Clinical characterizations and genetic analysis of metabolic liver disorders in local Population

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2023

Clinical characterizations and genetic analysis of metabolic liver disorders in local population

PhD Dissertation

A dissertation submitted in fulfillment of requirements for degree of Doctor of Philosophy in Zoology (Molecular Biology)

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2023

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ACKNOWLEDGEMENTS

Praise is to **Almighty Allah**, the Cherisher and Master of the world, who showed His countless blessings upon us. I would like to thank Almighty Allah for giving me the strength, knowledge, ability, and opportunity to undertake this research study and to complete it satisfactorily. Without his countless blessings, this would not have been possible.

I would like to extend my sincere gratitude to all who made this possible. I am heartily thankful to my respectable supervisor, **Dr. Sabika Firasat** for her continuous support, motivation and enthusiasm. Her expertise, her valuable suggestions and patience added considerably to my experience and enabled me to complete this project. She always has been continuous source of encouragement for me.

I am in great debt to our respected chairperson **Dr Amina Zuberi** and Dean Biological sciences **Dr Sarwat Jahan** for facilitating this task.

I would like to thank **Ms. Nagina Zulfiqar** for her precious time, support and data analysis.

I owe my greatest gratitude to all the **families** who participated in this study gave their consent and samples. I am thankful to all the staff of respected hospitals who helped me and facilitated me during my study.

I am highly thankful to clerical and technical staff for their undeniable contribution to my study and support.

I am Thankful to my **colleagues** at Molecular Biology Lab, for their cooperation and assistance.

I am highly indebted to my **Parents** and **family members** for prays and encouragement, I achieved this task only because of their support and endless sacrifices.

Bushra Gul

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List of Abbreviations

IMLDS	Inherited metabolic Liver disorders
IEMs	Inborn errors of meta
WD	Wilsons disease
GSD	Glycogen storage disease
ICP	Intrahepatic cholestasis
ATP7B	ATPase copper binding pump
ABCB4	ATP Binding Cassette Subfamily B Member 4
ALDOB	aldolase, fructose-bisphosphate B
GBEI1	1,4-Alpha-Glucan Branching Enzyme 1
FAH	fumarylacetoacetate hydrolase
ASL	Argininosuccinate Lyase
SLC25A13	Solute Carrier Family 25 Member 13
LIPA	Lysosomal Acid Lipase
SERPINA1	Serine Protein INhibitor-A1
CFTR	cystic fibrosis transmembrane conductance regulator
HFE	Homeostatic Iron Regulator
ALMS1	Alstrom syndrome protein 1
mg	Mili gram
BAL	British anti lewisite
MXXM	Methionine cluster motif
CXXC	Cysteine cluster motif
ATPase	Adenosine 5'-TriPhosphatase
ROS	Reactive oxygen species
HGMD	Human gene mutation database
XIAP	X linked inhibitor of apoptosis
PNPLA3	Patatin Like Phospholipase Domain Containing 3
NAFLD	Nonalcoholic fatty acid liver disease
ATOX1	Antioxidant Protein 1
MTHFR	methylenetetrahydrofolate reductase
COMMD	Copper metabolism containing domain
SAHH	S adenosylhomocysteine hydrolyse
DMT	Divalent metal transporter
MRI	Magnetic resonance imaging
CNS	Central nervous system
KF	Keyser Fleischer
Cu	Copper

