronary artery blockage,

atherosclerosis, myocardial infection, organ function, biliary mechanism, intestinal permeability, thyroid hormone, and adrenal illness. Factors may affect cholesterol are: Diet, weight, exercise, age & gender, diabetes, heredity, medications.

HIGH CHOLESTEROL LEVEL: Due to increasing the levels of cholesterol, the risk of certain diseases i.e. Hypolipoproteinaemia's, Nephrotic syndrome, Jaundice and Diabetes mellitus.

LOW CHOLESTEROL LEVEL: Due to decreasing the levels of cholesterol from their normal range, the risk of certain diseases i.e. Anaemias, Haemolytic jaundice, Severe malnutrition and Acute infections.

Lipid Test	**Desirable**	**Borderline High**	**High**
TC	Less than 200	200-239	Greater than and equals to 240
LDL	Less than 130	130-159	Greater than and equals to 160
HDL	Greater than and equals to 50	40-49	Less than 40
TG	Less than 200	200-399	Greater than and equals to 400

Table 3. Normal, Intermediate and high values of lipid profile

Proteins in the bloodstream carry cholesterol. A lipoprotein is a mixture of proteins and cholesterol. Different forms of cholesterol exist depending on what the lipoprotein carries. They are high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) (Priya et al, 2013).

- **HDL (High Density Lipoprotein)**

Because it transports cholesterol from other regions of your body to your liver, it is generally known as "good" cholesterol. The cholesterol in your body is subsequently removed by your liver. Causes of low level of HDL cholesterol are: obesity, type2 diabetes, elevated triglycerides and a lack of physical activity. Causes of high level of HDL are: thyroid disorders, inflammatory diseases and alcohol consumption (Priya et al, 2013).

- **Low Density Lipoprotein (LDL)**

Because a high LDL level causes plaque to build up in your arteries, it is commonly referred to as "bad" cholesterol. Causes of high level of LDL cholesterol are: heart attack and stroke. Causes of low level of LDL cholesterol are: Liver disease, thyroid issues, Anaemia and Malnutrition (Priya et al, 2013).

- **Very Low Density Lipoprotein (VLDL)**

VLDL is also a "bad" cholesterol since it adds to plaque accumulation in your arteries as well. VLDL and LDL, on the other hand, carry triglycerides whereas LDL contains cholesterol. Causes of low level of VLDL cholesterol are: Cancer, Haemorrhage stroke and Depression. Causes of high level of VLDL cholesterol are: Alcoholism, hyperthyroidism, Kidney disease and obesity (Priya et al, 2013).

- **TRIGLYCERIDE**

Triglycerides are a kind of lipid that the body uses to store calories that are not used immediately. These triglycerides circulate in the bloodstream, providing energy to your muscles. Extra triglycerides are seen in the blood after eating. Your triglyceride level may be elevated if you consume more calories than your body requires. Very low-density lipoproteins transport triglycerides throughout your body (VLDLs). The triglyceride level test is used to figure out how much triglycerides are within your blood. It is a form of blood fat. The examination can help you figure out how likely you are to acquire heart disease. A "triacylglycerol test" is another name for this procedure.

The medical name for high triglyceride levels in the blood is hypertriglyceridemia. Your cholesterol might be elevated if your triglyceride levels are high. Hyperlipidaemia is a term for this disorder. It's possible that your triglyceride level is excessive. Some are caused by triglyceride-raising behaviours. The list includes things like smoking, sitting still all day, being overweight or obese, drinking more alcohol, and eating a diet heavy in carbs and low in protein. High triglyceride levels might also be caused by medical disorders. These include hepatic cirrhosis, poorly managed diabetes, hyperlipidaemia, hypothyroidism, genetic predispositions, nephrotic syndrome or kidney disease, and pancreatitis. Causes of low amount of triglycerides are: low-fat diet, hyperthyroidism, malabsorption syndrome and malnutrition (Priya et al, 2013).

1.3.2 APOLIPOPROTEIN A1 (APOA1)

The protein Apo-A1 has both structural and functional properties found in HDL, accounting for around 70% overall protein. It is produced in the intestines and liver, and it activates peripheral tissue lecithin-cholesterol acyltransferase (LCAT), which breaks down free cholesterol into cholesterol ester and facilitates its transfer to the liver, where it is eliminated. When Apolipoprotein B (Apo-B) levels are raised, a low Apo-A1 level suggests a higher risk of cardiovascular disease. The following are the Apo-A1 reference ranges: 80–175 mg/dL for adult females and 75–160 mg/dL for adult men. Causes of low APOA1 level due to Chronic liver and kidney diseases, obesity, smoking and levels of TG high. Causes of rise APOA1 amount are: Pregnancy and Alcoholism (Priya et al, 2013).

1.3.3 GLYCOSYLATED HEAMOGLOBIN (HbA1c)

Hemoglobin is the oxygen-carrying pigment that gives blood its red colour, as well as the most common protein found in red blood cells. Hemoglobin A makes up around 90% of the blood (the "A" stands for adult type). Although 92 percent of haemoglobin A is made up of one chemical component, the remaining 8% is made up of small chemically distinct components. Hemoglobin A1c, A1b, A1a1, and A1a2 are some of the lesser components. HbA1c (haemoglobin A1c) is a glucose-binding component of haemoglobin. Glycated, glycosylated haemoglobin, or glycohemoglobin are all terms used to describe HbA1c.

Normal	<5.7%
Prediabetes	5.7% to 6.4%
Diabetes	6.5% or greater

Table 4. HbA1c levels

Causes of high level of HbA1c are: Diabetes, overweight, smoking, iron, chronic disease and sleep disorders. Causes of low level of HbA1c are: Alcohol, Liver disease and genetic haemoglobin disorders (Gautam Rawal et al, 2016)

1.3.4. ATHEROGENIC INDICES

An efficient indicator of plaque and coronary heart disease risk is the atherogenic index of plasma (AIP). Antiatherosclerotic lipoprotein particle size is linked to the AIP. As a result, this metric reflects the ratio of protective to atherogenic lipoproteins (Zhu et al, 2018 and Zhen Li et al, 2018).

1.4 DNA EXTRACTION

Whole genomic Dna isolation from biomolecules is an essential initial step in many molecular biology processes, including as linkage analysis, polymerase chain reaction (PCR), restriction enzyme analysis, genotyping, and mutation detection (Phillips et al, 2000; Wang et al, 2003). Additionally, the extraction of DNA from blood samples is a vital requirement for many diagnostic and prophylactic treatments, epigenetic study, and the identification of genetic abnormalities (Angelini et al, 2000; Lewis et al, 2005; Phillips et al, 2000; Wang et al, 2003).

1.5 **POLYMERASE CHAIN REACTION (PCR)**

The purpose of the PCR reaction was to verify that the genomic DNA was undamaged and to find out whether there was any inhibitory substance obstructing the process. PCR are of many types but we used allele specific (AS) PCR or ARMS PCR (Amplification Refractory Mutation System). It's a straightforward way for detecting single-base alterations or minor deletions. ARMS works by using sequence-specific PCR primers to amplify test DNA only when the target allele is present. Electrophoresis of PCR products on an agarose gel with ethidium bromide (EtBr) separates them. Advantages of this PCR are this method can tell the difference between two alleles with pinpoint accuracy, it can identify SNPs (single nucleotide polymorphisms) and it is a PCR process that is accurate, quick, and dependable. Disadvantages are it incorporates advanced primer design and mismatch integration methods, it can only detect a small number of SNPs at a time, it can tell the difference between homozygous and heterozygous alleles, it is an essential approach for genotyping and allelic variation research and it is a more expensive method since it involves more primer sets (Gaudet et al,2009 and Darawi et al, 2013).

The Association of haptoglobin gene polymorphism was checked by using AS-PCR or ARMS-PCR. A glycoprotein called haemoglobin (Hp) binds to free haemoglobin in the plasma and aids tissue protection and oxidative damage prevention. In addition, it serves as a regulator. Such a protein is acute-phase whose quantity in the plasma varies with disease for which a test is routinely performed. It has various physicochemical features that set it apart from other proteins. Hp has 3 alleles i.e. hp2-1, hp2-2 and hp1-1. According to several research, the phenotype of haptoglobin can be used to determine a person's specific vulnerability to certain illnesses. Additionally, HP is prone to a range of PTMs (post-translational modifications). These include chemical modifications to alpha-chains and beta-chain glycosylation, as well as structural alterations (Dobryszycka,1997).

The gene structure of Hp is located on chromosome 16q22. It has two types Hp1 and Hp2, which represents two homozygous Hp1-1 & Hp2-2 alleles as well as one heterozygous Hp2-1 allele. Hp1 has 5 exons and due to the duplication of exon3 and exon4 hp2 allele has arisen and it has 7 exons it is only present in humans as shown in Figure 1. (Koch et al, 2002).

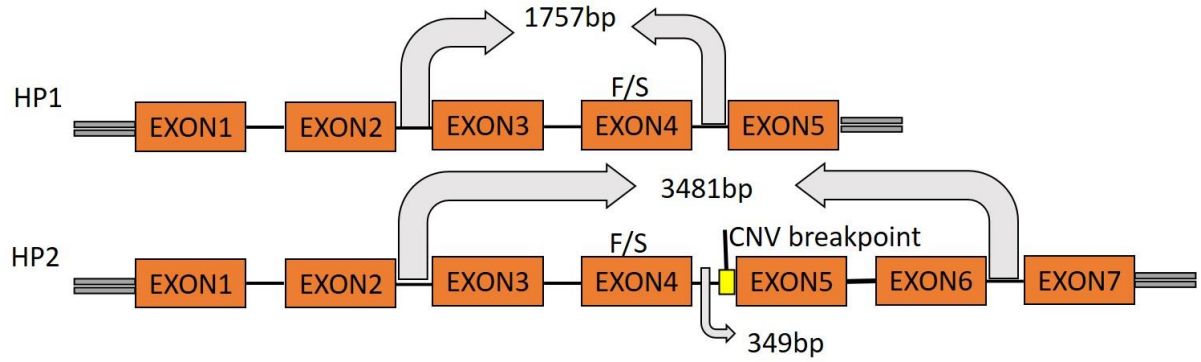


Figure 1. Haptoglobin Gene Structure

To amplify a 349bp Hp2-2 genotype locus, primers C (5'-CCTGCCTCGTATTAACTGCACCAT-3') and D (5'-CCGAGTGCTCCACATAGCCATGT-3') were used. Primer A annealing sites are found immediately upstream of the 1711-bp unit and the 1724-bp unit in Hp1 and Hp2, respectively. Both locus 188639 and 2781 in (Hp1) AC004682 & (Hp2) M69197 correspond to the nucleotide of the primer A at the end of 5'. Primer B has binding affinity slightly downstream of Hp 1 and Hp 2's 1711-bp segments. Both locus 186883 and 6261 in (Hp 1) AC004682 and (Hp2) M69197 correspond to the nucleotide of primer B at the end of 5'. The binding sites of Hp2 are two, while those of Hp1 are one. When using primers C and D in reactions, the Hp 2 template is the only one that produces a 349 bp PCR product; the Hp 1 template produces no product at all. The specific locus of the linking sites and the primers of C and D's 5'-3' orientation are responsible for this type of allelic specificity. The 349bp template of Hp2 product in M69197 stretches from nucleotide position 4352 to 4700, containing the primers C and D annealing sites. In the haptoglobin-related gene, additionally, primers C and D have a single binding site at the appropriate locations, however the primer and annealing site sequences are not entirely complimentary. Crucially, when using the HPr (Hp-related) gene act as a template, amplification reactions with C and D primers go towards opposite orientations, preventing the formation of a PCR product (Koch et al,2002).

Clinical significance: Anhaptoglobinemia and Hypohaptoglobinemia are caused by mutations in its regulatory locus. Additionally, this gene has been connected to inflammatory disease

behaviour, a decreased risk of Plasmodium falciparum malaria, coronary artery disease, Crohn's disease, diabetes, Parkinson's disease and inflammatory disease exposure.

1.6 Computational Approaches: In order to keep up with the rapid growth of bioinformatics data, computational approaches are a must. A simple and affordable method to find out if a SNP causes illness is through "in silico" prediction. When predicting outcomes, a variety of factors must be considered, such as populational, scientific, architectural, and bioinformatics. In silico modelling has evolved into a precise complementary, and often decisive, prognosis methodology as bioinformatics tools have improved and the number of current crystal structures has increased. Components like the extensive rules of protein configuration, three-dimensional structure, and identity in amino acids residue between distinct species or linked proteins are all taken into account at different levels by different imputation programmes. There are coding and non-coding regions in the haptoglobin gene. However, not all of the code components are required. Missense amino acids, or non-synonymous SNPs are also important. It causes changes in how translated amino acid sequences are classified. dbSNP, NCBI, SNPedia, and GWAS central are some of the freely available SNP databases. Nonetheless, the significance of genes could not be overstated. The relevance of SNP can be predicted based on the lack of empirical results on their functional effects. It is divided into five stages utilising computational tools: splicing, transcription, translation, post-translational, and protein stability (Duarte et al, 2018). A total of 59 SNPs were extracted randomly from dbSNP, NCBI, and SNPedia databases which include 34 missense variants, three inframe deletions, 27 intron variants, 4 non-coding transcript exon variants, five 5'UTR variants, one 3'UTR variant, and 3 intergenic variants. The aforementioned work is the first to attempt a comprehensive genotypic and bioinformatics investigation of non-synonymous (nSNP's), non-coding SNPs in the HP gene in the North Indian population.

CHAPTER – II

HYPOTHESIS

HYPOTHESIS

In diverse ethnicities and ethnic groups across the world, there are allelic and genotypic differences in Haptoglobin. Studies have reported that in India, (Maharashtra, Rajasthan, Delhi and Uttar Pradesh) the allelic frequency of Haptoglobin was measured by using starch gel electrophoresis, polyacrylamide gel electrophoresis, and the ELISA (enzyme-linked immunosorbent assay) technique from healthy people in order to conduct a caste-based study on control subjects. In India, there is no work on the molecular level and computational study related to Hp gene as well as in Diabetics and cardiovascular diseases. There is no work on relationship of Hp with anthropometric, lipid profile and BMI. This is the first extensive genotypic analysis in northern India. Some studies have shown that the Hp2-2 genotype raises the possibility of coronary diseases. The study hypothesized that cardiovascular disease is substantially more likely to occur in diabetics who are homozygous for the haptoglobin 2 genotype (Hp 2-2). The impact of Hp2-2 protein in both cardiovascular and diabetes diseases was being investigated for the first time in this work. Our findings conclude that a 349bp band is seen in diabetes patients, cardiovascular patients, or diabetics with CVD. It indicates that the HP2-2 allele genotypes were discovered in patients but not in healthy people in our population. The Hp 2-2 genotype is a CVD risk factor in type 2 diabetes patients in the North Indian population. Furthermore, the sequencing of these patients showed novel A>C mutations is present in diabetes, cardiovascular disease as well as cardiovascular with diabetes participants. The data show that Hp genotype may be used to stratify patients for optimal CVD therapy in people with type 2 diabetes.

CHAPTER – III

OBJECTIVES

OBJECTIVES

In this research, the following objectives are being pursued:

- To evaluate the anthropometric parameters like BMI, weight, height, waist-to-hip ratio, blood pressure and Biochemical parameters like blood glucose level, Apolipoprotein A1, and lipid profile in diabetes, cardiovascular patients, diabetes/CVD patients & control in North India.
- To study the genetic polymorphism in 349bp HP2 allele-specific sequence in diabetics, cardiovascular patients and diabetes/CVD patients.
- To study changes in the structure of heme and haptoglobin protein due to observed SNP's by using bioinformatics tools.
    i. Data mining for identification of SNP's in heme and haptoglobin for Diabetes and CVD.
    ii. Mapping of selected SNP's on their respective structures.
    iii. Predicting the effect of mutations in their respective structures and functions.

CHAPTER – IV

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Haptoglobin (HP) is a sialoglycoprotein. In addition to the liver cells, other tissues such the lung, brain, gut, artery vessels, spleen, kidney, and skin also generate it (Hamdy et al, 2014 and Galicia et al, 2011). Haptoglobin is found in all mammals e.g. adipose tissue of cattle, also found in the zebrafish as well as in some birds (Langlois and Delanghe, 1996). When the intravascular hemolysis occurs, about 10% RBC breakdown occurs i.e. 650mg per day or 27mg per hour. The free hemoglobin in the plasma moves via the glomerular filter leading to renal impairment. Haptoglobin is binding with free hemoglobin with the highest affinity i.e. KD-10^{-15}M, therefore avoiding its build-up in the kidney's proximal tubule cells and glomeruli, as well as avoiding oxidative tissue damage (Levy et al, 2010). Haemoglobin-haptoglobin molecule bind to CD163 scavenging molecule, it initiates the production of HMOX1 protein and the discharge of IL-10 in ex-vivo human macrophages and tissue macrophages (Asleh and Levy, 2005 and Philippidis et al, 2004). The Hb-scavenging receptor CD163 is found on the surface of macrophages and belongs to the SRCR family class B. Its expression is upregulated in the anti-inflammatory factors i.e. glucocorticoids & IL-10, its downregulated in proinflammatory cytokines i.e. lipopolysaccharide, interferon-α and TNF-α. During inflammation reaction Hp level is increase and decrease due to deficiency of hepatocellular and in a haemolytic conditions (Philippidis et al, 2004 and Dobryszycka, 1997).

4.1 Haptoglobin Structure

The structure of haptoglobin protein is similar with the immunoglobin protein because it has two polypeptide chains i.e. alpha and beta chains linked by two disulphide bonds i.e. HPα and HPβ as shown in Figure 2. The β-chain is heavier than the α-chain and it is identical in all types of HP proteins. α-chain and β-chain has 83 & 245aa residues and M.W. is 9100 & 40,000 (Langlois and Delanghe, 1996). The HP1F and HP1S structural gene is different in 1 amino acid i.e. the lysine amino acid of α1F domain in 54 position is replaced by the glutamic acid in α1S chain. The Hp-Hb binding is irreversible noncovalent binding i.e. 55 x 10^5 M^{-1} s^{-1} rate constant. HP forms a stable complex with Hb with a high affinity i.e. KD-10^{-15}M as shown in Figure 2. Each HP monomeric protein can interact with single Hbα-β dimer i.e. Hp is unable to link to deoxyhb. The HP β-chain has been mapped to the interaction site for Hb on HP. The β-globin chain of HB contains two binding sites for HP at β11-25 aa residues & β131-146 aa residue, whereas α-globin chain has single Hp interacting region i.e. α121-127. The size of α1 and α2 is~9 and~16 Kd respectively (Levy et al, 2010).

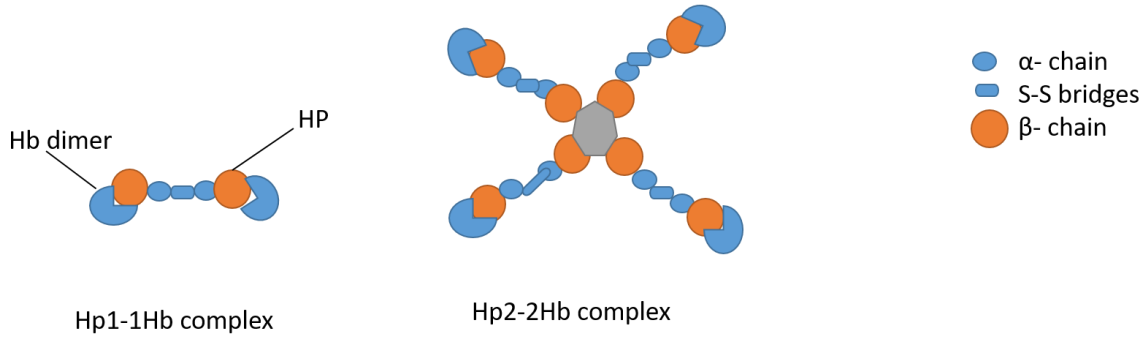


Figure 2. Structure of Haptoglobin-Haemoglobin complex.

Within the liver's parenchymal cells, the HB-HP molecule breaks. When Hp-Hb complex is assigned in macrophage cell it breaks into lysosomes where bilirubin, carbon monoxide, and iron are produced as a result of heme reactions by the enzyme heme oxygenase. Free Hb has iron heme component it produced hydrogen peroxide & generate Fenton reaction that causes damage to protein, DNA and lipids (Galicia et al, 2011). Hp is only bind with oxygenated haemoglobin dimers. Due to the oligomeric nature of this complex it's difficult to analyse the high or low affinity of this complex with CD163 scavenging receptor (Nielson et al, 2007). The β-chain is constant in both Hp1 and Hp2 is~45Kd. The Hb bind with β-Hp site. The Hpβ chain is N-glycosylated & carbohydrate side chains are terminal α2-6 sialic acid residues. The carbohydrate chain bind to the B-lymphocyte lectin CD22 molecule, this molecule depends upon the concentration of Hp protein. The binding of the Hb with the Hp is not only depend upon the serum Hp concentration but also types of HP protein (Galicia et al, 2011). Haptoglobin protein is different in size and shape i.e. Hp1-1 is smaller molecule than Hp2-1 and Hp2-2, when Hp interacts with Hb it makes Hp-Hb complex and Hp1-1Hb complex takes less time to discharge from the endothelial cells then Hp2-1Hb and Hp2-2Hb complexes and it has antioxidant properties. The binding of HB with HP1-1 is stronger, HP2-1 is intermediate & HP2-2 is weaker bonding as well as it does not effect on LDL and HDL molecule in healthy individuals as shown in Figure 3. When Hp-Hb complex bind to CD163 scavenging receptor, the Hp2-1Hb complex is seven times increase in anti-inflammatory mediators i.e. interleukin 6 & 10 than the Hp2-2Hb complex. Hp is also called as immune system modulator, it suppresses the T helper cell 1 & 2 (Th1 and Th2) that give rise to infection in the body (Hamdy et al, 2014, Galicia et al, 2011 and Levy et al, 2010).

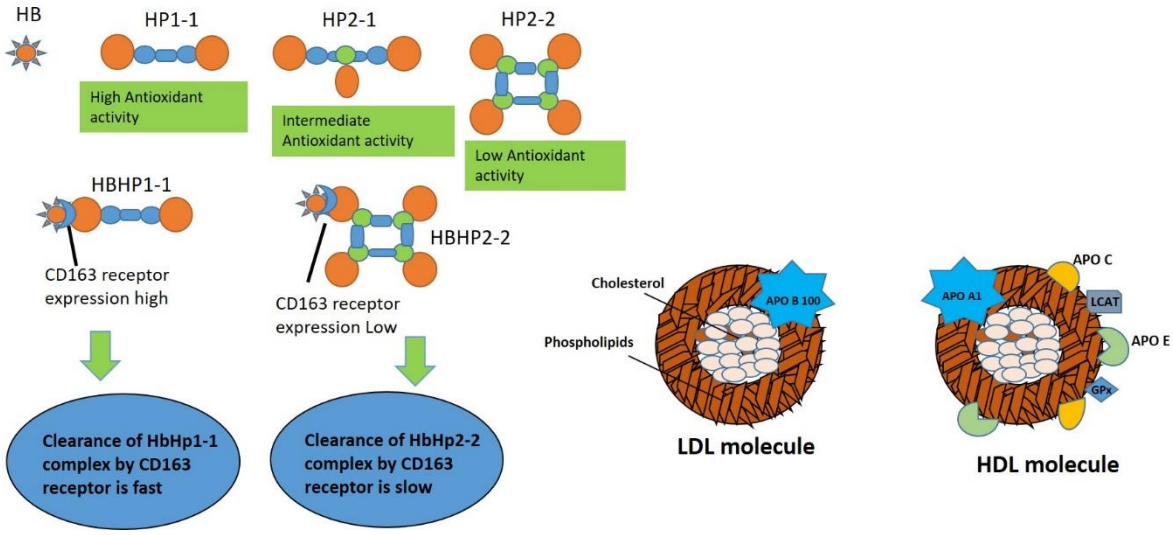


Figure 3. Role of haptoglobin protein in Healthy individuals.

In vitro, when in Chinese hamster ovary (CHO) cell induced with the Hp-Hb scavenging receptors, the Hp1-1Hb moves faster through endothelial cells than the HP2-2HB complex as illustrated by using rhodamine labelled and ^{125}I-tagged. However, it evaluated the half-life duration of ^{125}I-tagged Hp-Hb molecules in vivo in mice, which is~ 20min for HbHp1-1 molecules and~50min for Hp2-2hb molecules (Levy et al, 2010). In vivo, the Hp-Hb complex formation can contribute to inhibiting the Hb production of lipid peroxide and hydroxyl radical in inflammation zone. ROS (Reactive oxygen species) that are directly microbicide may be produced by this complex bound inside the phagolysosomes of phagocytic cells. The Hb-Hp complex shows a nitric oxide-derived relaxing substance from the endothelium that is inhibited (Levy et al, 2010 and Dobryszycka, 1997).

4.2 Haptoglobin gene

Haptoglobin gene is located on chromosome 16q22. Hp1 has 5 exons but during meiosis, through a non-homologous transition between the structural alleles, the HP1F and HP1S alleles fuse to form the HP2 allele. HP2 gene has 7 exons it is only present in humans as shown in Figure 4 (Dobryszycka, 1997, Boettger et al, 2016 and Koch et al, 2002). After the crossing over of two HPα2 allele, a rare HP Johnson type of HP arise, including other structural variants i.e. HP (Carlberg) and HP2-1 modified. The duplication of HP1 gene on chromosome 16 results in the HP related gene (HPr gene). The heterozygous HP2-1 can change the type of HP2 into HP2SS, HP2FF & HP2FS alleles (Dobryszycka, 1997).