ug	Micro gram
dl	Deci litre
h	Hour
CT	Computerized tomography
CSF	Cerebrospinal fluid
GAS	Global assessment scale
TM	Tetrathiomolybdate
QoL	Quality of life
G6Pase	Glycogen 6 phosphatase
GAA	Acid alpha glycosidase
M6P	Mannose 6 phosphate
GDE	Glycogen debranching enzyme
ASAT	Aspartate aminotransferase
AGL	Amylo-Alpha-1, 6-Glucosidase
BASD	Bile acid disorder
ALT	Alanine Transaminase
ASP	Aspartate Aminase
ALP	Alkaline Phosphatase
ATP8b1	ATPase phospholipid transporting 8B1
BRIC	benign recurrent intrahepatic cholestasis
BSEP	Bile salt export pump
EDTA	Ethylene Diamine Tetraacetate
ESS	Exonic Splicing Silencers
EtBr	Ethidium-bromide
FIC	Familial intrahepatic cholestasis 1 protein ;
FXR	Farnesoid-X receptor
GGT	Gamma-glutamyl transferase
HSF	Human splicing finder
ICP	Intrahepatic cholestasis
IUD	Intrauterine death
LPA	Lysophosphatidic acid
MDR2	Multidrug resistance-associated protein 2
MSAF	Meconium staining of amniotic fluid
NTCP	Sodium- dependent taurocholate cotransporter peptide
PC	Phosphatidylcholine
PFIC	Progressive familial intrahepatic cholestasis
SDS	Sodium Dodecyl Sulphate
TC	Taurocholate
TE	Tris-EDTA
USCD	Ursodeoxycholic acid
MRP2	Multidrug resistance related protein 2

FXR	Farnesoid X receptor
BSEP	bile salt export pump
ABCC2	ATP Binding Cassette Subfamily C Member 2
RDS	Respiratory distress syndrome
PIMS	Pakistan institute of medical sciences
CHL	Children Hospital Lahore
SPSS	Statistical package for social sciences
DNA	Deoxyribonucleic acid
rpm	Rounds per minute
mM	Mili molar
TBE	Tris Boric acid EDTA
dNTPs	Deoxy nucleotide tri phosphate
G6PC	glucose-6-phosphatase catalytic
ARMS	Amplification refractory mutation system
PCR	Polymerase chain reaction
EtBr	Ethidium bromide
LFT	Liver function test

Table of Content

Abstract

Most of metabolic liver diseases are caused by mutation in a gene/s encoding an enzyme or transport protein and biochemical analysis of serum analytes do not provide specific diagnosis. Specific and differential diagnosis therefore require liver biopsy for enzyme assay or histological analysis for protein transporters, which is performed in few laboratories around the world and require shipment of samples under dry ice which adds to the cost and is not often feasible for long distances. Thus, such diseases remain undiagnosed and hence untreated mainly in low-income countries with poor health facilities like Pakistan. To study the clinical characterization and molecular analysis of metabolic liver disorders in Pakistan, ethical approval was approved from Bioethical review board of Quaid-i-Azam University, Islamabad Pakistan. In the present study cases of three metabolic liver disorders i.e., Wilson's disease (WD), Glycogen storage disease type 1a (GSD 1a) and Intrahepatic cholestasis of pregnancy (ICP) were enrolled after informed consent.

Inherited mutations in the *ATP7B* gene cause Wilson's disease (WD), which is an autosomal recessive disorder. Diagnosis can be challenging because of wide range of the ages affected and the clinical heterogeneity that includes neuropsychiatric and hepatic signs. Patients suffering from WD can face severe form of disabilities or even decease. So, the inclusive purpose of this research was characterization of WD cases on clinical and genetic basis from Pakistani population. 80 patients with WD were included in study from the Pediatric Department and Liver Unit of Children Hospital Lahore (CHL), Pakistan institute of medical sciences (PIMS), over the period January 2018 to January 2020. Clinical data was recorded for demographic and statistical analysis of clinical variants using SPSS 20.0. Patients showed a wide range of clinical variability, with some common diagnostic features such as lower serum levels of ceruloplasmin and elevated 24-hour urinary copper defecation. Mean age at the diagnosis was 11.3 years. Parental cousin marriage was observed among 80 patients. Kayser-Fleischer ring was observed in 47 patients in their eyes due to copper deposition in decement membrane of the cornea. Family history of WD was observed in 47.5%. Statistical analysis showed significance between