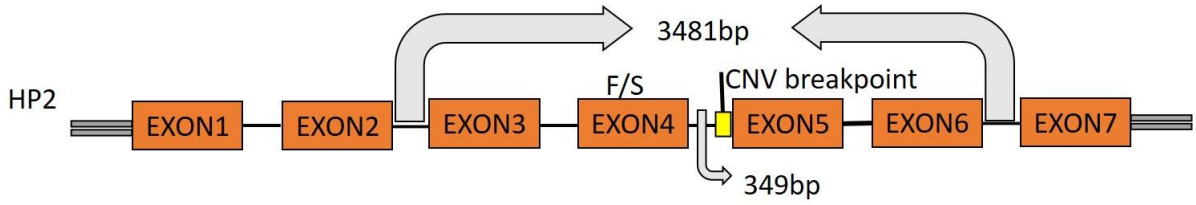


Figure 4. Structure of Haptoglobin 2 Gene.

HP was first described by the Jayle and Judas in 1946 and in 1955 smithies identified the three major types of HP. Haptoglobin has HP1 and HP2 alleles. By performing the starch gel electrophoresis, it has three phenotypes HP1-1, HP2-1 and HP2-2. Due to the molecular mass of the haptoglobin, it shows slow and fast migrating in the gel electrophoresis i.e. the three α-chains i.e. α^s (slower), α^f (faster) and α2 chains (slow-migrating) (Langlois and Delanghe, 1996). The HP1-1 phenotype expresses α^1 chains and the HP2-1 and HP2-2 expresses α^2 chains (Asleh et al, 2005 and Boettger et al, 2016).

4.3 *In silico* structure of Haptoglobin-Haemoglobin complex

PSIPRED tool was used to examine the secondary arrangement of haptoglobin as shown in Figure 5. The tertiary or three dimensional (3D) structure of human haptoglobin analysed by the UniProt online database as shown in Figure 6 (Wan et al, 2021).

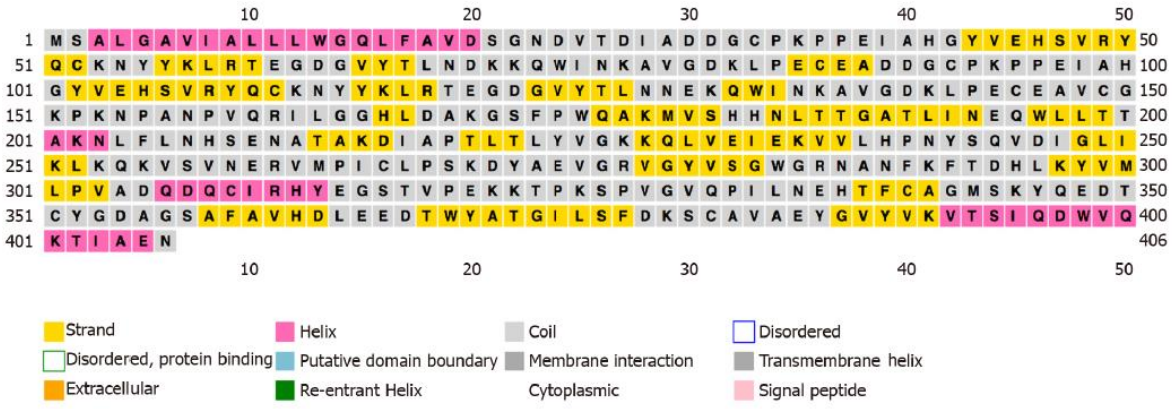


Figure 5. Secondary structure of haptoglobin by PSIPRED tool (Wan et al,2021).

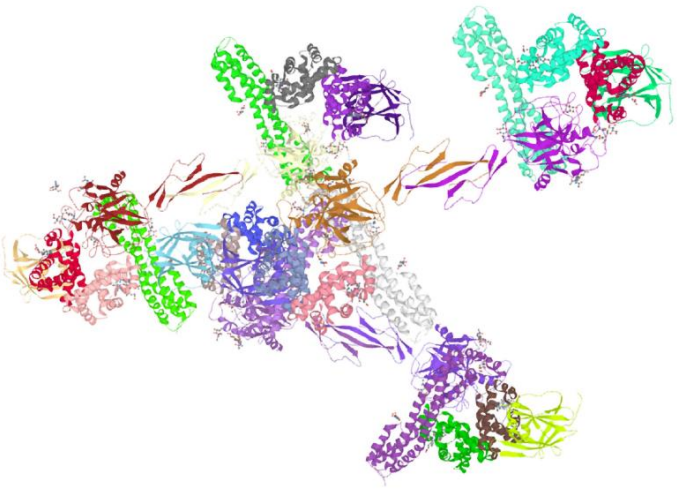


Figure 6. Three Dimensional structure of haptoglobin protein (Wan et al,2021).

The two kinds of domain present in haptoglobin i.e. CCP (Complementary Control Protein) and SP (Serine Protease) domain. A partial catalytic defect in HP prevents it from becoming an active protease. The crystal formation of Hp-Hb molecule is absolved from blood of porcine i.e. from pig or swine blood at 2.9A° resolution. A pig Hp-Hb structure reveals 82% similarity to the human Hp-Hb complex i.e. barbell shape with twice rotational arrangement throughout its venture as shown in Figure 7. HP CCP domain contains residues (33-90) & β-sandwich symmetry as compared to complement factors C1r and C1s. Commonly, CCP domain include four residues of cysteine (Cys) forming to two disulphide bonds but CCP domain of Hp has lack Cys residue. Cys33 residue joined with 2CCP domain. B1and B2 strand bind to form original B1/B2 string forming antiparallel β-sheet. This sheet is joined with other B1/B2 of CCP domain, it is called unnamed admixture structure of CCP domain including six-stranded β-sheet at centre position.

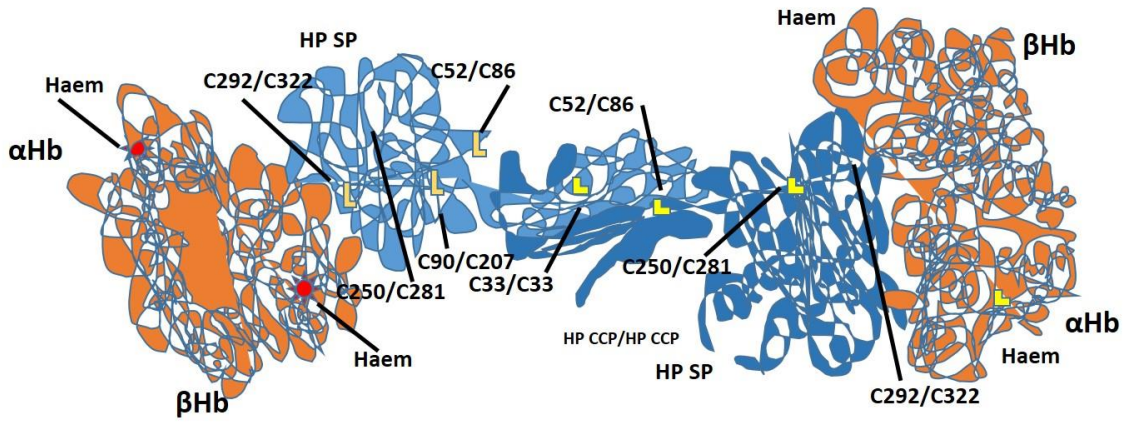


Figure 7. Porcine structure of haptoglobin (Andersen et al,2012).

The tyr^{56} shared H-bond with glu^{135} bind with ala^{88} & val^{199} chain also tyr^{55}, ala^{88}, val^{199} & glu^{201} engaged in Vander Waal interactions. Serine Protease domain is similar with chemo trypsin serine protease; it has two antiparallel β-string main domain which contain six β-strand & three α-helices. The surface of loop demonstrate various functions & structures as compared to serine protease i.e. serine proteases contain sequences (258–274) that are prolonged in loop 3. Due to the mutations occurs in CD163 molecule at four residues i.e. thr^{264}, val^{259}, glu^{261} & lys^{262}, the Hp breaks the CD163 binding. The αHb linkage between loop 3 residues (267–271) may disrupt the loop's structure and regulate the binding of the CD163 scavenging receptor. The absorbance bands α & β observed by UV/VIS spectrophotometer of crystallized porcine Hp-Hb complex at 575nm & 538nm spectra and υ_4 raman mode at 1,377cm^{-1}. The various Hp sites for Hb loops are responsible for the binding i.e. A (121-127), D (226-234), 1 (283-289), 2 (318-327) & 3 (253-277) residues. The Hp serine protease domain's amino terminal region (104-110) residues were responsible for Hb binding. The carboxyl terminal of αHb resemblance enzyme or product observed in complementary factor C1r as well as αHb Arg141 residue is present on the S1 pocket. The Interaction of a functional serine protease with αHb C-terminus in the Hp-Hb complex as well as Hp contacts with both Hb subunit from αHb through the primary sites of helix G, H & FG loop and βHb through helix C, G & FG loop (Andersen et al, 2012 and Bensi et al, 1985). The resolution of αβHb formation for Hb tetramer are lower 1,980A^0 as compared to Hp-Hb interaction i.e. 2,954A^0. The other molecule of Hp binding with Hb include αVal^{1}, αVal^{96}, αLys^{99}, αTyr^{140}, αArg^{141}, βTrp^{37}, βTyr^{145} and these involved in the formation of the oxidation modification in Hb by the hydrogen peroxide.

Hp is linked close to the heme molecule & it keeps the globin moieties in this area stable, that may stop the distribution of heme molecule. The tyr42 residue of αβHb helps in the movement of radical from α1 or α2Hb to β1 or β2Hb but Hp blocks this movement by the dissociation of Hb tetramer. The structure of Haptoglobin related protein (HPr) gene is different with HP1 gene i.e. the first intron has 1.3kb length in Hp1 and 9.5kb long in HPr. Secondly, the HPr Alu residue is found frequently at position -195 within 5'flanking locus, and it contains the RTVL-I (element like retrovirus) (Bensi et al, 1985 and Maeda et al, 1986). The impotency of the HPr gene is caused by RTVL-I, which contains a primer binding site that is similar to the 3'end of isoleucine tRNA. At the 3'end of Hp and HPr genes 620bp nucleotide sequence are identical and other regions of gene are different by an 6.4%. The amount of expression of the upstream components of the HP gene is impacted by tandem repeats or additional copies of HPr (Maeda et al, 1986).

4.4 HP and HPr gene expression

During liver development, the gene expression is switched off and on. Various gene is active in both fetal and adult liver (i.e. α1-antitrypsin, retinol binding protein, APOA1 etc.). While the transcription of α-fetoprotein is shut off in the foetal liver, other genes encoding for α1-acid glycoprotein in adults have active gene expression. HP and others gene expression switched off depending upon the hormonal changing. In vivo, in adult liver nuclei, the gene is expressed, while it is inactive in foetal liver nuclei. Secondly, Human hepatoma cell lines exhibit Hp gene expression i.e. Unlike other hepatoma cell lines, HepG2 i.e. Hep3B. HepG2 is present in the adult hepatocytes and Hep3B is present in the fetal adult hepatocytes. The quantity of interlayer mRNA changes in Hep3B tissue in response to acute phase reactant. In the Hep3B cells Hp gene is not found but the promoter region of this gene has a sensitivity site of DNase-I while Hp in Hela cells lacks Hp mRNA, the HepG2 gene is active (Oliviero and Marrone, 1987).

HPr gene is closely linked with Hp gene demonstrated by the investigation at the DNA level. Two polymorphism occurs i.e. intragenic or partial duplication and complete duplication. In partial duplication presents Hp2 allelic variants i.e. 1700bp band observed with various restriction endonuclease enzyme, on the basis of molecular weight of protein it showed polymorphism. In the complete duplication it showed the polymorphism of two genes i.e. HPr and HP. The Restriction fragment length polymorphism showed the increase or loss of a restriction site without corresponding changes to the restriction map or point mutations found in the EcoRI site for a specific enzyme in HP2 allele and second point mutation occur in PstI in HPr gene. Mutation occur in EcoRI in first intron of HPr and PstI at or near the third exon. The distance between these two mutations are 9500-10,000bp (Oliviero et al, 1985). Yang et al, showed the similarity of mouse Hp gene with human Hp gene. Mouse Hp gene (Chromosome 8) shared 80% identity with human Hp gene (chromosome 16q22). During inflammation the cytokine IL6 expression is lower than the human Hp gene but its expression is high when the human guanine amino acid is replaced to the adenine at position -160 in rat Hp gene as shown in Figure 8 (Yang et al, 1993).

Figure 8. Homology sequence of haptoglobin of human (H), mouse (M).

4.5 Different roles of Haptoglobin

Haptoglobin plays an important role in angiogenesis process in both in vitro and in vivo pattern through the production of gelatine-based matrix to increase the cell formation. Haptoglobin supresses the heat & stress activate precipitation of isolated proteins by making higher molecular weight compound in misfolded proteins. Chaperon functions is different in different kinds of Hp proteins i.e. HP1-1 has highest efficacy than HP2-2 molecule. When HP2-2 molecule bind with T4 antigen i.e. a receptor presents on the surface of the streptococcus bacterium, due to agglutination it suppresses the growth of bacteria. The Hp-Hb molecule bind on the surface of Trypanosome brucei parasite, it takes up the heme molecule to increase their growth & blocking the oxidative activity of host. Haptoglobin related protein (HPr) linked with Hb molecule is appliance with TLF i.e. Trypanosome lytic factor, a poisonous compound concerning parasites (Galicia et al, 2011).

4.6 Different techniques to determine the Hp phenotypes and genotypes

By using either starch or polyacrylamide gel electrophoresis, a non-denaturing gel electrophoresis is performed after Hb treated with 10µl serum to produce the Hp types in the forms of bands in the gel. The advantage of this method is to allow only use of plasma or serum of blood and disadvantage is requiring 0.10mg/ml Hp concentration in the serum, as well as time consuming process (Levy et al, 2010). Second technique is PCR amplification, it determines the Hp genotypes Hp1 and Hp2. The identification of HP 2 allele is determined by the presence of exon4 - exon3 junction, the disadvantage is it identification of HP1 is more problematic due to the production of large 1.7kb fragment i.e. 1757bp represented HP1 allele, 3481bp represented Hp2-1 allele and 349bp represented HP2-2 (Koch et al, 2002). Third is ELISA method; it detects the Hp polymorphism on the basis of the antibodies of same binding specificity of various Hp types. It shows 99% similarity with gel electrophoresis technique as well as it shows result >500 samples in one-time operator. But it has disadvantage also the Hp protein binds with the excessive amount of antibody, it produces insensitive test to Hp concentration in the blood sample (Levy et al, 2010).

4.7 Geographical distribution of Hp alleles

The genotypic frequencies of haptoglobin alleles varies in ethnicities. In India, caste wise the frequency of Hp1-1 was 0.05, 0.35 of Hp2-1 and 0.5 of Hp2-2 present. The study on three

population categories i.e. a tribe (Kabui) on 131 individuals, a caste group (Meitei Brahmins) on 262 individuals and a religious group (Muslims) on 335 individuals. The Hp allele was discovered to be strongest in Manipur due to inbreeding rather than a selection mechanism as all three populations live in the same area yet differ greatly from one another (Asghar et al, 2009). Similar results were found when studied on southern Gujrat population on eight tribal populations showed 1% HP2 allele frequency in Pavagadhi Chaudhary & 1.00 minimum frequency found in gamit i.e. 0.812 (Khurana et al, 2011) as shown in Table 5. To determine the different types of Hp gene in type2 diabetic patients, type2 diabetics with cardiovascular patients and control individuals in Egypt population as shown in Table 6. Hp2-2 allele was more prone to risk of cardiovascular disease in type2 diabetics observed on 160 participants. The frequency of Hp1-1 was 16.7%, 40.8% of Hp2-1 and 42.5% of Hp2-2 as well as Hp2-2 showed greater frequency in type2 patients with cardiovascular disease (Hamdy et al, 2014, Levy et al, 2002, Wobeto et al, 2011, Adinortey et al, 2011, Moussa et al, 2014, Kyle et al, 2019 and Pechlaner et al, 2014).

Country (year)	Sample size	Caste	Hp1-1, Hp2-1 (wild type allele frequency%)	Hp2-2 (mutant allele frequency%)	Technique used
India (1976) (Punjab region)	197	Brahmin, Bania, Khatri & Jat	0.05, 0.35	0.5	Starch gel electrophoresis (Sunderland et al, 1976)
India (1969) Western India Northern India	1058 (780) (194) (84)	Western India [Koukani (Saraswats, Christians) Gujarati (Hindus, Parsis) Marathis] Northern India [Hindus	1, 37 2, 51 8, 97 8, 41 5, 30	73 112 380 72 35	Starch gel electrophoresis (Baxi et al, 1969)

Southern India		Muslims] Tamil	3, 31	49	
India (Manipur) 2009	728 (Healthy)	335 Muslims 262 Meitei Brahmins and 131 Kabuis.	2.09,12.83 3.82,25.19 0.76, 18	85.37 70.99 85.50	Polyacrylamide disc gel electrophoresis (Asghar et al, 2009)
Gujarat (2011)	Healthy	Chaudhary, Gamit, Vasava, Dubla, Dhodia, Konkana	3, 37	3	Polyacrylamide gel electrophoresis (Yang et al, 1993)
Mumbai (2018)	Healthy	-	0,80	350	Polyacrylamide gel electrophoresis (Joshi et al, 2018)
Dadra nagar, Haveli (India) (2008)	Healthy	-	2, 4	94	Polyacrylamide gel electrophoresis (Dasgupta et al, 2008)
Singapore (Indian population (2016)	200(160 T2DM,40 Healthy)	-	-	93 (80.87) 22 (19.13)	RT-PCR (Dalan et al, 2016)

Table 5. Haptoglobin allele in India.

Country (year)	Sample size	Hp1-1, Hp2-1 (wild	Hp2-2 (mutant	Technique used	P value

		type allele frequency)	allele frequency)		
Egypt	160 T2DMWCVD	16.7%, 40.8%	42.5%	Starch gel electrophoresis	0.721
Israel (2002)	260CVDWDM, 260 healthy	Hp1-1 (32%in cases, 33% in controls), Hp2-1 (64%in cases, 44% in controls)	50%in cases, 16% in controls	Polyacrylamide gel electrophoresis	0.002 (Hp2-2 vs Hp1-1 & 0.010 (Hp2-2 vs Hp2-1)
Brazil (2011)	50CVDWDM, 70CVD (120 patients) & 142 Controls	Hp1-1 (24.2%in cases, 25.3% in controls), Hp2-1 (47.5%in cases, 42.3% in controls)	28.3%in cases, 32.4% in controls	Allele specific PCR	0.674
Ghana (2011)	290 T2DM, 108 healthy	1.39 odds ratio & 4.54 odds ratio in T2DM	18.27 odds ratio in T2DM	Polyacrylamide gel electrophoresis	0.01
Egypt (2014)	72 T2DM, 48 CVDWDM (120 patients) & 40 healthy	Hp1-1 (16.7%in cases, 40% in controls), Hp2-1 (40.8%in	42.5%in cases, 15% in controls	Allele specific PCR	T2DM vs Healthy is non-significant, CVDWDM vs healthy is p=0.01 &

		cases, 45% in controls)			CVDWDM vs DM is p=0.05
Tunisia (2014)	256 significant coronary stenosis (SCS) & 144 without significant coronary stenosis (SCS)	21.88%, 47.26% (with SCS) 29.16%, 51.39% (without SCS)	30.86% with SCS, 19.45% without SCS	Allele specific PCR	0.033
USA (2017)	385 Diabetes	21%, 46%	33%	Allele specific PCR	0.37
Austria (2014)	826 CVDWDM	10.3%, 41.7%	48%	Allele specific PCR	0.46

Table 6. Haptoglobin allele in other countries.

4.8 **Cardiovascular disease and Diabetic mellitus**

The haptoglobin protein plays a major role in the production of cardiovascular disease. The Munich Stent Study on 935 DM patients found a very strong relation with the genotype (Hp2-2) and an increased risk of serious adverse cardiac events (Costacou et al, 2012). Hp2-2Hb molecule is redox active so it interferes with the oxidative damage. Both HP1-1HB and HP2-2HB complex scavenge the nitric oxide, it acts as an endogenous antioxidant molecule that manage the blood vessels, smooth muscle & platelets activation. In vascular wall, NO dioxygenation of oxy-Hb results in the production of nitrate (NO_3^-) and ferric Hb (Fe_3^+) and when NO to non-liganded ferrous haemoglobin (Fe_2^+) directly binds to iron, it causes iron nitrosylation of deoxyhb. Both reactions result in a drop in NO and explain extracellular Hb acute vasoactivity, which is lowered by HP. Free Hb mediate its deoxygenation or makes it antioxidant (Tripathi, 2007). But the higher steady state concentration of HP2-2, the nitric oxide level is lower, so it allows the HP-HB molecule bind with the other proteins i.e. HDL, APOA1, triglyceride and LDL in serum and to change the functional and structural modifications as shown in Figure 9 (Moreno et al, 2008).

Methemoglobin (metHb) and the LDL molecule were both eliminated from the HP1-1 protein molecule, however HP2-2 still has some of this variant. LDL oxidizes both lipids and protein

as a result of the restricted heme transport from LDL to the HbHP2-2 complex. When Hp protein linked with the molecule of HDL (High density lipoprotein) cholesterol and it binds with the APOA1 (ApolipoproteinA1) where LCAT (Lecithin acyltransferase) enzyme present, it decreases the activity of the enzyme and retard the reverse cholesterol transport causing HDL molecule more immature and proatherogenic. The cardiovascular patients carrying Hp2-2 allele due to collecting of atherosclerosis lesion in the arteries (Frank et al, 2001 and Cahill et al, 2015).

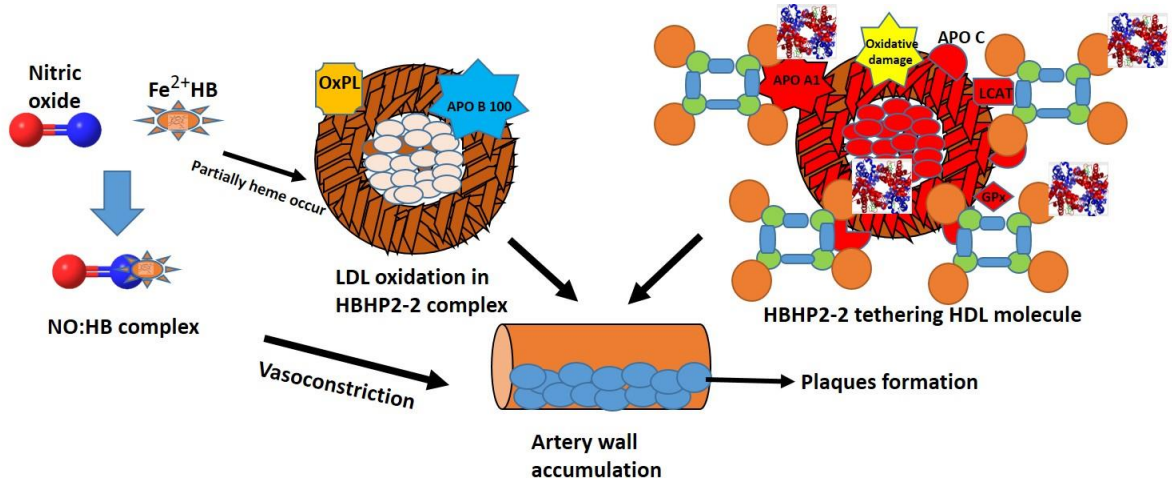


Figure 9. Role of haptoglobin protein in Cardiovascular disease.

The HbA1c (glycosylated haemoglobin) heme pocket's reduced capacity of HP2-2 protein to store heme molecules. In diabetes, compared to the Hb-HP1-1 complex, Hb-HP2-2 molecules are removed more gradually. An elevated level of plasma redox-active Hb-Hp complex might lead to an increased risk of cardiovascular disease in persons who also had the HP2-2 genotype and HbA1c≥6.5%. Additionally, connected to the HDL (high-density lipoprotein) molecule, this complex also reduces activity of antioxidant and elevated oxidation of lipoprotein from transport of heme while impairing HDL's ability to stimulate reverse cholesterol transport (RCT) as shown in Figure 10 (Carew et al, 2020).

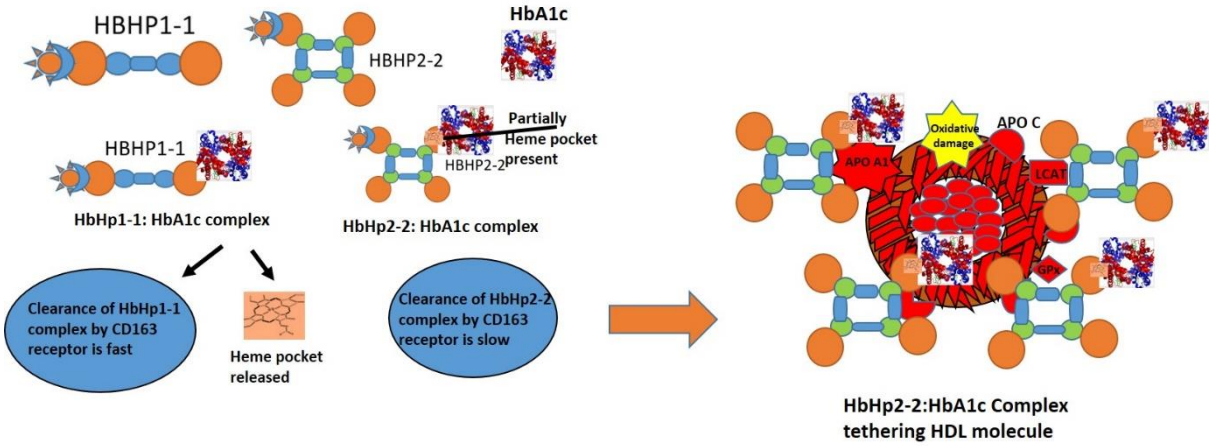


Figure 10. Progression of Cardiovascular disease in Diabetic mellitus.

But the Bruneck study does not confirm these findings (Pechlaner et al, 2014). The another issue for the production of type2 diabetics were oxidative damage. Hp1-1 has antioxidant properties, Hp2-1 is less antioxidant and Hp2-2 is oxidative. Hp2-2 allele is more prone to type2 diabetics and found statistical difference with control subject in northern Han Chinese populations observed on 1274 participants. The high level of lipid profile, blood pressure fasting glucose, systolic blood pressure and low level of diastolic blood pressure observed. The frequency of different alleles was Hp2-2 is 51.7%, Hp2-1 is 39.7% and Hp1-1, 8.6% respectively (Shi et al, 2012). Same results found rather than frequencies in Ghanian on 398 individuals (Adinortey et al, 2012). In the Brazilian population observed on 120 participants, Hp1-1 allele is more prone to refractory hypertension in type2 diabetic patients found significantly with higher systolic, diastolic blood pressure, total cholesterol and low density lipoprotein (Wobeto et al, 2011).

4.9. Relation of Apolipoprotein in Diabetes mellitus and Cardiovascular disease

A 267-aas prepro-apolipoprotein called apolipoproteins A-I is the first form of the protein and it is released in an immature state when the first 18 amino acids are removed, resulting in pro-ApoA-I (amino acid 19-267). Eventually, the pro-peptide is eliminated during the anagen phase, leaving the final ApoA-I with 243 amino acids as well as a 28 kDa molecular mass (aas 25-267). It is the most abundant protein within HDLs, accounting for responsible for the synthesis of HDL, making over 70% protein, restructuring, and processing. In addition to its structural relevance, ApoA-I plays a crucial role in reverse cholesterol transport by boosting the outflow of cholesterol into HDLs from surrounding cells and encouraging LCAT. In the course of reverse cholesterol transport, ApoA-I goes through a process of particle

reconfiguration in which it transforms from a lipid-free enzyme to spherical HDL particles. To begin, in order to produce immature HDLs without lipids, ApoA-I uses ABCA1 to get phospholipid and good cholesterol. Second, LCAT produces mature, spherical HDLs and cholesteryl ester (CE) by esterifying cholesterol on developing HDL. Rabbits were injected with identical VHDLs (VHDL high in ApoA-I, HDL3) slowed atherosclerotic plaque development and promoted atherosclerotic plaque reduction without changing HDL-cholesterol plasma levels in pioneering experiments. ApoA-I is also in charge of the liver SRB1 receptors detection of HDLs. ApoA-I levels, out of all the apolipoproteins, are linked to HDLs' atherosclerosis-preventive effects. The ApoA-I profile was investigated using proteomics and lipidomics, and significant alterations in its transcript dispersion pattern were discovered in diabetic individuals. Patients with diabetes have a markedly elevated level of ApoA-I (1-38). After translation, ApoA-I undergoes several environmentally favorable alterations. These changes include oxidation and fatty acid acylation. The generation of advanced glycation end products (AGE) and non-enzymatic protein glycation have attracted a lot of attention in diabetic patients. Both are associated with high blood sugar levels in diabetic mellitus. AGE conjugates to ApoA-I and prevent it from turning on LCAT, an enzymatic process that transforms proliferating HDLs into adult HDLs. AGEs appear to hasten the progression of CAD. The fewer evolved isoforms of the protein are the most fundamental forms, according to tests on ApoA-I that changed the protein. Diabetics were seen to have a boost in the less established, more basic kind of ApoA-I. HDL linked with ApoA-I is degraded both at the terminals N and C in coronary patients by macrophage metalloproteinase. Surprisingly, ApoA-I residues (1-38), which lacks residues (1-38), is discovered linked with LDL lipoproteins rather than VLDL or HDL micelles in blood samples from diabetic individuals. Research conducted in vitro indicates that the simulated removal of ApoA-I residues 1–43 leads to a fewer constant tertiary shape than the complete Apolipoprotein-A1. Removal variants lacking amino acids 1-41 and 1-59 in ApoA-I shown a decreased ability to bind lipids related to wild-type ApoA-I. The stability of soluble ApoA-I is assumed to depend on the 44-aa sequence located at the N-terminal of ApoA-I and amino acid 35–49 have recently been linked to ApoA-I flexibility to HDL particle size. Consequently, the loss of residue 38 initial amino acids (aas) present in ApoA-I (1-38) may influence how lipid-free ApoA-I transforms into particle in sphere form of HDL, impeding reverse cholesterol transfer and boosting LDL particle attachment. In contrast to the extended ApoA-I, in vitro tests have shown that the shortened ApoA-I form has a decreased capacity to attach to HDLs while having a significant increase in its ability to bind to LDLs. Because ApoA-I (1-38) is not associated with VLDLs, our findings

suggest that following truncation, ApoA-I (1-38) is selectively bound to LDLs rather than being incorporated into LDLs during particle production. HDL particles become less stable when the N-terminal amino acids are missing because they are essential to the formation of huge HDL complexes. Prior studies have shown that ApoA-I is present in LDL particles, despite the fact that it has previously been connected to HDLs as the main protein element in these micelles. In the current study, independent of baseline medication, those with diabetes exhibited significantly lower total ApoA-I blood levels as compared to healthy individuals, as measured by ELISA. The existence of cleaved ApoA-I variations is linked to the development of chronic illnesses such diabetes, atherosclerosis, and ageing. The increase in ApoA-I (1-38), a 26 kDa N-terminally shortened version of ApoA-I that is associated with bigger LDL granules in diabetic individuals, may affect the turnover of lipoprotein particles, make LDL more susceptible to oxidation, and raise cardiovascular risk (Cubedo et al, 2015 and Amoorthy et al, 2017). The emergence of chronic diseases including ageing, atherosclerosis, and diabetes is associated with the existence of variations of cleaved ApoA-I. Diabetics often have lipid problems, especially type-2 diabetics. Diabetics with dyslipidemia are more susceptible to coronary heart disease (CHD) and associated atherosclerotic effects. Hyperlipidemia is the most prevalent diabetic consequence, and it puts people at risk for early atherosclerosis and macrovascular problems. High triglycerides, raised serum LDL, raised serum cholesterol, and reduced serum HDL are all common lipid abnormalities in diabetes. Dyslipidemia's significant influence on cardiovascular problems necessitates undivided care throughout the phase of the disease (Zhang et al, 2017).

4.10. **Relationship of Haptoglobin with Anthropometric variables and lipid levels**

In comparison to other Hp2-1, Hp1-1, the Hp2-2 genotype carriers among non-diabetic controls had greater FPG (fasting plasma glucose), SBP (systolic blood pressure), LDL cholesterol, cholesterol and lower DBP (diastolic blood pressure) values. FPG is the most significant indicator for T2DM. While the FPG level among carriers of the HP2-2 genotype was within the normal range, it was noticeably greater than that of carriers of other HP genotypes. It was reported that the HP2-2 a biomarker for the development of FPG. Additionally, HP2-2 genotype was linked to higher ranges of lipid profile, both of which are danger signs of T2DM i.e. $p<0.05$ (Shi et al, 2012). In another study, Hp2-2 patients with or without diabetes tended to have lower FPG levels, this variation was not statistically noteworthy. In either the diabetes group or the control group, participants with various haptoglobin phenotypes had similar mean blood cholesterol, triglyceride, and LDL cholesterol levels. However, compared to the other

subject groups, control participants with the Hp2-1 phenotype had considerably lower HDL cholesterol levels. The blood triglyceride level, which was two times higher in the diabetes participants (p=0.001), was the main difference between the diabetic and control subjects (Quaye et al, 2006).

4.11. Role of Vitamin E in haptoglobin protein

Some diabetes people use vitamin E supplements, which drastically reduce lipid peroxides of HDL molecule and increase the Hp 2-2 DM individual's serum capacity to conciliate cholesterol release in vitro. By identifying patients who would benefit from vitamin E supplementation, HP genotyping may provide an individualized approach to healthcare, significantly lowering the burden of CVD in diabetic disease (Hochberg et al, 2017). In the initial clinical trial, the Israel Cardiovascular Vitamin E research was evaluated in 1434 diabetics with the Hp2-2 genotype. After 18 months, vitamin E recipients' rates of the major endpoints (stroke, MI, and cardiovascular mortality) were considerably lower (2.2%) than those who received a saline (4.7%). This was further supported by the meta-analysis, which showed the vitamin E possessed beneficial impacts on the heart endpoints in T2DM and the Hp2-2 allele. Consequently, vitamin E's cardioprotectivity may benefit patient groups with more severe diseases with elevated levels of oxidative damage, including those receiving hemodialysis (Seferovic et al, 2018).

4.12. Haptoglobin SNP's

The three promoter SNP's rs5467, rs5470 and rs5471 of Hp were genotyped in diabetic heart study i.e. diabetic with cardiovascular disease patients. The two SNP's rs5470 and rs5471 were linked with altered the Hp expression levels and rs5471 were also associated with Hp2-1 M phenotype in USA observed on 1208 participants (Soejima et al, 2020). The Hp copy number variants (CNV) and cis-acting expression were showed the strongly correlated with cholesterol levels in 22,288 participants in USA. Recurrent deletions into Hp2 allele formed a new Hp1 allele called low linkage disequilibrium (LD) between individual SNP's and Hp alleles. When Hp molecule interrelate with APO E protein, it forges APO E an antioxidant protein. The SNP's rs2000999 and Hp2 increased the level of LDL and total cholesterol (TC). As a result of HP1F and HP1S non-homologous recombination to create the HP2FS i.e. Hp2 allele. It demonstrated how SNP's haplotypes may be used to designate HP subtypes. Two methods to reduce the low LD and nearby SNP's haplotypes were recombination and recurrent deletions. In recombination process, If the structural variation's left and right SNPs had low LD with each

other, then the low LD were formed by homologous recombination close to the Hp CNV zone. In recurrent deletions, If the structural variation's left and right SNPs had low LD with each other, then the high LD were formed by homologous recombination close to the Hp CNV zone. It found that several pairings of SNPs on the opposing the CNV sides were highly related to one another i.e. LD (r^2>0.950) (Boettger et al, 2016, Wan et al, 2021 and Froguel et al, 2012). rs35283911[T], which has an allele frequency of 14.3% and is only found in the HP2 background. The intronic SNP rs2000999[A] in the adjacent gene (HPR) rs35283911[T] has been linked to decreased levels of haptoglobin and is significantly connected with it (Bjornsson et al, 2017).

CHAPTER-V

MATERIALS AND METHODS

MATERIALS AND METHODS

All study participants were required to provide written informed consent, and the study procedure received approval from the Institutional Ethics Committee. On a pre-designed proforma, all research participants provided information on demographic factors, illness history, and other pertinent information. The patients were then separated into four groups: type2 diabetes mellitus, cardiovascular, cardiovascular with type2 diabetes mellitus and controls. Standard methodologies were used to perform the major anthropometric and physiometric measures. All of the research participants blood was drawn and analyzed for DNA (deoxyribonucleic acid) and serum separation. Haptoglobin genotyping was performed by Allele specific or ARMS (Amplification Refractory Mutation System-Polymerase Chain Reaction) PCR and Sanger sequencing. The Samples were outsourced to Sequencing Department, Bioserve Biotechnologies India Pvt Ltd. Hyderabad, Telangana. Various computational approaches were used to determine the structural and functional consequences of haptoglobin polymorphisms. A total of 54 SNP's were extracted randomly from dbSNP, NCBI, and SNPedia databases which include 34 missense variants, three inframe deletions, 27 intron variants, 4 non-coding transcript exon variants, five 5'UTR variants, one 3'UTR variant, and 3 intergenic variants. Standard methods were also used to quantify lipid profile variables.

5.1. Sample size calculation

A significant sample size is required to conduct genetic studies. The sample size for this investigation was estimated by using the formula: $n=Z^2*P*Q/D^2$, based on assumptions of an 8.8% diabetes prevalence in India, cardiovascular disease prevalence in India is 11% and an odds ratio of 1.5 ($p\leq0.05$, CI-95%) and Cardiovascular with Diabetics is 21.4%. Diabetic mellitus (DM) were 123 patients, Cardiovascular (CVD) were 150 patients, Cardiovascular with Diabetics (CVDWDM) were 64 and control (N) were 130 participants were included.

5.2. Performa designing

An extensive review of the literature on haptoglobin, T2DM, cardiovascular and cardiovascular with type2 diabetes was conducted. A questionnaire was created to collect important data from T2DM, cardiovascular and cardiovascular with type2 diabetes patients and healthy controls. To reach the patients, a survey of several local hospitals, clinic as well as laboratories were conducted.

5.3. Ethical clearance and informed consent

The Institutional ethical committee of lovely professional university, Phagwara, (LPU/IEC/2018/01/01) and Punjab Institute of Medical Sciences (PIMS/IEC/21/07) investigated and confirmed the research proposal for this study and each participant provided written informed consent.

5.4. Inclusive criteria

The patients diagnosed with diabetes mellitus on the basis of American Diabetes Association criteria (ADA, 2015) on the basis of glycosylated haemoglobin (A1C) ≥6.5% and aged 26-75 years, were enrolled (males and females) from North India. Healthy individuals without history of any chronic disease were included in this study (Shi et al,2012).

5.5. Exclusive criteria

Patients with a history of diabetic retinopathy, gestational diabetes was excluded from the study. The study eliminated participants who were from east, west and south Indian states, pregnant ladies, nursing mothers and with age <26 and >75 y and children were excluded from the study (Hamdy et al,2014).

5.6. Data collection

After discussing the research's goal, process, advantages, and dangers, all study participants (or their associated person, if the subjects were unable to provide it) signed a written voluntary informed consent form. On a pre-designed proforma, all research participants filled out information on different demographic factors, illness history, smoking/alcohol use status, and other relevant information. Name, gender, age, father/name, mother's siblings, and home address were among the demographic information. The patient/accompanying person and/or clinical records gave information on the patient's illness history and medicines. Anthropometric and physiometric measures were also taken of the subjects. All participants taken from the clinic, labs as well as hospitals. (Adinortey et al, 2011 and Wobeto et al, 2011).

5.7. Collection of blood sample

All research participants had 5 ml of venous blood collected from their antecubital vein through venipuncture after fasting for 8-9 hours. 1 ml of blood was transferred to an EDTA-coated vial for DNA isolation, and the rest was transferred to a plain vial for serum separation (Levy et al, 2002). For future record keeping, the blood samples were correctly labelled with pre-determined unique codes. Blood samples were transported in ice packs from the hospital to the

laboratory and then maintained at 4°C until further examination. The Biochemical Waste Management and Handling Rules were used to handle all biochemical waste.

5.8. Anthropometric and physiometric parameters

Anthropometric measurements were taken with subjects in light clothing and without shoes. It includes height, weight, waist circumference (WC), hip circumference (HC) was taken using standard techniques. Steel tape was used to determine height. Participants were asked to stand with their backs to the wall, without shoes. Participants were weighed using a calibrated weighing machine while dressed in light clothing and without shoes. After exhaling at a location halfway between the iliac crest and the lower rib edge, the participant's WC was measured in standing posture. With the person standing upright and both feet together, HC was measured around the broadest area of the buttocks. The usual equations were used to compute the body mass index (BMI) and waist hip ratio (WHR) from the above data. Following that, the subjects were divided into two groups: normal and overweight/obese, based on BMI, WC, and WHR.

A typical mercury sphygmomanometer with the proper cuff size was used to measure physiometric variables such as systolic blood pressure (SBP) and diastolic blood pressure (DBP).

5.9. Serum separation from blood

Blood was collected from participants by the method of venipuncture described by Hill and Buckle (1956), in order to minimize the risk of hemolysis. Immediately after the collection of sample, keep the blood at room temperature for about 10 minutes to form blood clot. After the serum was isolated from whole blood by centrifugation at 2000 rpm for 10 minutes. Remove the serum from the clot by gently pipetting off into a clean tube using micropipette. Then, label with donor name and date of collection of blood. Store at -20°C.

5.10. Biochemical parameters

Biochemical parameters like blood glucose level, Apo-lipoprotein A1, HbA1c and lipid profile.

5.10.1 Total Cholesterol

The total cholesterol in the trial participants' serum samples was determined using the kit's manual technique. This test is based on the idea that cholesterol and fatty acids are formed from the hydrolysis of cholesterol esters in the presence of the enzyme cholesterol esterase (CE). In

the presence of cholesterol oxidase (CHOD), the cholesterol generated is further oxidised to yield cholestenone and hydrogen peroxide (H2O2). In the presence of peroxidase (PDO), the H2O2 is recognised by phenol-4-aminoantipyrine (4AAP), a chromogenic oxygen acceptor, leading in the production of a red coloured complex, quinoneimine. The amount of quinoneimine generated is related to the amount of cholesterol present in the blood sample.

The estimation of cholesterol involves the following enzyme catalysed reactions.

- Cholesterol ester $\xrightarrow{CE}$ Cholesterol + fatty acid
- Cholesterol + O_2 $\xrightarrow{CHOD}$ cholest-4ene-3one+H_2O_2
- $2H_2O_2$ + 4AAP + Phenol $\xrightarrow{POD}$ $4H_2O$ + Quinoneimine

CE: Cholesterol esterase

CHOD: cholesterol oxidase

4AAP: M4-aminoantipyrine

Methodology: (CHOD/PAP method)

Reagents: cholesterol reagent and cholesterol standard (200mg/dl).

Method:

Pipette into tubes mark	**BLANK(µl)**	**STANDARD(µl)**	**TEST(µl)**
Cholesterol reagent	500	500	500
Distilled water	5	-	-
Cholesterol standard	-	5	-
Sample	-	-	5

Table 7. Procedure of total cholesterol

Mix and well incubate at 37°C for 5 min. aspirate blank followed by standard and tests. Read the absorbance of standard and each test tube against blank at 505nm on biochemical analyser.

5.10.2 Triglyceride

The instructional technique included with the commercially available kit was used to estimate TG. This test is based on the idea that triglycerides are digested to create glycerol and fatty acids in the presence of the enzyme lipase. Glycerol kinase converts the glycerol generated into glycerol-3-phosphate. In the presence of glycerol phosphate oxidase, the glycerol-3-phosphate is then oxidised to dihydroxyacetone phosphate (DAP) and hydrogen peroxide. In the presence

of peroxidase, the freed H2O2 is recognised by chlorophenol-4-aminoantipyrine, a chromogenic oxygen acceptor, leading in the creation of a red colored complex called Quinoneimine. The amount of Quinoneimine produced is related to the amount of triglycerides in the blood sample.

Triglyceride + H_2O $\longrightarrow$ Glycerol + free fatty acid

Glycerol + ATP $\longrightarrow$ glycerol-3-phosphate + ADP

Glycerol-3-phosphate +O_2 $\longrightarrow$ DAP + H_2O_2

H_2O_2 + 4AAP + 3,5 DHBS $\longrightarrow$ Quiononeimino + $2H_20$

LPL: Lipoprotein lipase

GK: glycerol kinase

GPO: glycerol phosphate oxidase

DAP: dihydroxyacetone phosphate

ATP: adenosine triphosphate

4'AAP: 4- aminopyrine

DHBS: 3,5- Dichloro-2-hydroxy benzene sulfonate

Methodology: (CHOD/PAP method)

Reagents: Triglyceride reagent and triglyceride standard (200mg/dl).

Method:

REAGENT	BLANK(µl)	STANDARD(µl)	TEST(µl)
Working reagent	500	500	500
Distilled water	5	-	-
Standard	-	5	-
Serum	-	-	5

Table 8. Procedure of Triglyceride

Mix and well incubate at 37°C for 5 min. Aspirate blank followed by standard and tests. Read the absorbance of standard and each test tube against blank at 505nm on biochemical analyzer.

5.10.3. High Density Lipoprotein (HDL)

Calculated the HDL level by using Friedewald formula: HDL= Cholesterol/5

5.10.4. Very Low Density Lipoprotein (VLDL)

Calculated the VLDL level by using Friedewald formula: VLDL= Triglyceride/5

5.10.5. Low Density Lipoprotein (LDL)

Calculated the LDL level by using Friedewald formula: LDL= TC-HDL-TG/5

5.10.6. Apolipoprotein A1

It's a turbidimetric immunoassay for apoa1 determination that works on the agglutination reaction concept. Allow the test material to react with Quantia-Apo A1 activation buffer (R1) and antibody reagent (R2). When ApoA1 is present in the test material, it forms an insoluble compound that causes an increase in turbidity, which is detected at 340nm. The concentration of ApoA1 in the test material correlates to the rise in turbidity.

Methodology: Immunoturbidimetric method (Quantia kit).

Reagents: APOA1 reagents and APOA1 calibrator.

Method:

Blank set with zero. Took 400µl APOA1 working reagent (R1) added into 10µl diluted serum (450µl normal saline + 50µl serum). Mix and well incubate at 37°C for 5 min. Then, added 100µl APOA1 working reagent (R2) mix and read the absorbance of test tube against blank at 340nm on biochemical analyzer.

REAGENT	BLANK(µl)	Normal Saline(µl)	TEST(µl)
Working reagent (R1)	400	50	-
Working reagent (R2)	100	-	-
Diluted Serum	-	450	50

Table 9. Procedure of APOA1.

5.10.7. HbA1c

It's a turbidimetric immunoassay for determining HbA1c that works on the agglutination reaction concept. After treatment with haemolysing solution, the test specimen is allowed to react with latex reagent (R1). Total Hb and HbA1c attach to latex particles with high affinity. The quantity of binding is proportional to both compounds' relative concentrations in blood. After allowing the reaction mixture to react with anti-human HbA1c antibody (R2), the agglutination response is evaluated at 630nm absorbance. The rise in absorbance in the test specimen corresponds to the concentration of HbA1c.

Methodology: Immunoturbidimetric method (Quantia kit)

Reagents: HbA1c reagents and Haemolysing solution.

Method:

Blank set with zero. Took 500µl haemolysing solution added into 10µl whole blood. Mix and well incubate at 37°C for 5 min. Then, added 400µl HbA1c working reagent (R1) mix and rest at room temperature for 5 min. again added HbA1c working reagent (R2) mix and read the absorbance of test tube against blank at 630nm on biochemical analyzer.

REAGENT	BLANK(µl)	Haemolysing solution (µl)	TEST(µl)
Working reagent (R1)	400	-	-
Working reagent (R2)	100	-	-
Haemolysing solution	-	500	10

Table 10. Procedure of HbA1c.

5.10.8. Blood Glucose Level

Blood Glucose level was measured by a standard glucometer machine.

5.10.9. Atherogenic Indices

The following equations were used to generate the atherogenic indices from the lipid profile data listed above. This calculated by the following formula:

Atherogenic index of plasma (AIP) = TG/HDL-C

5.10.10. Diagnosis of Dyslipidemia

According to the American Diabetes Association (ADA) recommendations (2014), dyslipidemia was defined as LDL-C levels >110 mg/dl, triglyceride levels >150 mg/dl, and HDL-C values <50 mg/dl (for females) and <40 mg/dl (for males) in each participant.

5.11. DNA extraction and quantification

With slight adjustments, DNA was extracted from peripheral blood mononuclear cells using an inorganic technique (Miller et al., 1988). Yield gel electrophoresis was used to measure DNA.

5.12. DNA extraction from Blood

400µl of blood were combined with 1200µl of RBC lysis buffer and vortexed on a rocker until a transparent, glossy solution was formed. The fluid was then centrifuged for 2 minutes at

10,000rpm. After discarding the supernatant, a white pellet of white blood cells (WBCs) was recovered. The vial was then filled with 300µl of WBC lysis buffer, and the pellet was adequately disrupted using a micropipette. To the aforementioned solution, 20 µl of 10% sodium dodecyl sulphate (SDS) was added and incubated at 56°C for 30 minutes in an incubator. The solution was then centrifuged at 13,000rpm for 15 minutes after 150 µl of ammonium acetate was added. After centrifugation, the clear supernatant was transferred to a new vial, followed by the addition of a double volume of chilled ethanol, and invert to precipitate then centrifuged at 10,000rpm for 15 minutes. The supernatant was removed, and the DNA pellet recovered was washed in 70% ethanol (150µl). The centrifugation was done for 15 minutes at 10,000rpm. The supernatant was then discarded, and the pellet was dried for 10-15 minutes at room temperature. After that, the dry pellet was suspended in 50 µl of Tris-EDTA buffer and incubated for 15 minutes at 65°C. Until further processing, the extracted DNA was kept at -80°C.