stage of illness and deceased-alive status of patient. of the cases. Association of stage of disease with risk factors like age, therapy received, and symptom reversal showed significant relationship. Based on availability of funds and willingness to participate in molecular analysis, blood samples of 20 patients were collected and investigated for PCR based Sanger sequencing of both the forward and reverse directions of the exon 1, 2, 3, 4, 5, 8 & 13 of the *ATP7B* gene for disease causing variants, the software PROVEAN, MutationTaster, Polyphen 2, SIFT, and HSF were used to predict the pathogenic effects of the variants that were found. Study predicted the pathogenic effects of the identified mutations. The study identified 22 variants in selected exons and intron-exon boundaries, 7 disease causing variants and 15 polymorphisms. Out of **7** identified disease-causing variant (6 homozygous and 1 heterozygous), 3 (p.V761E, p.I976N, p.I774L are novel and 4 (L227Yfs*35, p.D765N, p.A1003A and p.S986T) are reported. Out of 15 polymorphisms 5 are known and 10 are novel. The results of the investigation revealed the clinical presentation of WD patients in Pakistan and identified sequence variants in the *ATP7B* region that were tested.

Type I glycogen storage disease (GSD I) is a rare autosomal recessive disease; hence it is the most likely diagnosis when a baby is born with a genetic problem. Glucose-6-phosphatase complex malfunction causes glycogen storage disorder type Ia (GSD Ia), an extremely rare autosomal recessive congenital mistake in carbohydrate metabolism (*G6PC*). Glycogen storage disorder type Ia (GSD Ia; Von Gierke Disease; MIM #232200) is caused by deficiency in this enzyme and is typically characterized by the hypoglycemia, hepatomegaly, hyperuricemia, lactic acidemia, and hyperlipidemia. Disease causing variants in the *G6PC* gene, located on the chromosome 17q21 result in glycogen storage disease type Ia (GSD Ia). Age of onset of GSD 1a ranges from 0.5 to 25 years with presenting features including hemorrhage, hepatic, physical and blood related abnormalities. The overall aim of present study was clinical and genetic characterization of GSD 1a cases from Pakistani population. This study included forty GSD1a cases presenting with heterogeneous clinical profile including hypoglycemia, hepatomegaly, lactic acidosis i.e., pH less than 7.2, hyperuricemia, seizures, epistaxis, hypertriglyceridemia (more than180 mg/dl) and sometimes short stature. All coding exons and intron-exon boundaries of *G6PC* gene were screened to identify pathogenic variant in

20 patients based on availability of funds and willingness to participate in molecular analysis, on availability of DNA samples. The PCR-Sanger sequencing technique was used for mutation analysis, and the softwares PROVEAN, MutationTaster, Polyphen 2, SIFT, and HSF were used to predict the pathogenic effects of the variants that were found. Overall, 21 variants were detected including 8 novel disease causing variants i.e., p. Ala37Pro, p. Asp69Asn, p.Gln82Lys, p.Thr108Pro, g.50_51insT, p.Gln24Pro, p.Val45Leu and p.Cys284Tyr in the screened regions of *G6PC* gene. Out of 13 identified polymorphisms, 4 were identified in heterozygous condition while 9 were found in homozygous condition. This study revealed clinical presentations of GSD1a cases from Pakistan and identification of novel disease-causing sequence variants in coding and intron-exon region boundaries of *G6PC* gene.