5.13. Quantification of extracted DNA

Yield gel electrophoresis on a 0.8 percent was used to quantify the freshly isolated DNA samples. 0.32 g agarose and 40 ml of 1 X TAE buffer were used to make the agarose gel. After heating the materials for 2 minutes, a clear gel solution was produced. The solution was then heated to 40-45°C before adding 3µl of ethidium bromide (EtBr) to it. The gel was put into a pre-assembled casting tray with combs and left aside for 30-40 minutes to polymerize. The gel was then placed in an electrophoretic chamber with 1 X TAE buffer. Following that, 5 µl of DNA and 2 µl of gel loading dye were mixed together and placed in to each well. The standard marker was 2µl of DNA put into the first well. For 20 to 25 minutes, the gel was run at 100 V. The gel was removed and photographed once electrophoresis was completed.

5.14. Genotyping of Haptoglobin

The genotyping analysis of haptoglobin was determined by using AS-Polymerase chain reaction (AS-PCR) as described by Koch et al,2002 with some variations. Primer C (5'-CCTGCCTCGTATTAACTGCACCAT-3') and Primer D (5'CCGAGTGCTCCACATAGCCATGT-3') were used to amplify an HP2-2 allele specific sequence of 349bp. PCR products were resolved by 1.5% agarose gel electrophoresis (Pechlaner et al, 2014). AS-PCR is a technique that relies on allele-specific primers that may be used to successfully assess single nucleotide polymorphism (SNP) such as transition, transversion, and insertion/deletion polymorphism. It has been applied in illness research,

molecular diagnostics, and forensic biological evidence. The particular primers in this strategy are intended to allow DNA polymerase amplification only if the nucleotide at the 3'-end of the primer exactly complements the base at the variant or wild-type sequences.

Conditions	Temperature	Time
Initial Denaturation	94°C	3min.
Denaturation	94°C	30sec.
Annealing	69°C	30sec.
Extension	72°C	30sec.
Final Extension	72°C	7min.

Table 11. Conditions used for AS-PCR

Components	Stock solution	Working solution
PCR master mix	Thermofisher scientific	10µl
PCR water	Thermofisher scientific	8µl
Forward primer	Saha gene	0.5µl
Reverse primer	Saha gene	0.5µl
DNA template	50ng	1µl
Total reaction	-	20µl

Table 12. Components of PCR and its concentrations

5.15. Sanger sequencing

The most extensively used sequencing technology was developed by Frederick Sanger and colleagues (1977). It includes DNA polymerase selectively incorporating chain-terminating dideoxynucleotides (ddNTPs) during in vitro DNA replication, and it was initially marketed by Applied Bio-systems. DNA template, dNTPs, a primer, DNA polymerase, and ddNTPs are the essential components of Sanger sequencing. For signal identification on automated sequencers, the ddNTPs are tagged fluorescently or with radiolabels.

5.16. To study changes in the structure of heme and haptoglobin protein due to observed SNP's by using bioinformatics tools:

- **Data mining for identification of SNP's in heme and haptoglobin for Diabetes and CVD.**

The SNP's of haptoglobin and heme were retrieved by using dbSNP, NCBI and SNPedia databases(http://www.ncbi.nlm.nih.gov/snp/andhttps://www.snpedia.com/index.php/SNPedia

) with a minor allele frequency (MAF) of 0.05% (5%) by using Ensembl genome browser release 48 (http://www.ensembl.org/). The rare variant MAF≤0.05 emerged usually in coding regions than frequent variants MAF≥0.05.

To evaluate the alignment of heme and haptoglobin with their respective sequence structure by analyzing the Clustal omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/).

- **Mapping of selected SNP's on their respective structures.**

To retrieve the information about the protein sequence of heme and haptoglobin by using Uniprot database (https://www.uniprot.org/). The RCSB-PDB database was used to analyse the three dimensional structure of the protein (https://www.rcsb.org/) and the mutations was mapped by using Pymol offline software (www.pymol.org) (Duarte et al, 2018 and kalia et al, 2016).

- **Predicting the effect of mutations in their respective structures and functions**

A. Non-synonymous SNP's functional analysis

Non-synonymous SNPs (nSNP's) was analyzed by using online available servers i.e. SIFT (Sorting Intolerant from Tolerant), PolyPhen2 (Polymorphism Phenotyping v2), SNAP2 and Mutation accessor (Duarte et al, 2018, Hepp et al, 2015 and kalia et al, 2016).

SIFT: SIFT is a multiplex algorithm that cover a sequence homology-based approach to evaluate the amino acid residues. A total number of SNP's (rsIDs) added for search with default settings. SIFT results were retrieved as damaging (0.00-0.05), potentially damaging (0.05-0.10), borderline (0.10-0.20) or tolerant (0.21-1.00) (https://sift.bii.a-star.edu.sg/www/SIFT_dbSNP.html).

PolyPhen2: Polyphen2 tool was used to check the effect of an amino acid residue on both the shape and structure as well as role of the protein by prediction of multiple sequence alignment and protein 3D structure. Uploaded a fasta sequence of a protein (rsID) with default settings. Polyphen2 results were collected as probably damaging, possibly damaging or benign with the alignment score from (0–1). "Score" is the possibility of the substitution being damaging; "sensitivity" and "specificity" equal to prediction affirmation. (Duarte et al, 2018 and kalia et al, 2016).

Mutation accessor: The mutation accessor server based on evolutionary information (FIS) was analyse in a functional impact score. A list of Uniprot protein accession and rsID uploaded

with default settings. The results were retrieved as neutral, low, medium or high (Hepp et al, 2015).

SNAP2: The SNAP2 (screening for non-acceptable polymorphisms) program consists of evolutionary data, predicted protein configuration, and to make predictions about the process of mutated proteins. Put a fasta sequence of a protein and run prediction. The results were "having an effect" or "being neutral", and a score, associated with the strength of the change, is given for each substitution along with the percentage of awaited accuracy (Duarte et al, 2018).

B. Synonymous SNP's functional analysis

It predicts the consequence of non-coding SNPs at TFBS (Transcription factor binding site), consensus sequences (splice sites), exonic splicing enhancers (ESE's) and mRNA binding levels by using Regulomedb, PolymiRTS, and SNPinfo (FuncPred).

Regulomedb: It a database used to identify the protein binding sites, transcription factor binding domain and regulatory binding motif variations of nucleotide variants. A list of rsIDs search for prediction. The interpretations were variants are likely to affect binding and mutants are less likely to affect binding and variants have minimal binding evidence (Boyle et al, 2012 & kalia et al, 2016).

SNPinfo (FuncPred): It gives the prediction of SNP's may have effects at the functional level. Uploaded rsIDs and search with default settings. The outcomes were expressed as transcription regulation by affecting transcription factor binding sites activity (TFBS), changing of splicing pattern, exonic splicing enhancers (ESS), changing of protein structure and regulation of protein translation by affinity microRNA (miRNA) binding sites activity (Xu and Taylor,2009).

PolymiRTS: Polymorphism in microRNA predicted the information of SNPs at molecular physiological, behavioral and disease phenotypes level. SNP's rsIDs was listed as analyze information and acknowledge to PolymiRTS (v3.0). 3'UTR SNP's rsID creating or burst miRNA site was accessed (Ziebarth et al, 2012).

Statistical analysis

The significant difference in SNP's were evaluated by using statistical tool SPSS software. The categorical variables were detected by Chi-square test. The mean and standard variation were

detected by descriptive statistics as well as comparison of two different variables by student t-test.

CHEMICALS	FIRM
Agarose	Loba Chemie
Ammonium chloride	Loba Chemie
Ammonium acetate	Loba Chemie
Ethanol Absolute	Loba Chemie
Ethidium bromide	Loba Chemie
Ethylene diamine tetra acetate (EDTA)	Loba Chemie
Glacial Acetic acid	Loba Chemie
HCL	Loba Chemie
Lambda DNA	Thermofisher
Sodium dodecyl sulphate (SDS)	Loba Chemie
Sodium hydroxide (NaOH)	Loba Chemie
Trichloroacetic acid	Loba Chemie
100bp DNA ladder	Thermofisher

Table 13. List of Chemicals and kits available for purchase

Kits	Firm
Apolipoprotein A1	Quantia kit, Coral
Cholesterol estimation kit	Quantia kit, Coral
Glycosylated Haemoglobin (HbA1c)	Quantia kit, Coral
Triglyceride estimation kit	Quantia kit, Coral

Table 14. Commercially Available Kits

Preparations of buffers and reagent

DNA extraction Reagents

Tris-Hydrochloric acid (Tris-HCl) (1 M): 6.057g of Tris (hydroxymethane) aminomethane was dissolved in distilled water and pH was adjusted to 8 with 1N Hydrochloric acid. The final volume was made-up to 50 ml using distilled water.

Ethylenediaminetetra acetic acid (EDTA) (0.5 M, pH 8.0): 9.306g of EDTA, disodium salt was dissolved in distilled water and pH was adjusted to 8.0 with 1N sodium hydroxide solution. Afterwards the pH adjustment, the final volume was made-up to 50 ml using distilled water.

Ammonium chloride (NH4Cl) (1 M): 2.67g of NH4Cl salt was dissolved in 50 ml distilled water.

RBC lysis buffer (pH 8.0): 10mM Tris-HCl (0.5ml), 1mM EDTA (0.1ml) and 125mM NH4Cl (6.25ml) were dissolved in distilled water to make the final volume of 50 ml.

WBC lysis or Tris-EDTA buffer (pH 8.0): 10mM Tris-HCl (0.5ml), 1mM EDTA (0.1ml) were dissolved in distilled to make the final volume of 50 ml.

SDS solution (10%): 10.00 g of SDS salt was dissolved in pre-warmed distilled to make the final volume of 50 ml.

Ammonium acetate (7.5 M)

28.9g of ammonium acetate salt was dissolved in distilled to make the final volume of 50 ml.

Ethanol (70%): 70 ml of the ethanol (absolute) was added in distilled water to make final volume of 100 ml and store at 4°C.

Ethanol (95%): 95ml of ethanol (absolute) was added in distilled water to make final volume of 100ml and store at 4°C.

Agarose gel electrophoresis reagents

TAE buffer (50 X): 48.4 g of Tris base, 11.42 ml of glacial acetic acid and 3.72 g of EDTA were dissolved in distilled to make the final volume of 200 ml.

Agarose gel: Required amount of agarose (0.8%, 1.5) was dissolved in 1 X TAE buffer and heated for 2 min. After cooling down, 3µl of EtBr (10 mg/ml) was added and the gel was poured into the casting tray containing the combs. After that the gel was allowed to polymerize for 30-40 min.

Loading Dyes: 0.25 g of Bromophenol blue (0.25% w/v) added in 100 ml of distilled water.

100 bp DNA ladder: 10µl of 100 bp ladder stock (500µg/ml) and 16.6µl of loading dye (6 X) were added in distilled water to make the final volume of 100µl.

CHAPTER- VI

RESULTS

RESULTS

It is a case control study in which 467 volunteers participated. The statistical significance of variables and mean ± standard deviation was evaluated by student t-test as well as comparison of categorical variables were determined by chi-square test. The Basic demographic data, biochemical as well as physiometric of all participants as shown in [Table 15 & 16], the mean age of T2DM (52.9±10.733), CVD (50.92±11.761) and CVDWDM (55.72±11.471) and in healthy controls (45.46±13.45) were respectively. The percentage of gender i.e. female and male in T2DM (66% and 34%), CVD (45% and 55%), CVDWDM (53% and 47%) and in healthy controls (48% and 52%) were respectively. The percentage of smokers and non-smokers in T2DM (18% and 82%), CVD (30% and 70%), CVDWDM (23% and 77%) and in healthy controls (28% and 72%), the percentage of alcoholic and non-alcoholic in T2DM (20% and 80%), CVD (37% and 63%), CVDWDM (33% and 67%) and in healthy controls (46% and 54%), the percentage of tobacco takers and non-takers in T2DM (4% and 96%), CVD (24% and 76%), CVDWDM (22% and 78%) and in healthy controls (12% and 88%), the percentage of dietary habits i.e. Vegetarian and non-vegetarian in T2DM (55% and 45%), CVD (45% and 55%), CVDWDM (33% and 67%) and in healthy controls (38% and 62%) were respectively. The physical activity i.e. low, high and moderate in T2DM (47%, 8% and 45%), CVD (35%, 21% and 44%), CVDWDM (42%, 17% and 41%) and in healthy individuals (52%, 14% and 34%).

The Anthropometric and Biochemical parameters as shown in [Table 15& 16], among anthropometric parameters BMI in T2DM participants was found to be statistically significant as compared to CVD participants and controls i.e. p=0.018 & p=0.003. WC and HC in T2DM was found to be statistically significant as compared to CVDWDM i.e. p=0.001, CVDWDM was found to be statistically significant with CVD individuals as well as healthy controls i.e. p=0.001 & 0.007. WHR in CVDWDM was found to be statistically significant as compared to healthy and CVD i.e. p=0.02 & 0.03. The mean value of FBS was found to be higher and statistically significant in all parameters i.e. p=0.001, 0.002 & 0.003. The mean value of HbA1c was found to be higher and statistically non-significant in CVDWDM as compared to T2DM i.e. p=0.850. The lipid profile parameters i.e. TC and HDL were statistically non-significant in CVDWDM as compared to healthy and T2DM i.e. p=0.434 & p=0.401. TG and VLDL were

statistically non-significant in CVDWDM as compared to healthy individuals i.e. p=0.237. LDL was statistically significant in CVD as compared to healthy individuals, T2DM and CVDWDM i.e. p=0.001, 0.002 & 0.005.

The mean value of APOA1 was found to be higher and statistically significant in CVD individuals as well as CVDWDM participants as compared to healthy individuals i.e. p=0.002 & 0.038. MAP and PP were statistically non-significant in T2DM as compared to healthy i.e. p=0.651 & p=0.567. SBP and DBP were statistically non-significant in T2DM as compared to healthy i.e. p=0.902 & p=0.392 and DBP was also statistically non-significant in CVDWDM as compared to CVD i.e. p=0.065. AIP in T2DM participants was found to be statistically significant as compared to CVD participants, CVDWDM and controls i.e. p=0.008, 0.014 & 0.004. Majority of the participants were obese according to BMI categories. However, higher prevalence of obesity was observed in T2DM participants as compared to controls and it showed statistical difference i.e. p=0.048 as shown in Table 17.

The extracted DNA was evaluated by 0.8% agarose gel electrophoresis as shown in figure 11. Out of 467 participants, the high aspect of the extracted DNA utilized in PCR reaction obtained one fifty-three 349bp HP2-2 allele products, of which 63 were diabetics, 55 were cardiovascular, and 35 were cardiovascular with diabetic patients but no product was found in healthy individuals as shown in figure 12.

The correlation of anthropometric, physiometric as well as biochemical parameters with haptoglobin2-2 allele in T2DM, CVD and CVDWDM as shown in Table 18-26. In Diabetic participants; TC, HDL, TG, VLDL, smokers, non-smokers, tobacco takers, non-takers, physical activity and FPG were statistically significant i.e. p=0.001,0.002,0.003 & 0.004. In cardiovascular participants; smokers, non-smokers, tobacco takers, non-takers, physical activity and dietary habits were statistically non-significant i.e. p=0.2,0.6 & 0.8. In cardiovascular with diabetic mellitus participants; TC, LDL, VLDL, APOA1, smokers, non-smokers, SBP, DBP, MAP and PP were statistically significant i.e. p=0.001,0.002,0.003 & 0.004.

PARAMETERS	Type2 Diabetes mellitus(N=123)	Cardiovascular (N=150)	Cardiovascular with Diabetes mellitus (N=64)	NORMAL (N=130)
AGE	51.37±11.924	51.89±13.079	54.60±12.05	45.52±12.936
GENDER F **M**	81(66%) 42(34%)	67(45%) 83(55%)	34(53%) 30(47%)	62(48%) 68(52%)
SMOKERS **NON-SMOKERS**	23(18%) 101(82%)	45(30%) 105(70%)	15(23%) 49(77%)	37(28%) 93(72%)
ALCOHOLIC **NON-ALCOHOLIC**	24(20%) 99(80%)	55(37%) 95(63%)	21(33%) 43(67%)	60(46%) 70(54%)
TOBACCO-TAKERS **NON-TAKERS**	5(4%) 118(96%)	36(24%) 114(76%)	14(22%) 50(78%)	15(12%) 115(88%)
VEGETARIAN **NON-VEGETARIAN**	68(55%) 55(45%)	67(45%) 83(55%)	21(33%) 43(67%)	49(38%) 81(62%)
PHYSICAL LOW **ACTIVITY HIGH** **MODERATE**	58(47%) 10(8%) 55(45%)	52(35%) 31(21%) 67(44%)	27(42%) 11(17%) 26(41%)	67(52%) 18(14%) 45(34%)
BMI (kg/m2)	25.97±5.359	27.43±4.77	27.54±7.52	28.21±6.31
WC (in)	38.46±4.77	38.34±5.23	41.29±5.51	37.90±6.31
HC (in)	40.78±4.82	40.39±5.20	43.35±5.60	39.90±6.31
WHR (cm)	0.94±0.036	0.94±0.010	0.95±0.012	0.945±0.007

FBG (mg/dl)	173.73±84.03	100.99±38.71	217.37±72.45	88.66±10.54
HbA1c (mg/dl)	8.26±2.86	5.16±0.74	8.17±1.86	5.33±1.62
TC (mg/dl)	180.87±49.18	227.5±48.36	174.55±49.04	169.45±39.51
HDL (mg/dl)	36.17±9.83	45.5±9.67	34.91±9.80	33.89±7.90
LDL (mg/dl)	93.52±51.28	140.98±36.69	106.89±37.04	98.39±37.14
TG (mg/dl)	255.87±222.11	205.10±70.61	163.70±73.26	185.87± 140.36
VLDL (mg/dl)	51.17±44.42	41.02±14.12	32.74±14.65	37.17±28.07
APOA1 (mg/dl)	106.34±57.88	100.22±60.54	114.78±49.89	94.49±47.13
MAP	86.67±13.86	101.58±9.42	94.34±15.97	86.00±9.41
PP	33.31±27.02	57.94±20.50	44.26±36.32	33.83±12.87
SBP	108.88±28.36	140.20±20.01	123.85±36.95	109.22±12.55
DBP	75.5±11.37	82.26±8.54	79.59±11.82	74.39±10.46
AIP	0.755±0.27	0.641±0.144	0.65±0.150	0.67±0.23

Table 15. The Mean ± Standard deviation of all variables of patients and controls. HbA1c, (Glycosylated Haemoglobin); TC (Total Cholesterol); FBG (fasting blood glucose); HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol); VLDL, (very low density lipoprotein); APOA1, (Apolipoprotein A1); BMI, (Body Mass Index); HC, (Hip Circumference); WC, (Waist Circumference); WHR, (Waist –hip ratio), SBP (Systolic blood pressure), DBP (Diastolic blood pressure), AIP (Atherogenic index plasma), MAP (Mean arterial pressure), PP (Pulse pressure).

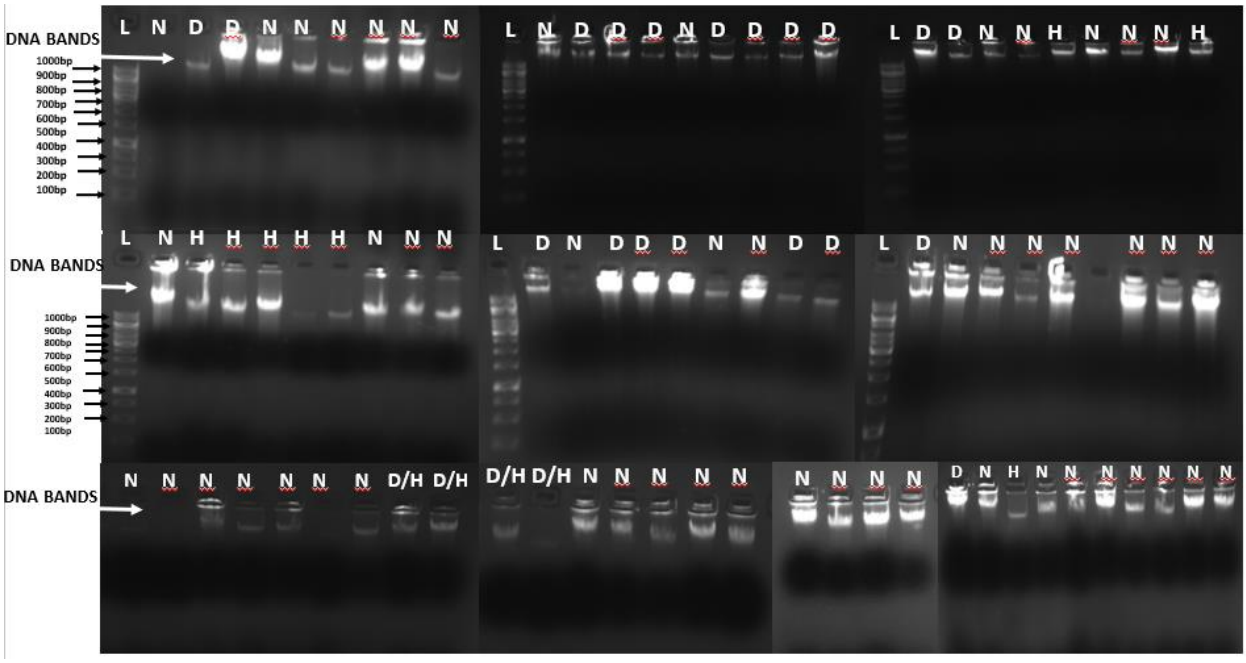
PARAMETERS	Type2 DM Vs CVD	CVDWDM Vs N	T2DM Vs N	CVDWDM Vs CVD	Type2 DM Vs CVDWDM	CVD Vs N
AGE	0.734	0.001	0.001	0.156	0.081	0.001
BMI (kg/m2)	0.018	0.513	0.003	0.902	0.100	0.240
WC (in)	0.840	0.001	0.432	0.001	0.007	0.530
HC (in)	0.528	0.001	0.220	0.001	0.001	0.480
WHR (cm)	0.108	0.020	0.099	0.032	0.060	0.748
FBG (mg/dl)	0.001	0.001	0.001	0.002	0.003	0.002
HbA1c (mg/dl)	0.001	0.002	0.023	0.001	0.850	0.030
TC (mg/dl)	0.002	0.434	0.042	0.001	0.401	0.005
HDL (mg/dl)	0.001	0.404	0.045	0.002	0.428	0.004
LDL (mg/dl)	0.001	0.135	0.387	0.002	0.060	0.005
TG (mg/dl)	0.009	0.237	0.003	0.002	0.004	0.015
VLDL (mg/dl)	0.008	0.215	0.003	0.003	0.002	0.014
APOA1 (mg/dl)	0.398	0.002	0.075	0.092	0.323	0.038
MAP	0.002	0.002	0.651	0.005	0.001	0.006
PP	0.005	0.001	0.567	0.001	0.020	0.005
SBP	0.008	0.002	0.902	0.004	0.001	0.009
DBP	0.015	0.002	0.392	0.065	0.025	0.002

AIP	0.008	0.561	0.014	0.490	0.004	0.145

Table 16: The statistical significance i.e. p value <0.05 of patients and control. T2DM, type2 diabetes mellitus; CVD, cardiovascular disease; HbA1c, (Glycosylated Haemoglobin); TC (Total Cholesterol); FBG, fasting blood glucose; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol); VLDL, (very low density lipoprotein); APOA1, (Apolipoprotein A1); BMI, (Body Mass Index); HC, (Hip Circumference); WC, (Waist Circumference); WHR, (Waist –hip ratio), SBP (Systolic blood pressure), DBP (Diastolic blood pressure), AIP (Atherogenic index plasma), MAP (Mean arterial pressure), PP (Pulse pressure).

	PATIENTS			
BMI CATEGORIES	**T2DM**	**CVD**	**CVDWDM**	**CONTROLS**
Underweight	8	3	7	5
Normal	43	45	20	42
Overweight	47	55	14	37
Obesity	25	47	23	46
χ^2 value	7.918	2.721	4.213	-
p value	0.048	0.437	0.239	-

Table 17. The correlation of obesity status of patients versus control participants.



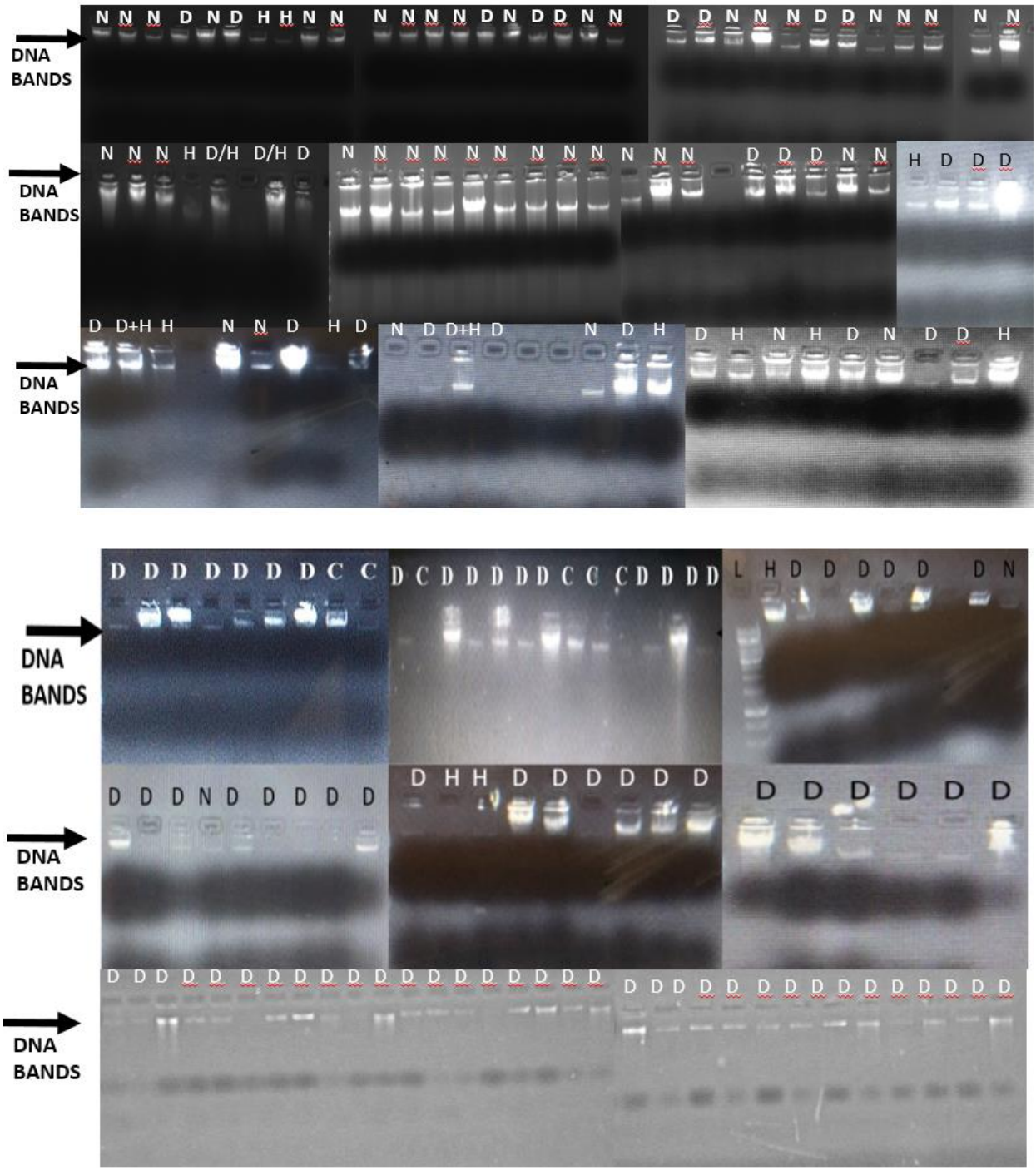
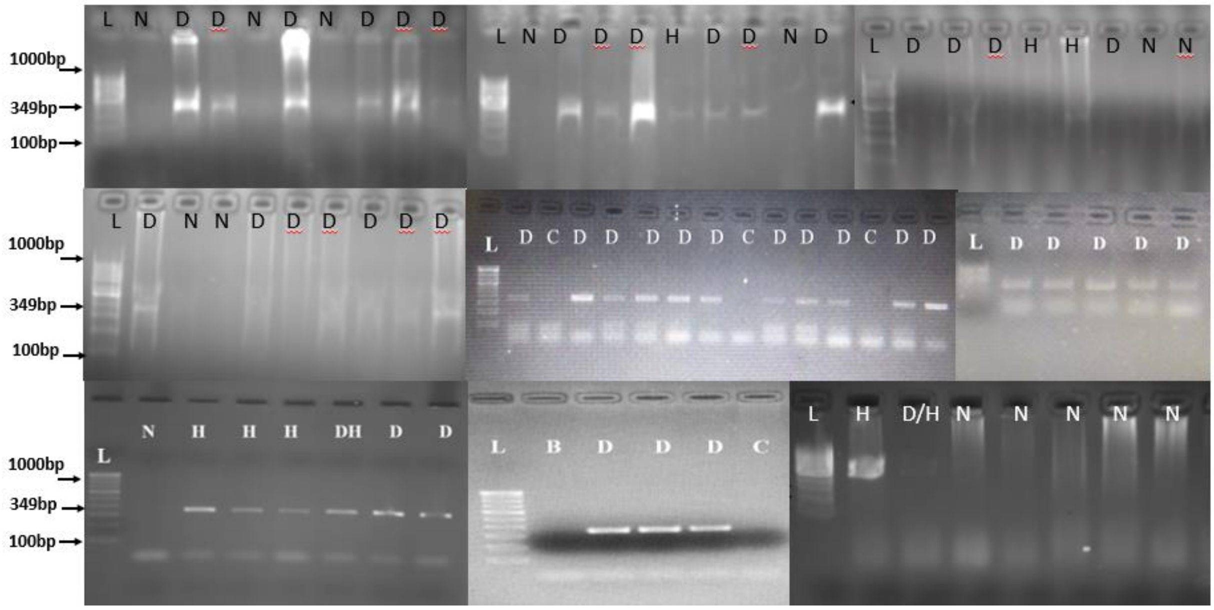
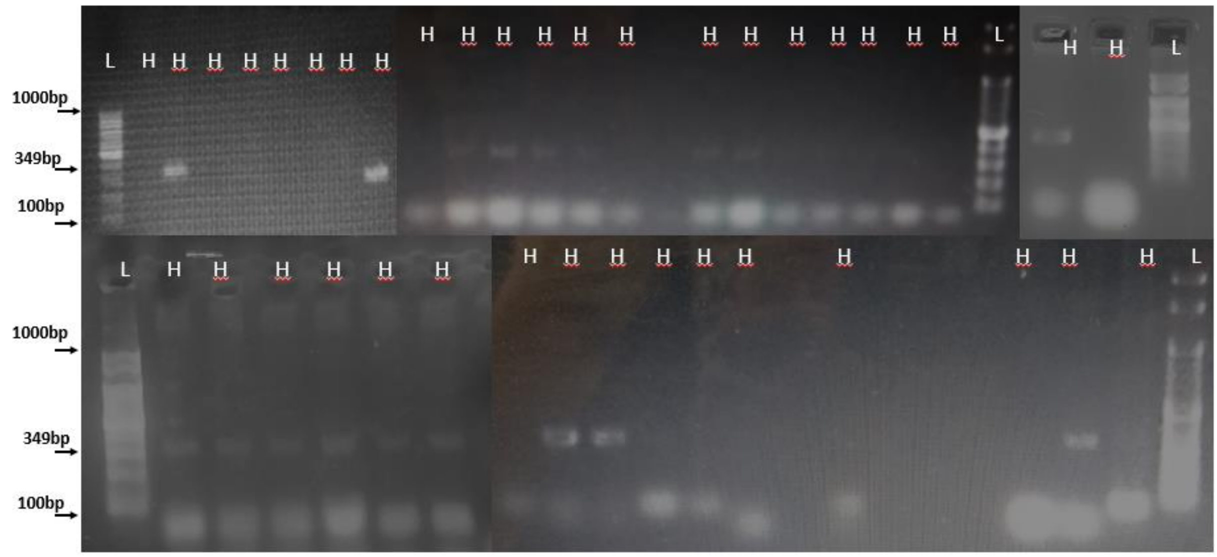
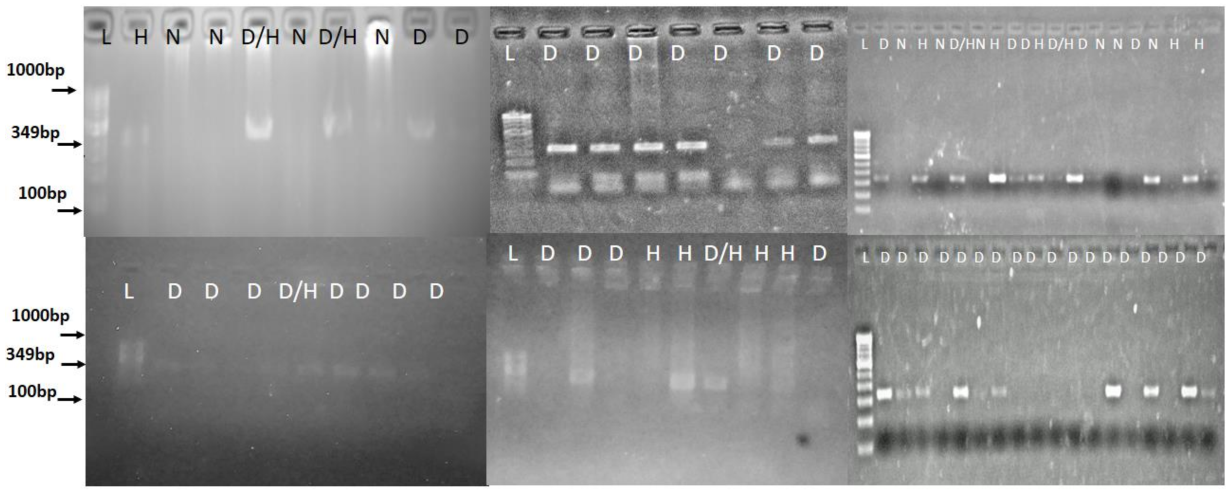


Figure 11. DNA smear: L representing 100bp to 1000bp DNA ladder. Where, N stands for normal; H stands for cardiovascular patients; D/H stands for cardiovascular with type 2 diabetes; D stands for type2 diabetes mellitus patients.


1000bp
349bp
100bp
L N D D N D N D D D
L N D D D H D D N D
L D D D H H D N N
L D N N D D D D D D
L D C D D D D D C D D D C D D
L D D D D D
L N H H H DH D D
L B D D D C
L H D/H N N N N N


1000bp
349bp
100bp
L H H H H H H H H
H H H H H H H H H H H H H L
H H L
L H H H H H H
H H H H H H H H H H L


1000bp
349bp
100bp
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L D D D D D D D
L D D D D/H D D
L D D D H H D/H H H D

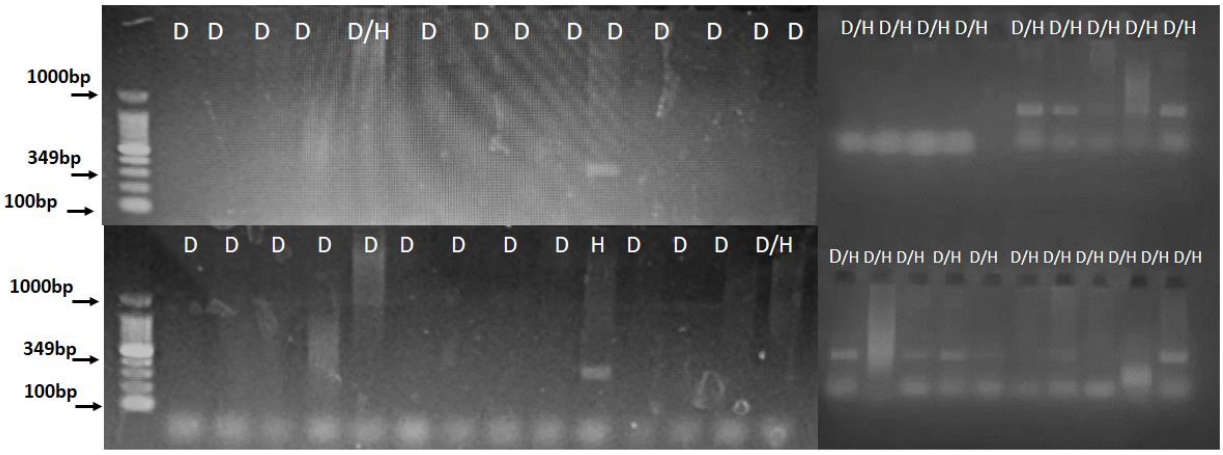


Figure 12. The amplification products of Hp2-2 genotype: H, D/H and D representing Hp2-2 (349bp) band and L representing 100bp to 1000bp DNA ladder. Where, N & C stands for normal or control; H stands for cardiovascular patients; D/H stands for cardiovascular with type 2 diabetes; D stands for type2 diabetes mellitus patients.

		HP2-2		Total	χ^2 value	p value
		NO	YES			
TC	BORDERLINE	6	19	25	13.864	0.001
	HIGH	3	10	13		
	NORMAL	51	34	85		
	Total	60	63	123		
LDL	BORDERLINE	9	8	17	0.143	0.931
	HIGH	2	2	4		
	NORMAL	49	53	102		
	Total	60	63	123		
HDL	BORDERLINE	6	22	28	14.078	0.001
	HIGH	51	34	85		
	NORMAL	3	7	10		
	Total	60	63	123		
TG	BORDERLINE	5	21	26	38.476	0.001
	HIGH	1	19	20		
	NORMAL	54	23	77		
	Total	60	63	123		

VLDL	BORDERLINE	13	13	26	39.091	0.001
	HIGH	6	38	44		
	NORMAL	41	12	53		
	Total	60	63	123		
APOA1	HIGH	0	5	5	5.081	0.79
	LOW	36	33	69		
	NORMAL	24	25	49		
	Total	60	63	123		

Table 18. The correlation of haptoglobin 2-2 allele with Lipid profile in Diabetic subjects.

		HP2-2		**Total**	**chi square**	**p value**
		NO	**YES**			
SMOKING	NO	44	56	100	4.891	0.027
	YES	16	7	23		
	Total	60	63	123		
TOBACCO	NO	60	58	118	4.964	0.026
	YES	0	5	5		
	Total	60	63	123		
ALCOHOL	NO	48	51	99	0.018	0.894
	YES	12	12	24		
	Total	60	63	123		
VEG	NON-VEG	26	29	55	0.091	0.764
	VEG	34	34	68		
	Total	60	63	123		
PHYSICAL	HIGH	8	2	10	6.88	0.032
	LOW	31	27	58		
	MODERATE	21	34	55		
	Total	60	63	123		

Table 19. The correlation of haptoglobin 2-2 allele with physiological variables in Diabetic subjects.

		HP2-2		**Total**	**p value**	**chi square**
		NO	YES			

SBP	ELEVATED	12	18	30	0.182	4.863
	NORMAL	45	38	83		
	HYPERTENSION Stage1	3	4	7		
	HYPERTENSION Stage2	0	3	3		
	Total	60	63	123		
DBP	NORMAL	41	46	87	0.049	6.018
	HYPERTENSION Stage1	7	13	20		
	HYPERTENSION Stage2	12	4	16		
	Total	60	63	123		
FPG	DIABETES	37	38	75	0.986	0.027
	HEALTHY	9	10	19		
	PREDIABETES	14	15	29		
	Total	60	63	123		
HbA1C	DIABETES	49	45	94	2.189	0.335
	HEALTHY	5	6	11		
	PREDIABETES	6	12	18		
	Total	60	63	123		
MAP	GRADE1	2	1	3	0.568	2.02
	GRADE2	1	1	2		
	MEDIATE	9	5	14		
	NORMAL	48	56	104		
	Total	60	63	123		
PP	GRADE1	0	5	5	0.021	7.702
	LOW	35	25	60		
	NORMAL	25	33	58		
	Total	60	63	123		

Table 20. The correlation of haptoglobin 2-2 allele with Glycemic status, hypertension and arterial pressure in Diabetic subjects.

		HP2-2		Total	P value	chi square
		NO	YES			
TC	BORDERLINE	33	11	44	0.001	56.347
	HIGH	58	12	70		
	NORMAL	4	32	36		
	Total	95	55	150		
HDL	BORDERLINE	49	17	66	0.001	57.732
	HIGH	4	32	36		
	NORMAL	42	6	48		
	Total	95	55	150		
LDL	BORDERLINE	35	16	51	0.001	25.632
	HIGH	41	7	48		
	NORMAL	19	32	51		
	Total	95	55	150		
TG	BORDERLINE	52	8	60	0.001	24.439
	HIGH	0	1	1		
	NORMAL	43	46	89		
	Total	95	55	150		
VLDL	BORDERLINE	38	25	63	0.002	24.021
	HIGH	46	10	56		
	NORMAL	9	22	31		
	Total	93	57	150		
APOA1	HIGH	0	10	10	0.001	30.065
	LOW	76	23	99		
	NORMAL	19	22	41		
	Total	95	55	150		

Table 21. The correlation of haptoglobin 2-2 allele with Lipid profile in Cardiovascular subjects.

		HP2-2		Total	chi square	p value
		NO	YES			
SMOKING	NO	67	38	105	0.034	0.855
	YES	28	17	45		

	Total	95	55	150		
TOBACCO USE	NO	69	45	114	1.612	0.204
	YES	26	10	36		
	Total	95	55	150		
Drinker	NO	69	26	95	9.646	0.003
	YES	26	29	55		
	Total	95	55	150		
DIETARY HABBIT	NON-VEG	54	29	83	0.239	0.625
	VEG	41	26	67		
	Total	95	55	150		
PHYSICAL ACTIVITY	HIGH	24	7	31	5.662	0.059
	LOW	35	17	52		
	MODERATE	36	31	67		
	Total	95	55	150		

Table 22. The correlation of haptoglobin 2-2 allele with physiological parameters in Cardiovascular subjects.

		HP2-2		**Total**	**Chi square**	**P value**
		NO	**YES**			
SBP	ELEVATED	2	7	9	29.527	0.001
	NORMAL	0	11	11		
	HYPERTENSION Stage1	31	14	45		
	HYPERTENSION Stage2	62	23	85		
	Total	95	55	150		
DBP	NORMAL	27	11	38	10.738	0.005
	HYPERTENSION Stage1	33	34	67		
	HYPERTENSION Stage2	35	10	45		
	Total	95	55	150		

FPG	DIABETES	4	17	21	21.907	0.001
	HEALTHY	47	24	71		
	PREDIABETES	44	14	58		
	Total	95	55	150		
HbA1C	DIABETES	0	5	5	11.413	0.003
	HEALTHY	71	31	102		
	PREDIABETES	24	19	43		
	Total	95	55	150		
PP	GRADE1	42	14	56	25.89	0.001
	LOW	0	13	13		
	NORMAL	53	28	81		
	Total	95	55	150		
MAP	GRADE1	37	11	48		
	GRADE2	3	0	3	8.65	0.034
	HIGHNORM	26	18	44		
	NORMAL	29	26	55		
	Total	95	55	150		

Table 23. The correlation of haptoglobin 2-2 allele with Glycemic status, hypertension and arterial pressure in Cardiovascular subjects.

		HP2-2		**Total**	**p value**
		NO	**YES**		
SMOKING	NO	18	31	49	0.013
	YES	11	4	15	
	Total	29	35	64	
TOBACCO USE	NO	25	25	50	0.155
	YES	4	10	14	
	Total	29	35	64	
DRINKER	NO	19	24	43	0.796
	YES	10	11	21	
	Total	29	35	64	
	NON-VEG	23	20	43	0.06

DIETARY HABBIT	VEG	6	15	21	
	Total	29	35	64	
PHYSICAL ACTIVITY	HIGH	7	4	11	0.404
	LOW	11	16	27	
	MODERATE	11	15	26	
	Total	29	35	64	

Table 24. The correlation of haptoglobin 2-2 allele with physiological parameters in Cardiovascular with type2 Diabetic subjects.

		HP2-2		**Total**	**p value**	**chi square**
		NO	**YES**			
TC	BORDERLINE	3	7	10	0.043	6.276
	HIGH	0	5	5		
	NORMAL	26	23	49		
	Total	29	35	64		
HDL	BORDERLINE	3	8	11	0.051	5.946
	HIGH	26	23	49		
	NORMAL	0	4	4		
	Total	29	35	64		
LDL	BORDERLINE	2	6	8	0.036	6.673
	HIGH	0	5	5		
	NORMAL	27	24	51		
	Total	29	35	64		
TG	BORDERLINE	0	8	8	0.013	8.677
	HIGH	0	1	1		
	NORMAL	29	26	55		
	Total	29	35	64		
APOA1	HIGH	1	3	4	0.048	6.084
	LOW	10	21	31		
	NORMAL	18	11	29		
	Total	29	35	64		
VLDL	BORDERLINE	10	14	24	0.005	10.78
	HIGH	0	9	9		

	NORMAL	19	12	31		
	Total	29	35	64		

Table 25. The correlation of haptoglobin 2-2 allele with Lipid profile in Cardiovascular with typ2 Diabetic subjects.

		HP2-2		**Total**	**p value**	**chi square**
		NO	**YES**			
SBP	ELEVATED	4	2	6	0.001	36.135
	NORMAL	20	3	23		
	HYPERTENSION Stage1	4	4	8		
	HYPERTENSION Stage2	1	26	27		
	Total	29	35	64		
DBP	NORMAL	19	9	28	0.005	10.658
	HYPERTENSION Stage1	6	12	18		
	HYPERTENSION Stage2	4	14	18		
	Total	29	35	64		
FPG	DIABETES	29	34	63	0.359	0.842
	PREDIABETES	0	1	1		
	Total	29	35	64		
HbA1C	DIABETES	28	28	56	0.132	4.044
	HEALTHY	0	1	1		
	PREDIABETES	1	6	7		
	Total	29	35	64		
PP	GRADE1	1	16	17	0.001	26.375
	LOW	13	0	13		
	NORMAL	15	19	34		
	Total	29	35	64		
MAP	GRADE1	0	17	17	0.001	28.576
	GRADE2	0	3	3		

	MEDIUM	3	7	10		
	NORMAL	25	9	34		
	Total	28	36	64		

Table 26. The correlation of haptoglobin 2-2 allele with Glycemic status, hypertension and arterial pressure in Cardiovascular with Diabetic subjects.

DNA SEQUENCING

DNA sequencing were done on six samples in which two were type2 Diabetics, two were Cardiovascular disease and two were Cardiovascular disease with type2 diabetic participants Following were Fasta sequences of forward as well as reverse primers of these samples: FP stands for forward primer and RP stands for reverse primer:

>24407-1-FP1(Diabetic)

CGCGAGTACATTCATCTTTCTTTAGAGAGAATGAATTATTGTAGCCCCTAGCCCT
TTCAATGAATTTCAGGGAATTGTGGAAATTCCTTTATTGGGATAATTGTTTAAAT
ATAATACAGTTCGCGAGCTTCTATTCGGGGTGGAACGATATTCCCAATAAAGGA
ATTTCCACAATTCCCTGAAATTCATTGAAAGGGCTAGGGGCTACCATAATTCATT
CTCTCTTAAGAAAAGATGAATCTTGAGCAACACTGGGTGGGAGCTCACATTTTGT
AGATGGGTGCAGTTAATACGAAGCAGGGAGG

>24407-5-RP1 (Diabetic)

TTTAGTTGCATTCATCTTTCTTTAGAGAGAATGAATTATTGTAGCCCCTAGCCCTT
TCAATGAATTTCAGGGAATTGTGGAAATTCCTTTATTGGGATAATTGTTTAAATA
TAATACAGTTCGCGAGCTTCTATTCGGGGTGGAAGGAGATTGCTAATCAAAGAAT
TTCCACAATTCCCTGAAATTCATTGAAAGGGCTAGGGGCTACCATAATTCATTCT
CTCTTAAGAAAGATGAATCTTGAGAAACACTGGCTGGAGCTCACATTTTGTAAAT
GGTGCAGTTAATACGAGGCAGGAGG

>24407-1-RP3 (CVD)

GCCAGGCATCAATATAATCCAGAGAGAATTGACATATATTGGAAACCCGCTACC
CCCTTTCCCTGAATTTCAGGGAATTGTGGAAATTCCTTTATTGGGATAATTGCTTA
CATATCATACAAATCCTCCCCTTTGTTTATATTTAAAAAAGTATCCCAAGAAAAG
AATTTCCACATTCCCTTGAATTCATTGGAAAGGGTAGGGGTACAAATTCATTCTC

TCTAAGAAGATGATCTAGAGCGCATTGGCTGGAGCTCAATTTTGTAAATGGTGCA
GGTAATACGAAGCAGGGGAG

>24407-1-FP4 (CVD)

GGGNTTCATCTTTCTTTAGAGAGAATGAATTATTGTAGCCCCTAGCCCTTTCAATG
AATTTCAGGGAATTGTGGAAATTCCTTTATTGGGATAATTGTTTAAATATAATAC
AGTTCGCGAGCTTCTATTCGGGGTGGAACGAGATTCCCAATAAAAGAATTTCCAC
AATTCCCTGAAATTCATTGAAAGGGCTAGGGGCTACAATAATTCATTCTCTCTAA
AGAAAGATGAATCTANAGCAACACTGGCTGGAGCTCACATTTTGTAGATGGTGC
AGTTAATACGAGGCAGGGA

>24407-5-RP5 (CVDWDM)

AGGCAGGAATGAAGAGAAGCCAGAGAGTTTGCTATTTGGAAATTGCTCCCCCTG
AACCTGGATTCACGGAATAGCGGAAATTCCTTTATTGGGATATCTGCTTCCACCC
CAAACAGAACTCGCGAACTGGATTATATTTAAACAATTATCCCAATAAAGGAAT
TTCCACAATTCCCTGAAATTCATTGGAAGGGGTAGGGGCTACCATAATTCATTCT
CTCTTAAGAAAGATGAATCTAGAGCAACACTGGGCTGGGAACGTCAGATTTTGT
AAATGGGTGCAGGGTAATACGAGGGCAGGGAAGGA

>24407-5-FP6 (CVDWDM)

TNGGTGATTCTCTTTCTTTAGAGAGAATGAATTATTGTAGCCCCTAGCCCTTTCAA
TGAATTTCAGGGAATTGTGGAAATTCCTTTATTGGGATAATTGTTTAAATATAAT
ACAGTTCGCGAGCTTCTATTCGGGGTGGAAGGAGATTCCCGATCAAAGAATTTCC
ACAATTCCCTGGAATTCATTGAAAGGGCTAAGGGCTACCATAATTCATTCTCTCT
TAAGAAATATGAATCTTGAGCAGCACTGCGCTGGAGCTCACATTTTGTAAATGGT
GCAGTTAATACGAGGCACGAG