Intrahepatic cholestasis (ICP), medically known as, obstetric cholestasis is pregnancy specific liver disease in which onset of symptoms occurs in third term. General pruritus, irregular liver function test and elevated serum bile acid are presenting symptoms. Increased load of pregnancy specific hormones and their metabolites obstructs the function of canalicular transporter proteins. Consequently, bile acid accumulates in blood serum. Intrahepatic cholestasis, although is of less significance for maternal health as it resolves after delivery, but it poses serious fetal risks. ICP has 0.2-2% prevalence worldwide. In Pakistan, 3% prevalence has been reported. ICP also exhibits familial clustering in certain cases that indicates role of the genetic factors. Mutations in canalicular biliary transport proteins disturb the mechanism of bile synthesis and flow. The candidate genes investigated for pathogenicity of ICP are: farnesoid X receptor *ATP8B1* (FIC1), bile salt export pump (BSEP), *ABCB11* and phospholipid translocator *ABCB4* (MDR3). To understand the molecular basis of ICP in our population, we screened known variants of exon 6 and exon 14 of *ABCB4* gene in 50 ICP patients presented at Wah General Hospital, during a course of six months (June 2019-November 2019). Preterm birth, meconium staining of amniotic fluid and stillbirth are some of the perinatal outcomes that have been documented alongside maternal pruritus and elevated blood bile acids. A molecular analysis of genetic variants of ICP was done by ARMS (Amplification Refractory Mutation System) PCR in 50 cases and 50 controls for the identification of known variants in exon 6 and 14 of *ABCB4* gene. The study identified disease causing variants i.e., c.504 C>T; in

17 cases, to be significantly associated with the disease. The variant c.1686 A>G showed non-significant association.

This study gives a way for improved understanding of molecular mechanism elucidating the phenotypic variability and genetic heterogeneity among patients with metabolic disorders i.e., Wilson's disease, Glycogen storage disease type 1a and Intrahepatic cholestasis of pregnancy. There are gaps in current knowledge about disease risk association of single nucleotide polymorphisms with physiological factors, potential target molecular pathway/s of metabolic dysfunction predisposing to specific clinical endpoints of liver dysfunction and their association to genetic attributes. The outcomes of this study provide further knowledge of genetic variants from our Pakistani population. Genetic counselling was provided to all families affected with WD and GSD 1a to refrain from cousin marriages that would help in decreased incidence of these devastating conditions in upcoming generations as ICP can affect subsequent pregnancies of affected women, therefore all the affected females enrolled in this study were provided with counseling to monitor the symptoms and seek proper screening in subsequent pregnancies. Furthermore, our findings help the clinicians to understand involvement of genetic factors and clinical heterogeneity of these conditions in our population, so that they may recommend the suspected cases for genetic analysis to get differential diagnosis for proper patient management. However further genetic studies are required to understand genetic basis of metabolic liver disorders, identification of prevalent sequence variants for establishment of cost effective molecular genetic tests for our local population.

CHAPTER 1
1. INTRODUCTION

The liver is chief organ for metabolism in vertebrates. It is responsible for an estimated 500 distinct functions, including general detoxification, the synthesis of proteins, the production of bile, the metabolism of lipids, carbohydrates, proteins, and bilirubin, storage of vitamin and mineral, and involved in immunological function (Kuntz & Kuntz, 2008). These tasks are executed by hepatocytes. More than 400 extremely uncommon monogenic disorders of the hepatic origin have been documented, which makes sense given the liver's wide range of responsibilities (Zabaleta *et al*., 2019).

Liver is a critical organ for most of the metabolic pathways and thus is target tissue for many inherited metabolic liver diseases (IMLDs). About 1 in 800 newborns are affected by IMLDs, a heterogeneous set of uncommon genetic disorders often caused by a single gene mutation (Pampols, 2010). All of these diseases share a defect in protein synthesis or function; these defects affect enzymes, receptors, and transporters involved in biochemical pathways critical to metabolism (Ferreira *et al*., 2019).