The alignment of patient samples was shown identical, similar and consensus sequences with haptoglobin gene as shown in Figure 13-14.

```
CLUSTAL O(1.2.4) multiple sequence alignment

Diabetic        GGCATGAAGAGAAGCCAGAGAGTTTGCTATTTGGAAATTGCTCCCCCTGAACCTGGATTC   60
Diabetic        GCCATAATCCAGAGAGAATTGAATATATTGGAAACCCGCTACCCCCTTTCCCTGAATTTC   60
HP              GTGTTCATCTTTCTTTAGAGAGATGAATTATTGTAGCCCCTAGCCCTTTCAATGAATTTC   60
CVD             ---ATTCTCTTTCTTTAGAGAGATGAATTATTGTAGCCCCTAGCCCTTTCAATGAATTTC   57
CVD             ---TTCATCTTTCTTTAGAGAGATGAATTATTGTAGCCCCTAGCCCTTTCAATGAATTTC   57
CVDWDM          ---TTCATCTTTCTTTAGAGAGATGAATTATTGTAGCCCCTAGCCCTTTCAATGAATTTC   57
CVDWDM          ---TTCATCTTTCTTTAGAGAGATGAATTATTGTAGCCCCTAGCCCTTTCAATGAATTTC   57
                    *           *      *   *                *** *         ***

Diabetic        ACGGAATAGCGGAAATTCCTTTATTGGGATATCTGCTTCCACCCCAAACAGAACTCGCGA   120
Diabetic        AGGGAATTGTGGAAATTCCTTTATTGGGATAATTGCTTACATATCATACAAATCCTCCCC   120
HP              AGGGAATTGTGGAAATTCCTTTATTGGGATAATTGTTTAAAAATAATACAGTTCGCGAGC   120
CVD             AGGGAATTGTGGAAATTCCTTTATTGGGATAATTGTTTAAATATAATACAGTTCGCGAGC   117
CVD             AGGGAATTGTGGAAATTCCTTTATTGGGATAATTGTTTAAATATAATACAGTTCGCGAGC   117
CVDWDM          AGGGAATTGTGGAAATTCCTTTATTGGGATAATTGTTTAAATATAATACAGTTCGCGAGC   117
CVDWDM          AGGGAATTGTGGAAATTCCTTTATTGGGATAATTGTTTAAATATAATACAGTTCGCGAGC   117
                * ***** * ********************  ** **  *    * ***   *

Diabetic        ACTGGATTATATTTAAACAATTATCCCAATAAAGGAATTTCCACAATTCCCTGAAATTCA   180
Diabetic        TTTGTTTATATTTAAAAAAGTA-TCCCAAGAAAAGAATTTCCACATTCCCTTGAATTCAT   179
HP              TTCTATTCGGGGTGGAAGGAGATTGATGTGCAGAGCAGCTCCCGCTCATCTGACTTTTCA   180
CVD             TTCTATTCGGGGTGGAAGGAGATTCCCGATCAAAGAATTTCCACAATTCCCTGGAATTCA   177
CVD             TTCTATTCGGGGTGGAAGGAGATTGCTAATCAAAGAATTTCCACAATTCCCTGAAATTCA   177
CVDWDM          TTCTATTCGGGGTGGAACGATATTCCCAATAAAGGAATTTCCACAATTCCCTGAAATTCA   177
CVDWDM          TTCTATTCGGGGTGGAACGAGATTCCCAATAAAAGAATTTCCACAATTCCCTGAAATTCA   177
                      *     *  **       *        *  * *  ***        *      *

Diabetic        TTGGAAGGGGGGGCTACCATAATTCTCTTAAGAAAGAGAATGAAACACTGGCTGGATTTT   240
Diabetic        TGGAAAGGGTAGTACAAATTCATTCTCTCTAAGAAGAGATCGAGCGCATTGCTGGCAATT   239
HP              CGGTTCACTAACTTCCAAATAGTTCTCTCTTTGCAGAGACGAACCCCCGAATTGCACATG   240
CVD             TTGAAAGGGCTAGCTACCATAATTCTCTCTTAAGAAATATGGAAGCACTGGCTGGCATTT   237
CVD             TTGAAAGGGCTAGCTACCATAATTCTCTCTTAAGAAAGATGGAAACACTGGCTGGCATTT   237
CVDWDM          TTGAAAGGGCTAGCTACCATAATTCTCTCTTAAGAAAGATGGAAACACTGGTGGGCATTT   237
CVDWDM          TTGAAAGGGCTAGCTACAATAATTCTCTCTAAAGAAAGATGNAAACACTGGCTGGCATTT   237
                  *                   *  ******      * * *   *          *    *

Diabetic        GTAAATGGGTGCGGGTATACGAGGGCAGGG 270
Diabetic        TTGTAAATGGGCGGTAATACGAAGCAGGGG 269
HP              GCTATGTGGAGCCTCGGTTCGCTACCAGTG 270
CVD             TGTAAATGGTCAGTTAATACGAGGCACGAG 267
CVD             TGTAAATGGTCAGTTAATACGAGGCAGGAG 267
CVDWDM          TGTAGATGGGGCGTTAATACGAAGCAGGGA 267
CVDWDM          TGTAGATGGTCAGTTAATACGAGGCAGGGA 267
                      *        * **      *
```

Figure 13. Alignment of samples with Haptoglobin gene.

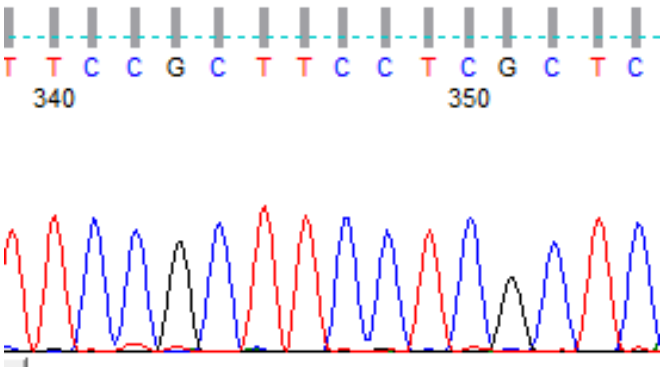


Figure 14. DNA sequencing of samples.

IN SILICO ANALYSIS

1. Data Retrieved: A total of 57 SNP's of Hb (27) and Hp (30) were extracted randomly from dbSNP, NCBI, and SNPedia databases which included 21 missense variants, three inframe deletions, 18 intron variants, 8 non-coding transcript exon variants, five 5'UTR variants, one 3'UTR variant, and 1 intergenic variant with a SNP's MAF value ≤0.05 as shown in Table 27. In our study maximum Hp SNP's has MAF value ≤0.05.

S.No.	SNP's	Chromosome	MAF	Variant	Position	Allele	Nearby Gene	Distance (bp)
1	rs1051308	16	0.49	3'UTR	4500302	A/G	HMOX2	33855||47
2	rs12162087	16	0.49	intron	70644808	A/G	DHODH||HP	-28352||-1201
3	rs12917999	16	0.49	Non-coding transcript exon	70646952	C/T	HP	943||5506
4	rs2000999	16	0.48	intron	70665594	A/G	HPR	10968||3052
5	rs2070937	16	0.49	Non-coding transcript exon	70647241	A/G	HP	1232||5217
6	rs2070938	16	0.50	Intron	70649518	A/T	HP	3509||2940
7	rs2071746	22	0.50	Intron	34106672	A/T	LOC100128526|| HMOX1	-32691||-415
8	rs2071747	22	0.08	Missense	34107185	C/G	HMOX1	98||13009
9	rs2071749	22	0.49	Intron	34113413	A/G	HMOX1	6326||6781
10	rs2236765	16	0.49	Non-coding transcript exon	70646666	A/G	HP	657||5792
11	rs2270363	16	0.50	5'UTR	4466293	A/G	NMRAL1||HMOX2	-1400||-154
12	rs28928876	16	0.0	Missense	163290	C/T	HBA2	415||418
13	rs334	11	0.14	Missense	5204808	-/T/G/A	HBB	1536||69
14	rs33916412	11	0.0	Missense	5204742	T/G/C/A	HBB	1470||135
15	rs33921589	11	<0.01	Missense	5204388	T/C/A	HBB	1116||489
16	rs33924775	11	0.0	Missense	5204421	T/G/C/A	HBB	1149||456
17	rs33931984	16	0.0	Missense	163164	C/G/T	HBA2	289||544

18	rs33946401	11	0.0	Missense	5204486	T/G/C	HBB	1214||391
19	rs33957286	11	0.0	Missense	5203462	T/G/A	HBB	190||1415
20	rs33976006	11	0	Missense	5204474	T/C/A	HBB	1202||403
21	rs33985510	11	0	Missense	5204402	T/G/C/A	HBB	1130||475
22	rs33993166	16	<0.01	Missense	163002	C/G	HBA2	127||706
23	rs34151786	11	<0.01	Missense	5204510	T/G	HBB	1238||367
24	rs34210688	11	0	Inframe deletion	5204410	-/GACGTGACACTGTTC	HBB	1138||467
25	rs34378160	11	0	Missense	5204570	G/A	HBB	1298||307
26	rs35213748	16	0	Missense	163203	C/T	HBA2	328||505
27	rs35461039	16	0.26	Non-coding transcript exon	70648127	A/G	HP	2118||4331
28	rs35553496	11	<0.01	Missense	5204436	-/T/TGT/G	HBB	1164||441
29	rs35672478	16	0	Inframe	163215	-/GTG	HBA2	340||493
30	rs36008922	11	<0.01	Missense	5204496	T/C	HBB	1224||381
31	rs36071424	16	0.43	Intron	70644101	-/T	DHODH||HP	-27645||-1908
32	rs3761439	22	0.48	Intergenic	34105889	A/G	LOC100128526||HMOX1	-31908||-1198
33	rs3794693	16	0.35	Non-coding transcript exon	70648167	C/G	HP	2158||4291
34	rs3794694	16	0.50	Missense	70648286	C/G	HP	2277||4172
35	rs3852780	16	0.29	intron	70650635	A/C	HP	4626||1823
36	rs398580	16	0.26	intron	70652954	G/A	HP||HPR	-496||-1672
37	rs41514946	16	0	Missense	163572	A/C	HBA2	697||136
38	rs434738	16	<0.01	Intron	70652834	G/A	HP||HPR	-376||-1792

39	rs437638	16	0.26	Intron	70652953	T/C	HP||HPR	-495||-1673
40	rs4788458	16	0.48	Non-coding transcript exon	70646176	C/T	HP	167||6282
41	rs5467	16	0.30	Intron	70645781	C/T	DHODH||HP	-29325||-228
42	rs5468	16	0.18	Intron	70645832	G/T	DHODH||HP	-29376||-177
43	rs5469	16	0.48	5'UTR	70645919	A/T	DHODH||HP	-29463||-90
44	rs5470	16	0.19	5'UTR	70645922	C/G	DHODH||HP	-29466||-87
45	rs5471	16	0.22	5'UTR	70645962	A/C	DHODH||HP	-29506||-47
46	rs5472	16	0.45	5'UTR	70645968	A/G	DHODH||HP	-29512||-41
47	rs5475	16	0.12	Missense	70651849	A/G	HP	5840||609
48	rs5478	16	0.28	Intron	70652813	T/C	HP||HPR	-355| |-1813
49	rs599806	16	<0.01	Intron	70651411	T/C	HP	5402||1047
50	rs63749918	11	0	Inframe deletions	5204752	-/CCA	HBB	1480||125
51	rs664134	16	0.49	Non-coding transcript exon	70649149	A/G	HP	3140||3309
52	rs7201866	16	0.50	intron	70644035	A/T	DHODH||HP	-27579||-1974
53	rs7202268	16	0.49	Intron	70644247	A/G	DHODH||HP	-27791||-1762
54	rs7203426	16	0.48	Intron	70644056	C/T	DHODH||HP	-27600||-1953
55	rs8056409	16	0.28	Intron	70652815	C/T	HP||HPR	-357||-1811
56	rs8062041	16	0.50	Non-coding transcript exon	70646465	C/T	HP	456||5993
57	rs111033605	16	<0.01	Missense	173003	G/A/C	HBA2	-

Table 27. SNP's of Haptoglobin and Haemoglobin.

2. Gene-Gene Interaction: The interaction of haptoglobin gene with other genes was predicted by gene mania tool as shown in Figure 15. It showed the physical interaction of haptoglobin with other genes was 77.64%.

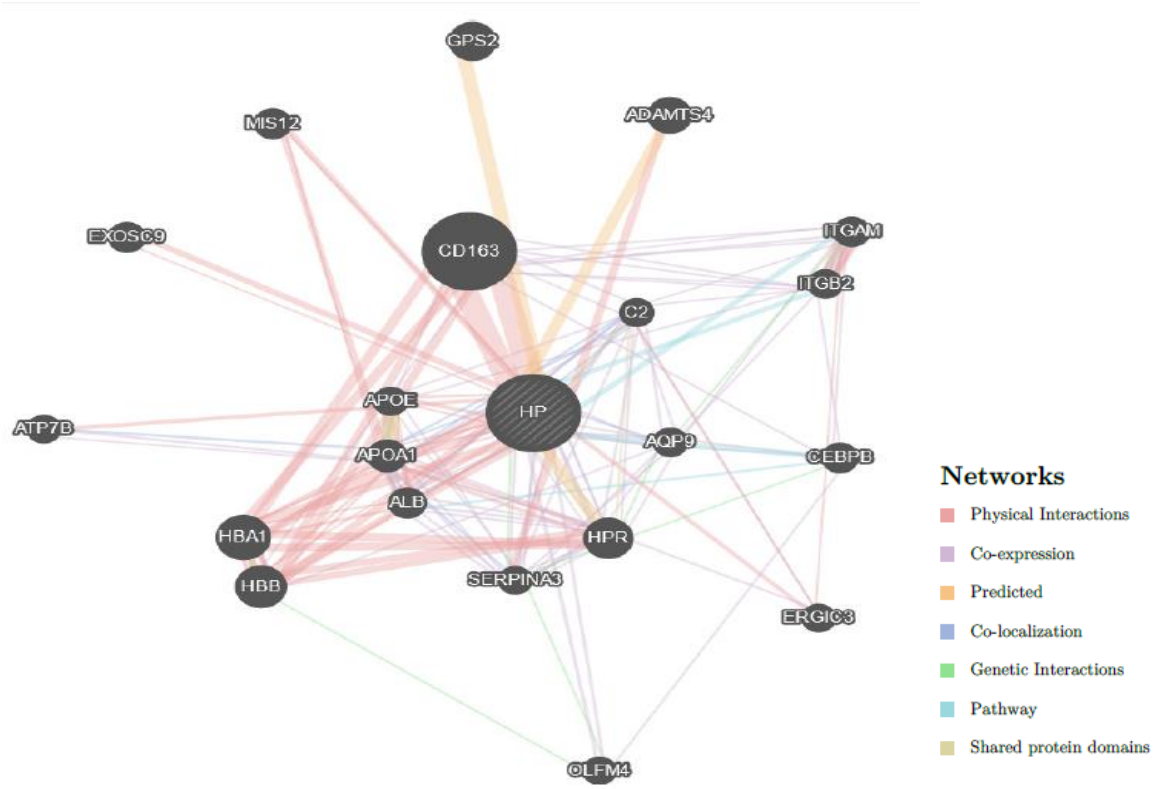


Figure 15. Interaction of Haptoglobin with other genes.

3.3. Alignment of Haptoglobin and heme sequences: The sequence alignment of heme and haptoglobin with their respective structure sequences by using the Clustal omega sequence alignment tool showed consensus, identical and similar sequences as shown in (Figure 16-17).

Figure 16. Showed the alignment of heme sequences with their respective structure PDB ID by Clustal omega. Here, dots represent the conserved sequences are identical and similar.

Figure-17. Showed the alignment of Hp sequences with their respective structure PDB ID by Clustal omega. Here, dots represent the conserved sequences are identical and similar.

3. Mapping of selected SNP's on their respective structures.

Searching a protein database using a protein query (BLASTn) against PDB and RCSB-PDB database for their 3D structure of the protein and results obtained are shown in (Table 28). The selection of that PDB-ID's structures sequence who have the highest sequence identification and query cover. Then, found the SNP's mutations from NCBI database and map these mutations on their respective structure and highlighted the mutations in a structure by using Pymol an open-source molecular visualization software as shown in (Figure 18).

S.NO.	Accession No. (rs)	PDB ID	Sequence Identification	Query Cover
1.	rs199474827	3IIE A	39%	69%
2.	rs121908088	1MYP C 4EJX D	47% 47%	43% 43%
3.	rs72554627	3CZH A 3RWL A	31% 32%	52% 47%
4.	rs12191297	4DVQ A 4ZGX A	98% 98%	41% 41%
5.	rs36008922	3AT5 B	51%	61%
6.	rs137853233 HP	4WJG C 4F4O C	94% 71%	10% 10%
7.	rs119469013	3F9P C 1CXP C	62% 62%	53% 53%
8.	rs28937573	2LDU A 5D5U B	69% 69%	33% 33%
9.	rs33957286 HBB	3HRW B 4MQK B	38% 48%	39% 30%

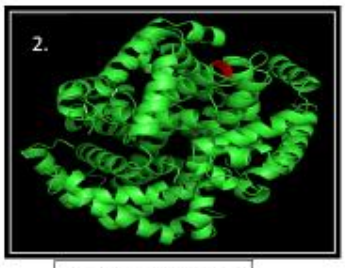
10.	rs28928876 HBA1	1Y0D A	98%	83%
11.	rs33944813	1RVW A	51%	85%
12.	rs33931984	2R80 A	68%	84%
13.	rs33924775	3AT5 B 1GBU B	56% 100%	54% 45%
14.	rs33921589	5ME2 B 1GBV B	100% 100%	43% 43%
15.	rs33916412	1DXT B	61%	66%
16.	rs34210688	2HHE B	81%	78%
17.	rs34378160	1Y2Z B	64%	87%
18.	rs35213748	1A4F A	70%	76%
19.	rs35672478	4MQH A	66%	72%
20.	rs41514946	1RVW A	89%	22%
21.	rs56378716	5MFA A	63%	76%
22.	rs63749918	1YE0 B	88%	75%
23.	rs72551322	3C6G A 3CZH A	32% 32%	39% 39%
24.	rs78950939	5MFA A	60%	71%
25.	rs104894302	1ZOY D 4YTP D	96% 96%	27% 27%
26.	rs104894557	1RZ8 B 1RZJ H	54% 54%	14% 14%
27.	rs119469014	5MFA A 4EJX D	88% 88%	47% 47%

28.	rs2071746	5JBH_2	100%	1%
29.	rs2071747	3J3F 5 5A8L A 4CXE 2	100%	3%
30.	rs2270363	3J3F 5 5A8L A 4CXE 2	100%	3%
31.	rs1051308 HM	3J7Y A	100%	1%
32.	rs17177078	4W21 5	15%	1%
33.	rs2071749	1N3U A 1NI6 A 5BTQ A 6EHA A	98.84%	25%
34.	rs10980508	6QKL N 5AN9 N	94.44%	1%
35.	rs33946401	6FF4 Z	97.22%	14%
36.	rs3761439 HMOX1	1RY1 O	100%	1%
37.	rs33976006	6FF4 Z	98.41%	12%
38.	rs33985510	5HU6 B 1Y85 B 2HHE B 1GBV B	100%	40%
39.	rs334	1GBU B 1CH4 A	100%	38%
40.	rs34151786	1Y85 B	88%	45%

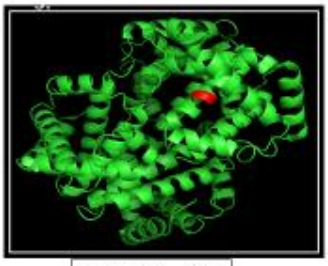
		6HBW B		
41.	rs111033605	1BZ1 A 3IA3 B	97.73%	45%
42.	rs35553496	1CH4 A 1Y7C B	100%	45%
43.	rs33993166	1BZ1 A 3IA3 B 1BAB_A	97.73%	45%
44.	rs9399137	4V19 A 4CE4 A	2(N)	15%
45.	rs7950726	4UE5 A	2(N)	36%
46.	rs2000999	4WJG 2	87.10%	9%
47.	rs8062041 HP	6IRS A	44.44%	16%
48.	rs5471	2ZUE B	100%	2%
49.	rs5470	2ZUE B	100%	2%
50.	rs104894517	3J3V A	86.36%	4%
51.	rs2070937	6QKL N	100%	1%
52.	rs434738	5H1S A	100%	2%
53.	rs599806 HP	31Y9 A	86.36%	5%
54.	rs 664134	4UE4 A	100%	2%

Table-28. Retrieve of all PDB-IDs, sequence identification and query cover of all SNPs by using BLASTx, BLASTn, and tBlastx tool.

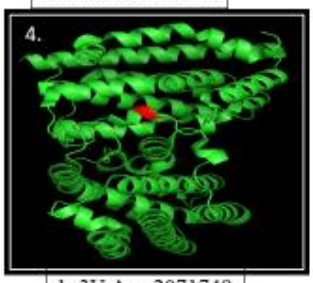
1bz1A rs33993166



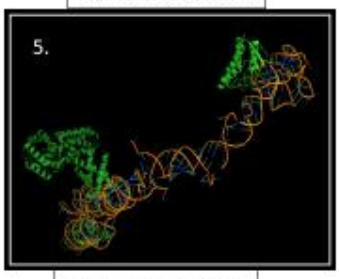
1ch4A rs35553496



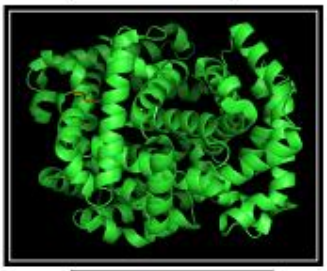
1GBU B rs334



1n3U A rs2071749

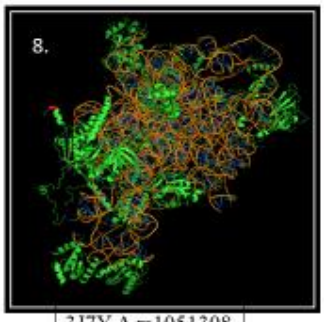


1R1Y O rs3761439

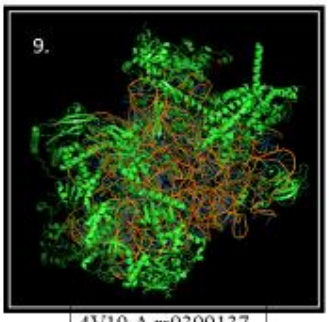


1Y85 A rs34151786

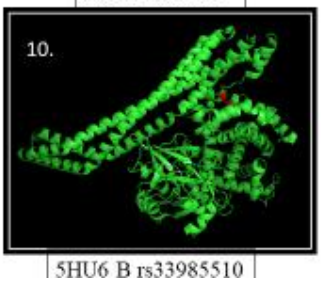
2ZUE B rs5471



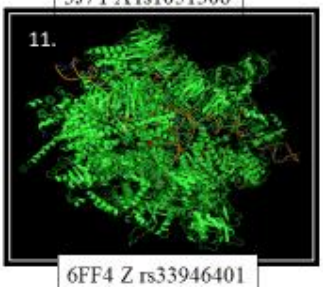
3J7Y A rs1051308



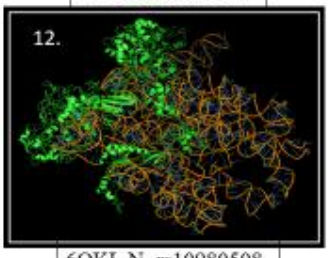
4V19 A rs9399137



5HU6 B rs33985510

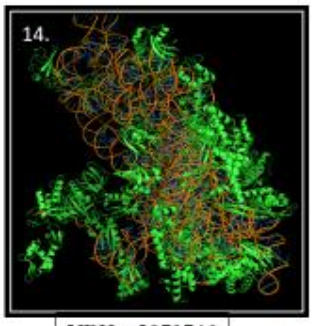


6FF4 Z rs33946401



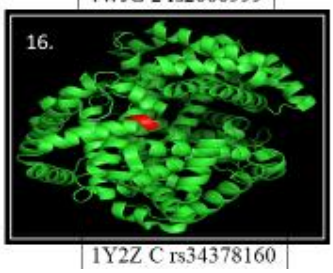
6QKL N rs10980508

4WJG 2 rs2000999

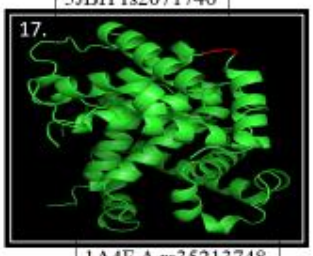


5JBH rs2071746

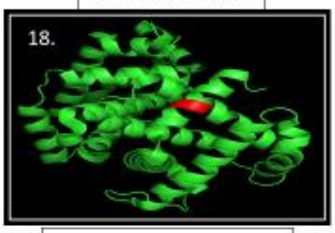
6IRS A rs8062041



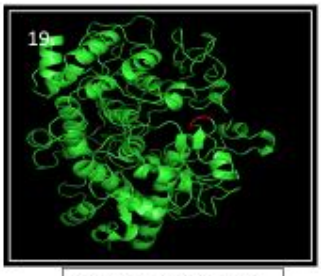
1Y2Z C rs34378160



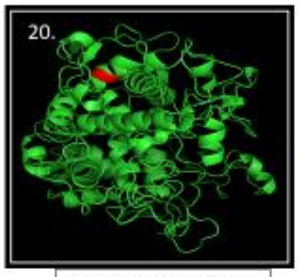
1A4F A rs35213748



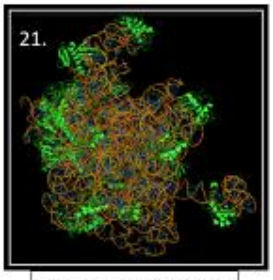
4MQH A rs 35672478



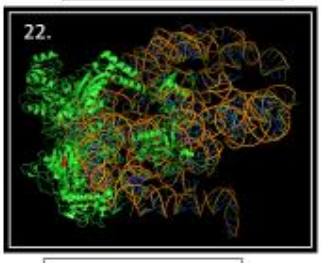
5MFA A rs56378716



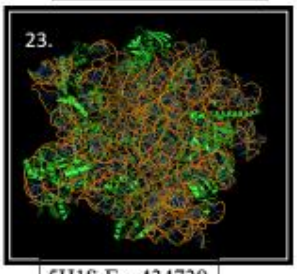
5MFA A rs 78950939



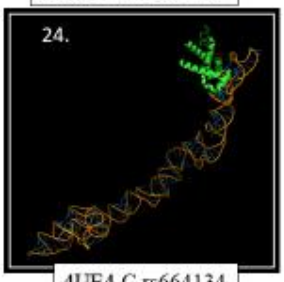
3J3V 5 rs104894517



6QKL B rs2070937



5H1S E rs434738



4UE4 C rs664134

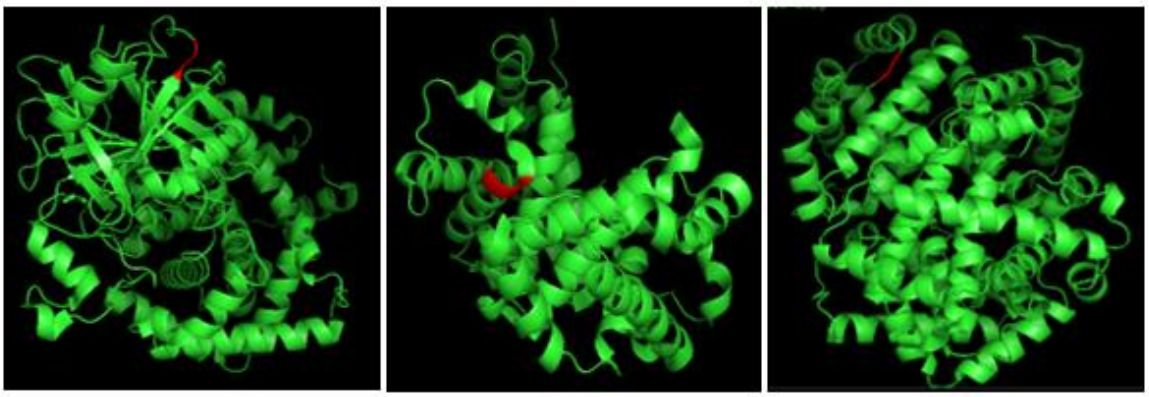


Figure 18. Showing identified mutations in red.

4. Mutations effect of nsSNP: It is predicting by using SIFT, PolyPhen2, Mutation accessor and SNAP2 servers.

4.1. **SNAP2 tool**: The protein sequence of 21 SNP's was submitted in SNAP2 server and results obtained are shown in (Table 29-30). Two SNP's viz., rs35553496 and rs2071747 have neutral or no effect at structural level and the rest of the mutations T-H, Y-C, A-D, G-D, V-E, E-V, E-Q, T-P and T-C have shown an effect on structure and function of these SNP's with the best percentage accuracy of 93, 82, 87, 53 and 97.

PDB ID	SNP's rsID	PREDICTED EFFECT	SCORE	EXPECTED ACCURACY
1Bz1 A	33993166	EFFECT	71	85%
1Bz1 A	111033605	EFFECT	74	85%
1CH4 A	35553496	NEUTRAL	-85	93%
1GBUB	334	EFFECT	67	80%
1n3U A	2071749	EFFECT	86	91%
1RY1 O	3761439	NEUTRAL	-70	82%
1Y85 A	34151786	EFFECT	26	63%
2ZUE B	5471	NEUTRAL	-78	87%
3J7YA	1051308	NEUTRAL	-7	53%
4V19A	9399137	EFFECT	81	91%

4WJG 2	2000999	EFFECT	70	85%
5HU6 B	33985510	NEUTRAL	-99	97%
5JBH 2	2071746	EFFECT	70	85%
6FF4 Z	33946401	EFFECT	58	75%
6IRS A	8062041	EFFECT	94	95%
6QKL	10980508	EFFECT	36	66%
1Y2Z B	34378160	EFFECT	81	91%
1A4F A	35213748	EFFECT	35	66%
4MQH A	35672478	EFFECT	37	66%
5MFA A	56378716	EFFECT	74	85%
5MFA A	78950939	EFFECT	71	85%
3J3V A	104894517	EFFECT	80	91%
6QKL N	2070937	EFFECT	66	80%
5H1S A	434738	EFFECT	71	85%
4UE4 A	664134	EFFECT	56	75%

Table-29. **List of SNP's effect analyzed by SNAP2.**

4.2. SIFT tool: Out of 21 SNP's; 16 SNP's showed deleterious effect with a score of 0, 0.01, 0.02, 0.03 and 0.016. If values vary from 0 and 0.05 is predicted to effect the function of protein. Five more SNP's i.e. rs2071747, rs33993166, rs111033605, rs35553496, and rs334 showed tolerated with a score of 0.2, 0.1 and 0.06 and it has no effect on the function of protein as shown in Table 30.

4.3. Polyphen2 server: 8 SNP's showed benign effect with a score of 0, 0.04, 0.02, 0.03 and 0.016 and rest 13 SNP's showed probably and possibly damaging effect score was nearer to 1. If value is nearer to 1, it is predicted to effect the structure of protein as shown in Table 30.

4.4. Mutation accessor server: Out of 21 nsSNP's; three SNP's rs35553496, rs5475, and rs2071747 shown neutral, six SNP's shown medium with a stability score were 0.6, 0.7, 0.8, and 0.9, and the rest 12 SNP's were shown high with stability score closer to 1. If value is 0 to 1, it is predicted that these variant is disease causing as shown in Table 30.

SNP's RsID	SIFT Score	SIFT prediction	PolyPhen2 Score	PolyPhen2 prediction	Mutation accessor Score	Mutation accessor Prediction	SNAP2 Prediction
rs33993166	0.292	Tolerated	0.278	Benign	0.948	High	Effect
rs111033605	0.292	Tolerated	0.278	Benign	0.948	High	Effect
rs35553496	0.11	Tolerated	0.005	Benign	0.008	Neutral	Neutral
rs334	0.063	Tolerated	0.007	Benign	0.94	High	Effect
rs34151786	0.016	Deleterious	0.53	Possibly damage	0.932	High	Effect
rs33985510	0.018	Deleterious	0.946	probably damage	0.98	High	Effect
rs33946401	0.03	Deleterious	0.875	possibly damage	0.891	Medium	Effect
rs34378160	0	Deleterious	1	Probably damage	0.993	High	Effect
rs35213748	0	Deleterious	0.984	Probably damage	0.994	High	Effect
rs33976006	0.01	Deleterious	0.886	Possibly damaging	0.875	Medium	Effect
rs2071747	0.17	Tolerated	0	Benign	0.096	Neutral	Neutral
rs41514946	0	Deleterious	0.636	Possibly damaging	0.953	High	Effect
rs33916412	0	Deleterious	1	Probably damaging	0.992	High	Effect
rs33921589	0	Deleterious	1	Probably damaging	0.993	High	Effect
rs33924775	0.01	Deleterious	1	Probably damaging	0.993	High	Effect
rs33931984	0.03	Deleterious	0.025	Benign	0.847	Medium	Effect
rs36008922	0.02	Deleterious	0.964	Probably damaging	0.914	Medium	Effect
rs33957286	0.03	Deleterious	0.028	Benign	0.633	Medium	Effect
rs28928876	0	Deleterious	0.99	Probably damaging	0.986	High	Effect

rs5475	0.43	Deleterious	0.049	Benign	0.085	Neutral	Effect
rs3794694	0.4	Deleterious	0.83	Possibly damaging	0.9	Medium	Effect

Table 30. Mutations effect of Coding SNP's.

5. Mutations effect of synonymous SNP: It is predicting by using FuncPred, Regulomedb, and PolymiRTS databases.

5.1. **FuncPred tool:** Several 36 SNPs were admitted to the FuncPred server and outcomes were found inside (Table 31). A total number of 24 SNPs were found to affect the function of which one 3'UTR SNP's rs1051308 was found to affect miRNA binding site and five 5'UTR variants rs5470, rs5469, rs5472, rs2270363, and other SNP's variants were obtained to be affecting transcription factor binding site.

5.2. **PolymiRTS server**: One 3'UTR SNP's rs1051308 showed two alleles G and A affecting the miRNA target sites shown in (Table 31).

5.3. **Regulomedb database**: 8 SNP's had minimum functional evidence (Category4, 5, and 6), two SNP's rs34210688 and rs36071424 had very likely to affect the binding for TF binding + matched TF motif + matched DNase footprint + DNase peak (2b) and 24 SNPs had regulomedb score of '1f' and 1b had elucidation for eQTL+ TF binding DNase peak and thus expected to have regulatory functions. Single SNP; s i.e. 599806 had a range of 7 for variants with no elucidation information found (Table 31).

SNP ID's	Regulomedb			FuncPred	PolymiRTS	
	Score	Category	Description		Alleles	miRNA
rs7201866	1b	Likely to affect binding and linked to expression of a gene target	eQTL +TF binding + any motif +DNase footprint +DNase peak	√	-----	
rs5469	1b			√	------	
rs5470	1b			√	------	
rs437638	1b			√	------	
rs398580	1b			√	------	
rs2270363	1f	Likely to affect	eQTL +TF binding/DNase peak	√	------	
rs1051308	1f			-----	G	has-miR-497-3p
	1f				A	has-miR-6894-5p
	1f					has-miR-7154-3p
	1f					
	1f					

	1f 1f 1f 1f 1f	binding &linked to expressio n of a gene target				has-miR-765 has-miR-766-5p has-miR-7847-3p
rs7203426	1f			√	------	
rs7202268	1f			√	------	
rs5467	1f			√	------	
rs5468	1f			√	------	
rs5471	1f			√	------	
rs5472	1f			√	------	
rs4788458	1f			√	------	
rs8062041	1f			√	------	
rs2236765	1f			√	------	
rs12917999	1f			√	------	
rs2070937	1f			-----	------	
rs35461039	1f			-----	------	
rs3852780	1f			√	------	
rs5478	1f			√	------	
rs8056409	1f			√	------	
rs2000999	1f			-----	------	
rs2071746	1f			-----	------	
rs34210688	2b	Likely to affect binding	TF binding + any motif + DNase footprint + DNase peak	-----	------	
rs36071424	2b			-----	------	
rs63749918	4	Minimal binding evidence	TF binding+ DNase peak	------	------	
rs35672478	4			√	------	
rs434738	4			√	------	
rs3761439	4			√	------	
rs664134	5	Minimal binding evidence	TF binding or DNase peak	------	------	
rs2070938	5			------	------	
rs3794693	5			------	------	
rs2071749	5			------	------	
rs12162087	6	Minimal binding evidence	Motif Hit	√	------	
rs599806	7	NA	NA	√	------	

Table 31. Mutations Effect of Non-coding SNP's.

3.5 Protein-Protein interaction: An outcome for the interaction of pdb files 4X0L; among haptoglobin from "Homo sapiens" (chain C) and haemoglobin alpha chain from "Homo sapiens" (chain A & B) is displayed. The collection of protein-protein interaction complexes, the normalised energy per residue values have been standardised, and it was discovered that the stabilising protein-protein complexes have values between -2 and -6 kJ/mol. as shown in

(Table 32). With a normalised energy value more than 1, the Top-9 residues in this belong to chain C and are described as potential hotspots because they exhibit the maximum level of interaction. With a normalised energy greater than 1, only three residues from chain A were among the Top-9 residues. Similarly, in the interaction of chain C with chain B four residues from chain C and five residues from chain B reported as potential hotspots with a normalized energy more than 1 as shown in (Table 33). All of the projected hotspots are plotted on the B-factor field with a score of "0 to 6" i.e. it is less fluctuate as showing in (Figure 19).

Interactions	Chain A with C	Chain B with C
Hydrogen Bond Energy	-36.19 kJ/mol	-23.86 kJ/mol
Electrostatic Energy	-21.84 kJ/mol	15.56 kJ/mol
Van der Waals Energy	-213.85 kJ/mol	-149.80 kJ/mol
Total Stabilizing Energy	-271.88 kJ/mol	-158.10 kJ/mol
Number of interface residues	86	68
Normalized Energy per residue	-3.16 kJ/mol	-2.33 kJ/mol
No. of Short Contacts	8	3
No. of Hydrophobic Interactions	4	1
No. of van der Waals Pairs	8494	6406
No. of Salt Bridges	4	0
No. of Potential Favourable Electrostatic Interactions	3	8
No. of Potential Unfavourable Electrostatic Interactions	7	7

Table-32. Interactions and Total Stabilizing Energy.

Res Num	Res Name	Chain	Res Num	Res Name	Chain
290	PHE	C	167	LEU	C
139	SER	A	41	ARG	B
383	VAL	C	102	GLU	B
2	VAL	A	100	ASP	B

352	TYR	C	98	HIS	B
328	VAL	C	289	ASN	C
135	THR	A	38	TRP	B
346	TYR	C	295	HIS	C
329	GLY	C	160	GLN	C

Table-33. **Potential Hotspots.**

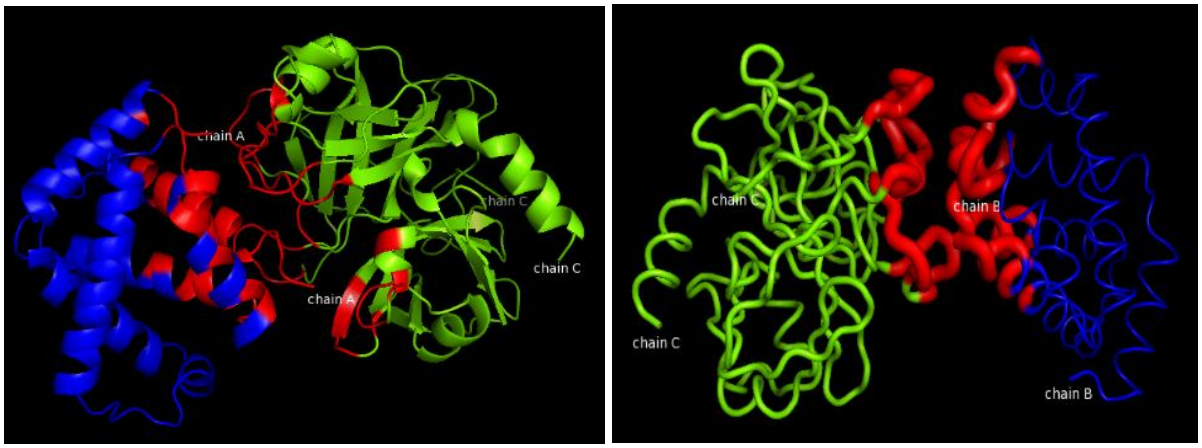


Figure-19. Illustrating B-factor of Chain A (Hb) interaction with Chain C (Hp) and Chain B (HB) interaction with Chain C (HP).

The CB-CB atom distance of Valine amino acid at 2 & 136 position or residue position of Chain A (2VALA and 136VALA) showing Hydrophobic interactions with Valine at position or residue number 383 & 328 of Chain C (383VALC & 328VALC) at distance 6.38A° & 6.07A°. Similarly, Valine amino acid at 97 position or residue position of Chain A (97VALA) have shown Hydrophobic interactions with Phenylalanine at position or residue number 290 of Chain C (290PHEC) at distance 4.94A°. Phenylalanine amino acid at 99 position or residue position of Chain A (99PHEA) are showing hydrophobic interactions with phenylalanine at position or residue number 290 of Chain C (290PHEC) at distance 6.49A°. In case of haemoglobin chain B interaction with haptoglobin chain C Leucine amino acid at 106 position or residue position of Chain B (106LEUB) showing Hydrophobic interactions with alanine at position or residue number 288 of Chain C (288ALAC) at distance 6.28A° as shown in (Table 34).

RESIDUE-1				RESIDUE-2				Type of H-Bond	Distance (D-A) Å
Res Num	Res Name	Chain-1	Atom Name	Res Num	Res Name	Chain-2	Atom Name		
95	ASP	A	OD1	286	ARG	C	NH2	SS	2.95
95	ASP	A	OD2	286	ARG	C	NH1	SS	3.03
135	THR	A	O	352	TYR	C	OH	BS	3.25
139	SER	A	O	379	LYS	C	NZ	BS	2.84
142	ARG	A	OXT	183	HIS	C	NE2	SS	2.71
41	ARG	B	O	160	GLN	C	NE2	BS	2.96
41	ARG	B	NH2	165	GLY	C	O	SB	2.93
44	GLU	B	OE2	160	GLN	C	NE2	SS	3.07
102	GLU	B	OE1	288	ALA	C	N	SB	3.16

Table-34. Potential Hydrogen Bonds

Here, it is reported that the charged residues, ASP and ARG, form salt bridges and that the side-chain nitrogen and oxygen atoms of two oppositely charged residues can be found to be closer together than 4A° i.e. Aspartic acid amino acid at 95 position or residue position of Chain A (95ASP) form salt bridge with Arginine amino acid at position or residue number 286 of Chain C (286ARG) at distance 3.80, 2.95, 3.03 & 3.70A° as shown in (Table 35). All charged interprotomer amino acid pairings with atomic distances of 10A° or fewer are taken into account and reported. Within this distance limit, both favourable (opposite charges) and unfavourable (similar charges) electrostatic interactions are documented as shown in (Table 36 & 37). A short contact is thought to be formed by all atoms that are closer to a distance that is equal to the total of their van der Waals distances, -0.40A°. Here, the short contacts of all the atoms is greater than -0.40A° as shown in (Table 38). These all residues showed protein-protein interactions in haptoglobin chain C with haemoglobin chain A & B.

RESIDUE-1				RESIDUE-2				Distance Å
Res Num	Res Name	Chain-1	Atom Name	Res Num	Res Name	Chain-2	Atom Name	
2	VAL	A	CB	383	VAL	C	CB	6.38

97	VAL	A	CB	290	PHE	C	CB	4.94
99	PHE	A	CB	290	PHE	C	CB	6.49
136	VAL	A	CB	328	VAL	C	CB	6.07
106	LEU	B	CB	288	ALA	C	CB	6.28

Table-35. Potential Hydrophobic Interactions

RESIDUE-1				RESIDUE-2				Distance Å
Res Num	Res Name	Chain-1	Atom Name	Res Num	Res Name	Chain-2	Atom Name	
95	ASP	A	OD1	286	ARG	C	NH1	3.80
95	ASP	A	OD1	286	ARG	C	NH2	2.95
95	ASP	A	OD2	286	ARG	C	NH1	3.03
95	ASP	A	OD2	286	ARG	C	NH2	3.70
76	ASP	A	CB	325	LYS	C	CB	9.69
95	ASP	A	CB	286	ARG	C	CB	9.97
95	ASP	A	CB	291	LYS	C	CB	9.70
98	HIS	B	CB	168	ASP	C	CB	9.33
100	ASP	B	CB	166	HIS	C	CB	7.89
100	ASP	B	CB	295	HIS	C	CB	7.75
100	ASP	B	CB	297	LYS	C	CB	5.90
102	GLU	B	CB	286	ARG	C	CB	9.75
102	GLU	B	CB	291	LYS	C	CB	7.69
102	GLU	B	CB	295	HIS	C	CB	8.89
102	GLU	B	CB	297	LYS	C	CB	8.04

Table-36. Potential Favorable Electrostatic Interactions

RESIDUE-1				RESIDUE-2				Distance Å
Res Num	Res Name	Chain-1	Atom Name	Res Num	Res Name	Chain-2	Atom Name	

93	ARG	A	CB	183	HIS	C	CB	8.83
100	LYS	A	CB	286	ARG	C	CB	9.45
100	LYS	A	CB	291	LYS	C	CB	8.19
140	LYS	A	CB	379	LYS	C	CB	9.17
142	ARG	A	CB	183	HIS	C	CB	9.04
142	ARG	A	CB	202	LYS	C	CB	9.25
142	ARG	A	CB	208	HIS	C	CB	9.98
98	HIS	B	CB	166	HIS	C	CB	7.07
98	HIS	B	CB	170	LYS	C	CB	9.50
100	ASP	B	CB	168	ASP	C	CB	9.51
100	ASP	B	CB	348	GLU	C	CB	7.72
102	GLU	B	CB	294	ASP	C	CB	8.72
102	GLU	B	CB	348	GLU	C	CB	8.60
105	ARG	B	CB	291	LYS	C	CB	8.25

Table-37. Potential Unfavorable Electrostatic Interactions

RESIDUE-1				**RESIDUE-2**				**Distance Å**
Res Num	**Res Name**	**Chain-1**	**Atom Name**	**Res Num**	**Res Name**	**Chain-2**	**Atom Name**	
96	PRO	A	HD2	286	ARG	C	HH22	1.80
138	THR	A	C	352	TYR	C	HH	2.39
139	SER	A	N	352	TYR	C	HH	2.29
139	SER	A	CA	352	TYR	C	HH	2.49
139	SER	A	O	379	LYS	C	HZ2	1.88
139	SER	A	HA	352	TYR	C	HH	1.83
139	SER	A	HB3	330	VAL	C	HG13	1.90
142	ARG	A	OXT	183	HIS	C	HE2	1.74
41	ARG	B	HH21	166	HIS	C	HA	1.95

102	GLU	B	OE1	287	ASN	C	HB3	1.91
102	GLU	B	OE1	288	ALA	C	H	2.14

Table-38. **Potential Short Contacts**

CHAPTER- VII

DISCUSSIONS

DISCUSSIONS

Despite major breakthroughs in diabetes prevention and medical care, CVD continues to disproportionately afflict diabetics across the world. As a result, the development of vascular problems in diabetes is clearly influenced by genetic susceptibility, and an efficient variation in the gene has been determined as an indicator of the chance of developing these issues. Early discovery of the condition and prompt treatment can lessen the disease's morbidity and death (Low wang et al, 2016 and Schmidt, 2019).

In our findings, the anthropometric variables i.e. BMI in T2DM (25.97±5.359) was found to be statistically significant i.e. p=0.018 as compared to CVD (27.43±4.77) patients and healthy participants (28.21±6.31) i.e. p=0.003. Similar findings were found in the research conducted by Sheriff et al. and Loureiro NSL et al. In our study, WC, WHR and HC were found to be statistical significance in CVDWDM (41.29±5.51, 0.95±0.012 & 43.35±5.60) as compared to healthy (37.90±6.31, 0.945±0.007 & 39.90±6.3), CVD (38.34±5.23, 0.94±0.010 & 40.39±5.20) and T2DM (38.46±4.77, 0.94±0.036 & 40.78±4.82). BMI is a risk factor of obesity and in non-communicable diseases. The high level of BMI is because of insulin resistance and due to the deposition of adipose mass near the area of waist and hips may cause obese person and CVD disease. These variables dependent on environmental factors, hereditary, dietary patterns and diminish extent of physical activity. Similar findings were found in the research conducted by Sheriff et al, Lampignano et al and Hosseini et al, 2022. Insulin resistance, the main cause of type 2 diabetes, may be contributing to the rise in BMI. Insulin sensitivity of cell membranes is significantly decreased. As a result, the crucial mechanism by which insulin helps glucose flow through the cell wall to be turned into energy is significantly hampered. As a result, extra glucose continues to circulate in the circulation, resulting in high blood sugar levels that are transferred to the liver. Once there, the sugar is transformed into fat and circulated throughout the body via the bloodstream. Obesity and weight increase are outcomes of this mechanism. The BMI and WHR measurements are dependent on dietary patterns and dwindling levels of physical activity. It has been hypothesised that these environmental influences, particularly in those people with metabolic genotype, reveal a hereditary vulnerability to obesity.

In our findings, the biochemical parameters such as TC, HDL-C were statistically non-significant in CVDWDM (174.55±49.04, 34.91±9.80) as compared to healthy (169.45±39.51, 33.89±7.90) and T2DM (180.87±49.18, 36.17±9.83). Our results were not found to be similar

with Rusdiana et al. TG and VLDL were statistically non-significant in CVDWDM (163.70±73.26, 32.74±14.65) as compared to healthy individuals (185.87± 140.36, 37.17±28.07) i.e. p=0.237. LDL was statistically significant in CVD (140.98±36.69) as compared to healthy (98.39±37.14) individuals, T2DM (93.52±51.28) and CVDWDM (98.39±37.14) i.e. p=0.001, 0.002 & 0.005. Similar results were found in the research conducted by Sheriff et al, Priya and Begum et al, 2020 and Mehtarian et al, 2021. The high levels of the lipid profile are related with the dietary or eating habits (Wang et al, 2022).