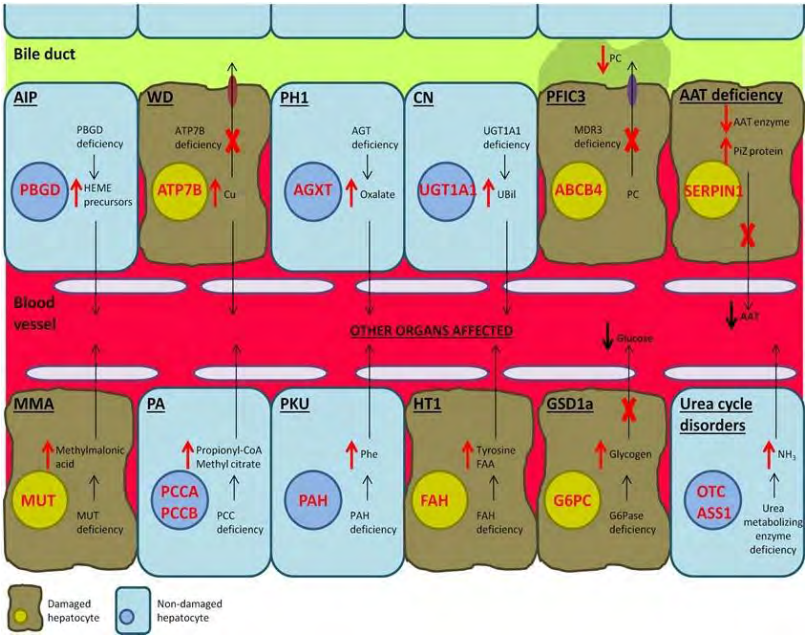


Figure 1.1. Schematic representation of IMLDs (Zabaleta *et al*., 2019)

Inherited liver disorders are clinically and genetically heterogeneous, with several different forms. These conditions predominantly follow a recessive mode of inheritance thus are more prevalent in developing countries where consanguinity is customary. In our In the

Pakistani community, around sixty percent of marriages are consanguineous, and more than eighty percent of those marriages are among first cousins (Hussain & Bittles, 1998). This high rate of consanguinity coupled by the large family size has greatly contributed to increased prevalence of all recessively inherited disorders (Hamamy, 2012). Thus, Pakistani population provides a valuable genetic resource for research on molecular basis of inherited liver conditions to fully understand their pathophysiology and search for treatment options. Though a lot of progress is observed on molecular characterization of different viral infections leading to liver dysfunction (Ali *et al*., 2011; Attaullah *et al*., 2011) in Pakistan but wide spread studies on genetic basis and molecular characterization of all kinds of inherited liver disorders including Wilson's disease, Intrahepatic cholestasis, Glycogen storage disease, Hemochromatosis, Congenital hepatic fibrosis etc. has not taken place and there are only few epidemiological studies regarding patients with these disorders (Aziz, 2010; Hafeez *et al*., 2016).

Genetic disorders known as inborn errors of metabolism (IEMs) cause an individual to accumulate toxic molecules that disrupt normal organ function and prevent the body from synthesizing essential compounds (Hafeez *et al*., 2016). Mutations in the *ATP7B, ABCB4, ALDOB, FAH, ASL, GBE1, SLC25A13, HFE* and *ALMS1* genes are reported to be associated with IEMs (Aziz, 2010; Scorza *et al*., 2014). Furthermore, these disorders may show their full-blown clinical symptoms in adulthood since the spectrum of clinical presentation in some of these conditions might be age specific (Clayton, 2002). In this work, we have focused on Wilson disease, Glycogen storage disease (GSD) and Intrahepatic cholestasis (ICP).

1.1. Wilson's Disease

Wilson's disease (WD) is a sporadic congenital disorder that involves metabolism of copper. Kinnier Wilson delivered the first thorough, clear depiction of both pathological and clinical details of the disease that is now known by his name (Kayser, 1902). In 1948 it was discovered that Wilson's disease occurs due to ceruloplasmin deficiency resulting in disturbance in metabolism of copper in the liver (Cumings, 1948; Scheinberg & Gitlin, 1952).

WD is a complaint related to copper metabolism that is autosomal recessive, with prevalence ranging from 1 in 35,000-100,000 live births (Gupta, Chattopadhyay, *et al*., 2007; Wan *et al*., 2006). Buildup of copper in several organs results in hepatic and neurological impairment, which are lethal if left untreated with copper-chelating agents such as penicillamine. In order to provide prompt and appropriate therapy, a correct diagnosis of WD in both the patient and any afflicted siblings is required (Gitlin, 2003; Schilsky, 2009). If a patient with WD is not correctly diagnosed, this could result in missed chances for preventative treatment or the incorrect administration of potentially harmful medicines (Gupta, Maulik, *et al*., 2007; Liver, 2012b; Schilsky, 2009).

Liver plays a very important role in copper disposition, as most of the copper from our diet passes through the liver. In the liver the copper can be used for energy production, or it can be eliminated from the body via biliary route. Copper performs a broad array of physiologic functions as it is essential for many enzymes (Roberts & Sarkar, 2008).

Typically, intake of copper in diet i.e., dietary copper ranges from 1-10mg depending upon number of legumes, chocolates, and meat consumed. The suggested intake is 0.9 mg/d, so the normal balance of copper is maintained by regulation of the excretion by Hepatobilliary route. In order to remove excess of copper almost 85% of copper is excreted daily (Roberts & Sarkar, 2008). Along with dietary copper, proximal small intestine also absorbs copper from gastric and pancreatic juice and saliva. Once the copper is absorbed, reversibly bound to the serum albumin and to different enzymes.

Copper is distributed throughout the body via copper-histidine and copper-albumin, with the liver being a primary recipient. In the kidney, copper that is only weakly linked to amino acids is filtered and reabsorbed in tubules. The copper in bile that is excreted is too complicated to be reabsorbed (Liver, 2012b; Tao & Gitlin, 2003).

Ceruloplasmin absorbs nearly all of the copper in the blood, around 95%. The presence of copper (non-ceruloplasmin-bound) in urine throughout the course of 24 hours is an important diagnostic indicator for Wilson illness (WD). Patients with WD despite rigorous treatment had low plasma amounts of ceruloplasmin as determined enzymatically (Ferenci *et al*., 2005; MacIntyre *et al*., 2004).

First identified by Samuel Alexander Kinnier Wilson in 1912, WD was first studied in a group of 12 people. However, the first examples of WD with leading tremor were documented by Westphal in 1883. Keyser fisher rings, a diagnostic feature of WD, were first described in 1902–1903 by Kayser and Fleischer. Followed by the discovery of instabilities in metabolism of copper as etiology of WD and autosomal recessive inheritance pattern. D-penicillamine was the initial orally available medication for WD, followed by zinc and trientine in 1961 and 1982 respectively. In 1971, Starzl and colleagues transplanted livers to treat WD. P-type ATPase implicated in copper transport (*ATP7B*) was first identified in 1993, and its genomic location was determined to be on chromosome 13q (Walshe, 2017). In Figure 1.2, we see a sequence of key findings that establish WD as an inherited disease of copper metabolism that results in pathological buildup of copper in a variety of organs, most notably the liver and brain, and manifests itself in a wide variety of symptoms.

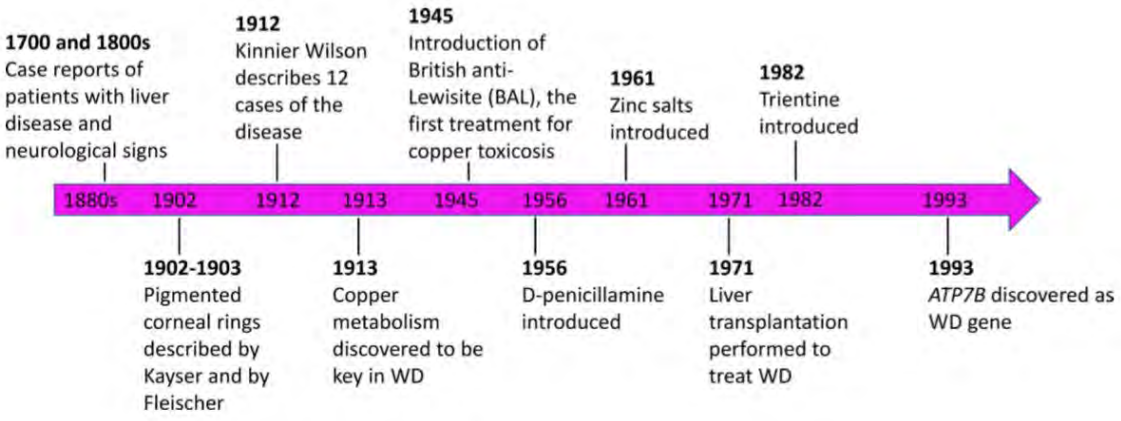


Figure 1.2. Key findings in WD Adopted from (Członkowska *et al*., 2018)