The major markers of Cardiovascular disease are APO A1, SBP, DBP, MAP, PP, AIP were examined in diseases and healthy participants. Mean arterial pressure, pulse pressure and systolic and DBP were found to be non-statistically significant in T2DM as compared to healthy participants. Similar results were found in the research conducted by Adegbenga AB, 2018, Wang man et al. DBP were found to be non-statistically significant in CVDWDM as compared to CVD participants. Similar results were found in the research conducted by Wang man et al. SBP, DBP, PP, MAP influenced by structural changes in blood vessels, wave reflection or arterial stiffness (Wang man et al, 2022 and Jiang et al, 2022).

Apo A1 is the part of cholesterol molecule so changes in the concentrations of the cholesterol molecule, it reflected in the serum levels of apolipoproteins. In our findings, Apo A1 were statistically significant in CVDWDM and CVD as compared to healthy participants. Similar results were found in the research conducted by sheriff et al. But not similar results with research conducted by villalpando et al, 2019. ApoA-I undergoes post-translational modifications, including fatty acid acylation and oxidation, which have been linked to the environment and metabolism. Non enzymatic protein glycation and Advanced Glycation End Product production have received a lot of interest in diabetic patients. Both are linked to hyperglycemia in type 1 and type 2 diabetes, where AGE adducts bind to ApoA-I and prevent it from activating Lecithin Cholesterol Acyltransferase (LCAT), the enzyme that converts nascent HDLs to mature HDLs. The development of Coronary Artery Disease (CAD) appears to be accelerated by AGEs. Modification experiments on ApoA-I have also revealed that the most basic versions are the protein's less mature isoforms. In most forms of amyloidosis, atherosclerosis, and neurodegenerative disorders, proteolysis is assumed to play a key role. In fact, HDL-associated ApoA-I is really destroyed by macrophage metalloproteinase in coronary patients at both the N and C termini. As a result, the transition from lipid-free ApoA-I to spherical HDL particles may be hampered by the lack of the first 38 amino acids present in ApoA-I (1-38). Possibly decreasing reverse cholesterol transit and making it easier for it to

attach to LDL particles. Large HDL complexes need the N-terminal amino acid residues, and their removal results in less stable HDL particles (Cubedo et al, 2015 and Amoorthy et al, 2017).

Fasting blood sugar and HbA1c are a marker of diabetic mellitus, it was found to be statistically significant in all variables of our study but HbA1c showed non-statistically significant in T2DM as compared to CVDWDM participants. Similar results were found in the research conducted by Kumar et al and Nnakenyi et al, 2022. According to reports, VLDL and LDL absorption in the liver is reduced in T2DM patients, which causes their amounts of these lipoproteins in the plasma to rise, especially in the postprandial period. This condition is most frequently seen in type T2DM patients who have severe insulin insufficiency or inadequate glycemic control. Additionally, it has been stated that the lower availability of LDL receptors contributed to the restricted LDL clearance. Previous research has connected DM and diabetic microvascular problems, such as nephropathy and retinopathy, to the Hp polymorphism, piquing researchers' interest in elucidating the function of Hp phenotypes in DM and related cardiovascular consequences.

The goal of this study was to find out the relation of hp2-2 genotype with lipid profile variables, APOA1, HbA1C, blood sugar levels in diabetic, cardiovascular disease and cardiovascular with diabetic disease. In our study, in T2DM the hp2-2 genotype showed positive or statistical significance i.e. $p< 0.05$ with TC, HDL, TG, VLDL, Smoking, Tobacco users, Physical activity as well as fasting plasma glucose. Similar results were found in the research conducted by Bhardwaj et al, 2020 but LDL showed negative results with this study. The relationship of hp2-2 genotype with CVD participants showed statistical significance i.e. $p<0.05$ with lipid parameters, Fasting plasma glucose, HbA1c, SBP, DBP, MAP, PP, alcohol use, APOA1. But negative results were found in the research conducted by Mehtarian et al, 2021. The relationship of hp2-2 genotype with CVDWDM participants showed statistical significance i.e. $p<0.05$ with TC, LDL, VLDL, Smoking, SBP, DBP, MAP, PP & APOA1. But negative results were found in the research conducted by Vasudevan et al, 2022. The participants of T2DM, CVD, and CVDWDM were shown to have the Hp2-2 genotype, but healthy participants did not.

The impact of this gene in infection and oxidation has sparked speculation about its possible link to cardiovascular disease, particularly in the presence of elevated oxidative stress [16]. It has been reported that diabetic people with the Hp2-2 phenotype have a five-fold higher chance

of having CVD than people with the Hp1-1 phenotype. It was discovered that the Hp 2-1 phenotype was connected to an intermediate risk of CVD. Individuals without diabetes mellitus, however, did not show any connection. The Hp 2-2 allele was linked to a very significant increase in the risk of severe adverse cardiac events in a study, on 935 DM patients. Firstly, the Hp2-2 protein molecule stays in the transmission for a longer period of time and is shown to have undergone greater oxidative damage owing to the bigger molecule of Hp1-1. Secondly, in the Hp1-1 protein molecule haem variation from methemoglobin (metHb) to LDL molecule was removed but partially occurs in Hp2-2. Due to that limited haem transport from the Hb-Hp2-2 molecule to molecule of LDL causes oxidation of LDL lipids as well as protein. The lower capability of Hp2-2 protein to preserve haem molecule in the haem pocket of HbA1c. Hence, Hb-Hp2-2 molecules in diabetes are removed more slowly than the Hb-Hp1-1 complex. When the participants have both Hp2-2 genotype and HbA1c≥6.5%, they may be at greater risk of CVD in plasma redox-active Hb-Hp complex. This complex is further linked to HDL molecule, thus results in damage to the activity of HDL in stimulating Reverse Cholesterol Transport (RCT), decrease antioxidant activity, and increase lipoprotein oxidation from heme transport.

The Hp 2-2 genotype is assessed in patients with diabetes and related consequences. Therefore, the Hp 2-2 genotype may be a valuable indicator of a person's likelihood of developing DM and its consequences. The low cost and ease of use of the genotyping technique are another advantage of this work. Another application is to detect Single Nucleotide Polymorphisms (SNPs), small insertions/deletions, and copy number variations is another benefit of genotyping approaches over whole genome sequencing and genome wide association studies (which is the case for haptoglobin).

The DNA sequencing data of diabetic, cardiovascular and cardiovascular with diabetic participants were aligned with haptoglobin sequence. It showed A to C mutation as well as haptoglobin SNP’s were found i.e. rs3852780 (intron region) and rs5471(5’UTR region). These two SNP’s are associated with transcription factor binding site and the variation is more likely to be a regulatory variable that affects gene expression.

In silico analysis

The extraction of SNPs of hemoglobin and haptoglobin genes by using SNPedia, dbSNP, and NCBI databases. These genes cover the coding and non-coding regions, not only coding SNP’s are relevant but also a missense variant, non-coding part changes in the amino acid sequence

(Kalia et al, 2016). SNP's are predicted with computational analysis with the manipulation of MAF. MAF is combined with a statistical power of a study, viz; sample size and MAF is used to discover the mutant allelomorph of an SNP in a mentioned population have an opposite reaction (Grover et al, 2007). SNPs with a frequency of MAF ≥ 0.05 are normally marked in the maximum number of genome studies, for occurrence, the international HapMap project (https://www.ncbi.nlm.nih.gov/projects/SNP/docs/rs_attributes.html).

The interaction of haptoglobin with other genes were found 77% i.e. 77 percent similarity were found with other genes. The effect of mutations in their respective structures and functions was predicted by using different tools i.e. to predicting the nsSNP by using SIFT, PolyPhen2, Mutation accessor and SNAP2 tool servers, and the synonymous SNP's predicting by FuncPred, Regulomedb, and PolymiRTS databases. The damaging, tolerated and deleterious effects were shown by SIFT and PolyPhen2. The SNAP2 server predicts the mutations at a functional and structural level of proteins some shown neutral and some shown effects with the best percentage accuracy. A neutral amino acid substitution may change the consensus sequence arrangement involved in mRNA splicing and lead to rare transcripts (Duarte et al, 2018). Mutation accessor predicts the alterations on proteins, these changes have an impact on both the stability and functionality of proteins.

Agundez et al, showed rs1051308 this 3'UTR SNP's affect the miRNA binding site i.e. the A allele of this SNP has been associated with the development of multiple sclerosis disease as well as rs1051308 is more likely to be a regulatory variable that affects gene expression and is marked with enhancer histone marks and connected to an eQTL and our result is similar with this study (Agundez et al, 2016). 37 SNPs were examined for functional need for candidate gene investigations based on these characteristics.

Ofon et al, showed the polymorphism of SNP i.e. rs8062041 of HP (present at intron 1-2), is associated with transcription factor binding site and the variation is more likely to be a regulatory variable that affects gene expression and our findings similar with this study. It showed T allele of rs8062041 has been linked to a decreased chance of getting the sleeping sickness and it is statistically significant (p=0.0002) in Cameroonian population (Ofon et al, 2017).

Abah et al showed the SNP rs12162087 (-1203G > A) is associated with severe malarial anemia as well as associated with low levels of HP. Due to its location upstream of the HP gene's transcriptional start site, the variation is more likely to be a regulatory variable that affects gene

expression. Moreover, this variation is marked with enhancer histone marks and connected to an eQTL. The HP gene's proximal upstream (promoter) region and distal upstream region may interact through DNA looping, which may impact the production of the protein our result is similar with this study (Abah et al, 2018).

De Feudis et al and Soejima et al showed this rs5467 SNP was connected with Hp concentrations, rs5472 has been shown to be strongly associated with Hp expression, impacting the serum protein levels. Three polymorphisms (rs5467, rs5469 and rs5472) were identified by genetic research of the Hp gene promoter area in GH patients (De Feudis et al, 2019 and Soejima et al, 2020). However, in our study these all SNP's variant is related to an eQTL by the prediction of Regulomedb database.

Previous studies showed the SNP rs17177078 was associated in connection with dietary heme iron consumption to T2DM (type2 diabetes mellitus) but it is not statistically significant (Pasquale et al, 2013). rs2071746 polymorphism was linked to clinical outcomes in individuals with atherosclerosis-related ischemic stroke. The A allele of this SNP is protected factor for patients with heart stroke and in T2DM patients, T(-413)A has connections with the incidence of subclinical atherosclerosis and diabetic nephropathy, respectively (Cao et al, 2014 and Lee et al, 2015). For T2DM patients, vascular atherosclerosis and diabetic nephropathy risk were related with rs3761439 G(−1135)A polymorphisms in the HO-1 promoter (Lee et al, 2015). However, in our study the functions of this SNPs is based upon their existence in a minimal binding evidence and transcription factor binding site.

The SNP rs2000999 is a likely to affect binding and linked to expression of a gene target as well as eQTL +TF binding/DNase peak. In other study, this SNP showed a potent genetic predictor of blood levels of HP and has been linked to decreased levels of total and low-density lipoprotein cholesterol. The rs2000999 was associated with cardiovascular risk factor. Given that these SNPs are upstream of the HP gene's transcriptional start site; the variation is more likely to represent a regulatory factor that influences gene expression (Froguel et al, 2012 and Soejima et al, 2014).

Protein-protein interaction can examine a variety of protein-protein interfaces and provide a quantitative representation of the degree to which interactions occur at the interface (Sukhwal and Sowdhamini, 2015). The collection of protein-protein interaction complexes, the normalized energy per residue values have been standardized, and it was discovered that the stabilizing protein-protein complexes have values between -2 and -6 kJ/mol. All hotspots are

displayed in one color in PPCheck, while the remaining amino acids are displayed in a different shade with a 70% accuracy rate, PPCheck can forecast these hotspots. The first two columns for the Top-9 residues include the residue number and name, while the third column lists the protein chain to which each of the Top-9 residues belongs as shown in (Table 33).

B-factors have been used to explore protein flexibility in vividly. To create a sequence-based predictor of local protein flexibility, B-factor information was utilized. Additionally, crystal packing connections and physiological protein-protein binding sites have been distinguished by using B-factors (Sukhwal and Sowdhamini, 2015 and Carugo et al, 2018).

If the CB-CB atom distance of any hydrophobic residue is smaller than 7A°, such as ALA, VAL, LEU, TRP, ILE, TYR, and PHE, hydrophobic interactions are observed to arise. It has been observed that all charged residues, including LYS, ASP, HIS, ARG, GLU and LYS, form salt bridges when the distance among the side-chain oxygen and nitrogen atoms of two oppositely charged residues is closer than 4A°. However, our result is similar with this study.

The case-control studies, regulatory and functional analyses of Hp SNPs: rs5467, rs5469, rs5472, rs1051308, rs8062041, and rs2000999 were documented. The other SNPs need significant study since they have not yet been investigated. As a result, scientists will be able to concentrate on the experimental data of these SNPs in a broad spectrum of inflammatory disease scenarios.

CHAPTER- VIII

CONCLUSIONS

CONCLUSIONS

BMI and Waist circumference is a risk factor of obesity, diabetes and cardiovascular disease in our study. Fasting blood glucose, HbA1c, MAP, PP, SBP are risk factor in diabetic state and progression of heart diseases in diabetic individuals. Total cholesterol, HDL, LDL, TG and VLDL are a risk factor in diabetes mellitus and cardiovascular disease as compared to healthy participants. APOA1 is a biomarker only in case of cardiovascular disease in our study. Atherogenic index plasma is showing a correlation only in diabetic mellitus individuals in our study.

Obesity is also a biomarker for the production of T2DM in our population. In case of diabetic mellitus individual's lipid profile i.e. TC, HDL, LDL, VLDL, physiometric parameters i.e. smoking, tobacco takers, sedentary lifestyle as well as fasting blood glucose are high and showing a positive correlation with haptoglobin2-2 genotype in our population.

In case of cardiovascular participant's lipid profile i.e. TC, HDL, LDL, VLDL, TG, physiometric parameters i.e. smoking, tobacco takers, sedentary lifestyle, drinkers as well as APOA1 are high and showing a positive correlation with haptoglobin2-2 genotype in our population.

In case of CVDWDM individual's lipid profile i.e. TC, LDL, TG, VLDL, physiometric parameters i.e. smoking, as well as SBP, DBP, MAP and PP are high and showing a positive correlation with haptoglobin2-2 genotype in our population.

Our results suggested that hp2-2 genotype is a marker for diseases. In our study, Hp2-2 genotype is detected in diabetic, cardiovascular as well as cardiovascular with diabetic diseases. If it is present in diabetic participants, then it is more prone to cardiovascular disease. If it is present in cardiovascular participants, then it is more prone to heart stroke, arteries blockage, myocardial infarction. If in some patients don't show hp2-2 genotype in diseases, then it is due to the dietary habits, environmental factors, duration of diseases, taking supplements of vitamin E & C.

The DNA sequencing data of diabetic, cardiovascular and cardiovascular with diabetic participants were aligned with haptoglobin sequence. It showed A to C mutation as well as haptoglobin SNP's were found i.e. rs3852780 (intron region) and rs5471(5'UTR region). These two SNP's are associated with transcription factor binding site and the variation is more likely to be a regulatory variable that affects gene expression.

An incredible number of SNPs accessible in the Hp gene that have been eminent. Some SNPs have shown neutral i.e. rs5471 and rs33985510 and some have shown having an effect of a mutation on their structure. Some SNPs rs3151786, rs33946401, rs34378160, rs35213748, rs56378716, and rs78950939 showed a deleterious and damaging effect, and alter the functionality of protein and their stability. Therefore, these mutants are associated with diseases i.e. HB O Padova, HB G, HB Valletta (Beta-thalassemia), HB S (Travis), HB J Europa, Hb Marineo, Myeloperoxidase deficiency, anhaptoglobinemia, Parkinson's disease, cholesterol storage disease, coronary artery disease, atherosclerosis, type2 diabetes, sickle cell anemia, lung cancer, and schizophrenia disease. This will become more relevant in the next-generation sequencing data analysis to study any type of disease.

The case-control studies, regulatory and functional analyses of Hp were related to these SNPs: rs5467, rs5469, rs5472, rs1051308, rs8062041, and rs2000999. The other SNPs need significant study since they have not yet been investigated. As a result, scientists will be able to concentrate on the experimental data of these SNPs in a broad spectrum of inflammatory disease scenarios.

Hence, mutation's effects concerning their structures and functions had to be understood and further detailed study will result in paving path towards more precise and accurate identification of the associated diseases.

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APPENDICES

School of Bioengineering & Biosciences

LOVELY PROFESSIONAL UNIVERSITY, PHAGWARA

CONSENT FORM

I have been explained the possible risks and benefits and also have understood the purpose which my blood samples are being sought by the School of Bioengineering & Biosciences of Lovely Professional University, Phagwara.

I am free from my pressure whatsoever and hereby give my own consent to

(i) The withdrawal of blood samples (about 5ml) blood by venepuncture; and

(ii) To all types of analysis of my blood samples for research purposes for acquisition of knowledge for the benefit of mankind by School of Bioengineering & Biosciences.

I will have the right to know the analyzed result of my sample and I am not giving my consent for disclosure of any personal information either direct or indirect from the analysis of my sample to anyone without my further consent.

Signature/Thumb Impression

Name of Subject:

Date:

Name of Investigator:

Date:

Signature:

Name of the Witness/Clinician

Date:

Signature:

School of Bioengineering & Biosciences
LOVELY PROFESSIONAL UNIVERSITY, PHAGWARA

CONSENT FORM

ਮੈਨੂੰ ਸੰਭਾਵਿਤ ਜੋਖਮਾਂ ਅਤੇ ਲਾਭਾਂ ਬਾਰੇ ਦੱਸਿਆ ਗਿਆ ਹੈ ਅਤੇ ਇਹ ਉਦੇਸ਼ ਵੀ ਸਮਝ ਗਿਆ ਹੈ ਕਿ ਮੇਰੇ ਲਹੂ ਦੇ ਨਮੂਨੇ ਲਵਲੀ ਪ੍ਰੋਫੈਸ਼ਨਲ ਯੂਨੀਵਰਸਿਟੀ, ਫਗਵਾੜਾ ਦੇ ਸਕੂਲ ਆਫ਼ ਬਾਇਓਇਨਜੀਨੀਅਰਿੰਗ ਐਂਡ ਬਾਇਓਸੈਂਸੀਅਨ ਦੁਆਰਾ ਮੰਗੇ ਜਾ ਰਹੇ ਹਨ।

ਮੈਂ ਆਪਣੇ ਦਬਾਅ ਤੋਂ ਮੁਕਤ ਹਾਂ ਅਤੇ ਇਸ ਨਾਲ ਮੇਰੀ ਆਪਣੀ ਸਹਿਮਤੀ ਹੈ

(i) ਵੈਂਪੰਕਚਰ ਦੁਆਰਾ ਲਹੂ ਦੇ ਨਮੂਨਿਆਂ (ਲਗਭਗ 5 ਮਿ.ਲੀ.) ਖੂਨ ਵਾਪਸ ਲੈਣਾ; ਅਤੇ

(ii) ਸਕੂਲ ਆਫ਼ ਬਾਇਓ ਇੰਜੀਨੀਅਰਿੰਗ ਐਂਡ ਬਾਇਓਸਾਇੰਸਿਜ਼ ਦੁਆਰਾ ਮਨੁੱਖਤਾ ਦੇ ਲਾਭ ਲਈ ਗਿਆਨ ਦੀ ਪ੍ਰਾਪਤੀ ਲਈ ਖੋਜ ਦੇ ਉਦੇਸ਼ਾਂ ਲਈ ਮੇਰੇ ਖੂਨ ਦੇ ਨਮੂਨਿਆਂ ਦੇ ਹਰ ਪ੍ਰਕਾਰ ਦੇ ਵਿਸ਼ਲੇਸ਼ਣ ਲਈ।

ਮੈਨੂੰ ਮੇਰੇ ਨਮੂਨੇ ਦੇ ਵਿਸ਼ਲੇਸ਼ਿਤ ਨਤੀਜੇ ਨੂੰ ਜਾਣਨ ਦਾ ਅਧਿਕਾਰ ਹੋਵੇਗਾ ਅਤੇ ਮੈਂ ਆਪਣੀ ਨਿੱਜੀ ਸਹਿਮਤੀ ਤੋਂ ਬਿਨਾਂ ਕਿਸੇ ਨੂੰ ਵੀ ਨਮੂਨੇ ਦੇ ਵਿਸ਼ਲੇਸ਼ਣ ਤੋਂ ਸਿੱਧੇ ਜਾਂ ਅਪ੍ਰਤੱਖ ਤੌਰ ਤੇ ਕਿਸੇ ਵੀ ਨਿੱਜੀ ਜਾਣਕਾਰੀ ਦੇ ਖੁਲਾਸੇ ਲਈ ਸਹਿਮਤੀ ਨਹੀਂ ਦੇ ਰਿਹਾ।

ਦਸਤਖਤ / ਅੰਗੂਠੇ ਦੀ ਪ੍ਰਭਾਵ

ਵਿਸ਼ਾ ਦਾ ਨਾਮ:

ਤਾਰੀਖ਼:

ਜਾਂਚਕਰਤਾ ਦਾ ਨਾਮ:

ਤਾਰੀਖ਼:

ਦਸਤਖਤ:

ਗਵਾਹ / ਕਲੀਨੀਸ਼ੀਅਨ ਦਾ ਨਾਮ

ਤਾਰੀਖ਼:

ਦਸਤਖਤ:

Department of Bioengineering and Biosciences

Lovely Professional University, Phagwara-Jalandhar, G.T. Road, Punjab

PERFORMA

Sample No. **Date:**

1. Name:
2. Sex: M/F
3. Age:
4. Father's Name:
5. Mother's Name:
6. Siblings:
7. Address:
8. Education:
9. Contact No.:
10. Smoking: Y/N
    - Past
    - Current

11. Tobacco: Y/N
12. Alcohol consumption: Y/N
    - Past
    - Current
13. Dietary habits: Veg./Non-Veg.
14. Physical activity: (1-very low, 2- low, 3-moderate, 4-high, 5- very high)
15. Anthropometric Parameters
    1. Height:
    2. Weight:
    3. BMI:
    4. Waist circumference:
    5. Hip circumference:
    6. Waist-hip ratio:
16. Clinical parameters
    1. Blood pressure:

       Systolic:

       Diastolic:

17. Laboratory Parameters

- Fasting blood glucose (FBG):
- Total cholesterol (TC):
- Triglycerides (TG):
- High density lipoprotein (HDL):
- Low Density lipoprotein (LDL):
- Very low Density lipoprotein (VLDL):
- Apo lipoprotein A1 (APOA1):
- Glycosylated Haemoglobin (HbA1c):
- Mean Arterial Pressure (MAP):
- Pulse Pressure (PP):
- Atherogenic Index Plasma (AIP):

18. Glycemic status

- Duration of diabetes:
- Family history of diabetes: Mother/Father/Spouse/ Siblings/Children/Grandparents

19. Heart status

- Duration of cardiovascular:
- Family history of cardiovascular:
- Other complications:

20. Use of medications:

Place from where sample collected:

Amount: Storage conditions:

RESEARCH PUBLICATIONS/ CONFERENCES/ WORKSHOPS/ PAPER PRESENTATIONS

LIST OF CONFERENCE/WORKSHOP/ABSTRACTS/ PAPER PRESENTATIONS

1. Participation in International Symposium "Sweet Revolution- Stevia", held on 21st April 2016, jointly organized by School of Biotechnology & Bioscience and Green Valley Stevia, Banga, held at Lovely Professional University, Phagwara.
2. Participation in Workshop on advanced research methodology, held on 5th and 6th December, 2016 organized by Human Resource Development Centre, Lovely Professional University, Phagwara.
3. Participation in Workshop on molecular marker analysis, held on 11th November,2017 at Lovely Professional University, Phagwara.
4. Participation in Workshop on Quantitative Data Analysis & statistical design of scientific experiments, held on 13th to 17th November, 2017 at Lovely Professional University, Phagwara.
5. Participation in National workshop on next generation sequencing data analysis, held on 30th & 31st march,2018 at Lovely Professional University, Phagwara.
6. Participated and Presented Oral presentation and abstract published "In silico analysis of Haptoglobin gene polymorphism in Diabetes & cardiovascular disease" in International conference on Innovative strategies for sustainable water management held on 17th,18th November,2017, organized by School of Bioengineering and Biosciences in collaboration with Department of Bioresource Engineering, McGill University Canada at Lovely Professional University, Punjab.
7. Participated and Presented Poster presentation "Structural and Functional analysis of haptoglobin gene: a marker of Diabetes and Cardiovascular disease." In International Conference on Biosciences and Biotechnology (ICBB-2019).
8. Participated and Presented Poster presentation "Haptoglobin2-2 is a marker of Diabetes and progression of Cardiovascular disease in North Indian population: A case control study." In Continuing Medical Education (CME) & Workshop "Technologies Update in Transfusion Medicine (2023) at Lovely Professional University, Phagwara.

9. Participation in Workshop on Protein Molecular Modelling and Computer Aided Drug Design, held on 8th and 9th November, 2019 organized by Human Resource Development Centre, Lovely Professional University, Phagwara.

RESEARCH PUBLICATIONS

Puneet, Upadhyay, A. K., Kaur, M., & Singh, R. D. (2023). Assessment of Haptoglobin 2-2 Genotype in Type 2 Diabetes and Cardiovascular Patients in North Indian Population: A Case-control Study. Journal of Clinical and Diagnostic Research, 17(1), 6–10. https://doi.org/10.7860/jcdr/2023/57707.17340.