Copper that is loosely attached to the albumin or histidine reaches liver via portal vein where this copper is up taken by sinusoidal plasma membrane into Hepatocytes (Petris, 2004). CCS1 guides the copper to the SOD1 *ATOX1* guides the copper towards the Wilson ATPase (Narindrasorasak *et al*., 2004; Singleton & Le Brun, 2007). Glutathione and Metallothioneins are found also in cytoplasm where they also react with the copper. Role of copper in the metabolism in mitochondria mainly involves cytochrome-c oxidase, and the associated proteins like Sco1 Sco2 and Cox17, Cox19. The main chaperon for the

targeted delivery of copper to the mitochondria is Cox17. Cox19 is also involved in the transport of copper across mitochondria's (Horng *et al*., 2005).

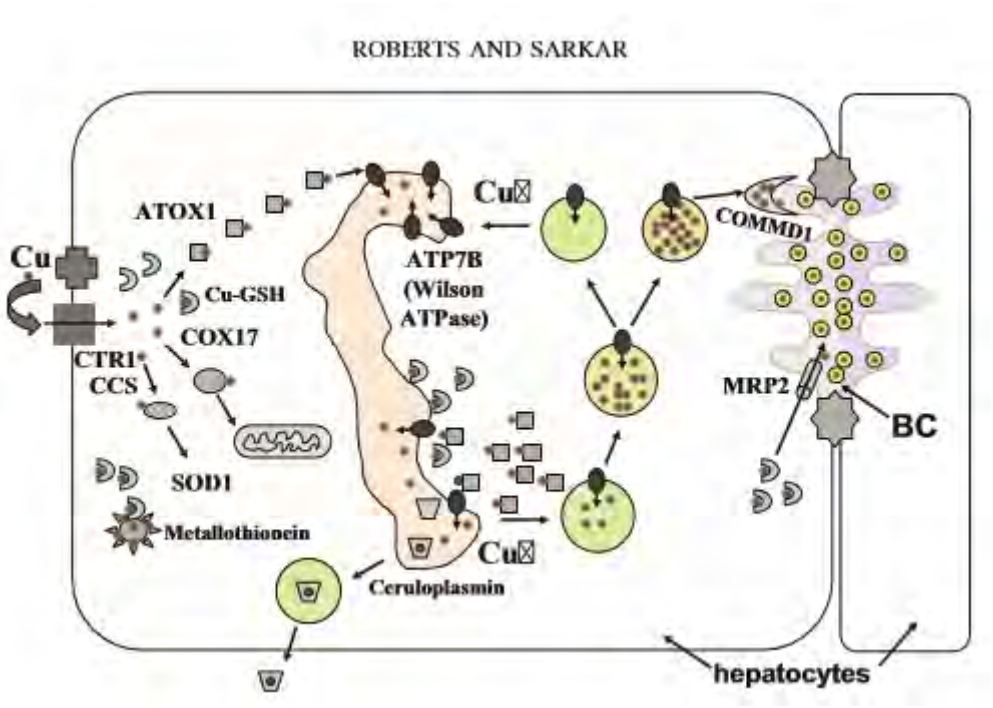


Figure 1.3. Cartoon portraying the web of proteins involved in disposition of copper within hepatocyte Adopted from (Roberts & Sarkar, 2008).

The disease my present a fulminant hepatic failure with intracellular hemolysis followed by leading to renal failure. Infrequently, people develop hepatocellular cancer. This heterogeneity in presentation is suggestive of drug-induced hepatotoxicity. Any of the aforementioned symptoms may indicate drug-induced liver damage (Figure 1.4).

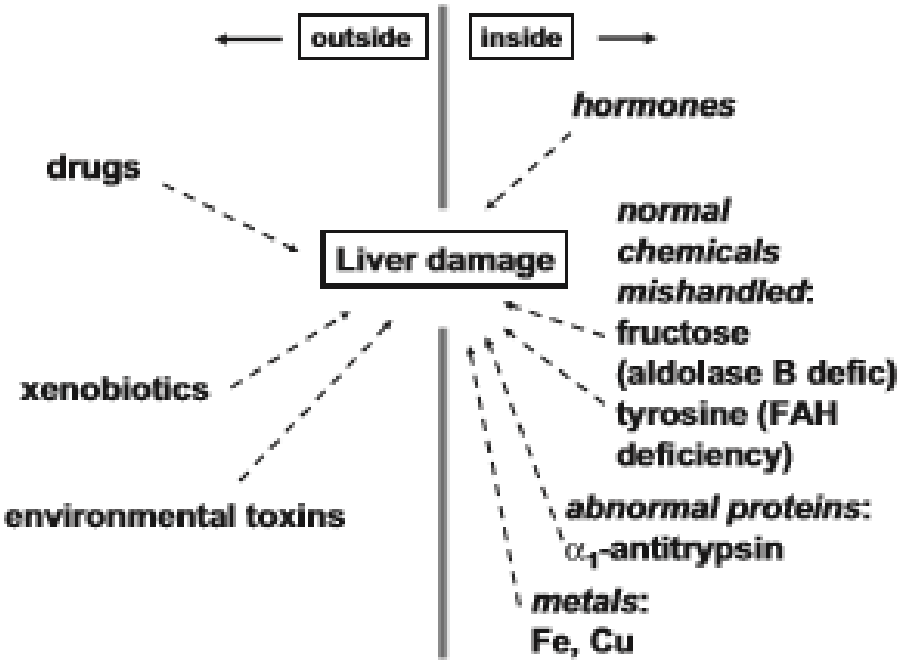


Figure 1.4. Liver ailments caused by different risk factors.

1.2. Pathophysiology of Wilson's disease

Free copper is very poisonous that can cause lasting harm to cells, despite its critical role in cellular activity. For this, complex systems that bind copper molecules have been created for secure transport of necessary copper to designated places and safe removal of excess copper by biliary system. Copper transport is aided by the ceruloplasmin and *ATP7B* proteins.

Failure of biliary copper defecation due to the *ATP7B* mutations and inactivation of the *ATP7B* transporter causes an imbalance in the body's copper levels. The *ATP7B* is responsible for lowering serum ceruloplasmin via transporting copper, which is required for the formation of active ceruloplasmin. Low ceruloplasmin formation may cause a decrease in total serum copper levels in WD, but an increase in harmful non-ceruloplasmin-bound copper is more common (Liver, 2012b). Tissue pathology and clinical symptoms in WD are caused by an excess of toxic copper in the liver and throughout the body.

Normally, to generate "ceruloplasmin," six copper molecules must be incorporated into six copper molecules in the apoceruloplasmin. The trans-Golgi network in liver cells is where this occurs (Hung *et al*., 1997). The *ATP7B* transporter is also relocated to cytoplasmic vesicles in the liver, where it transfers excess copper to the bile canaliculus via the hepatocyte's apical membrane, leading to bile excretion (Forbes & Cox, 2000; La Fontaine *et al*., 2001). In individuals with WD, *ATP7B* gene mutations results in malfunctioning *ATP7B* protein which is unable execute proper functioning. Thus, copper gradually collects within the liver cells that compromise liver function, also ultimately exceeded and unbound copper spills out of the liver and is deposited in other and tissues organs, leading to damage and dysfunction.

Pathological tissue alterations brought on by the toxic effects of excess copper are the primary cause of the clinical symptoms seen in WD (Cumings, 1948) (Figure 1.5).

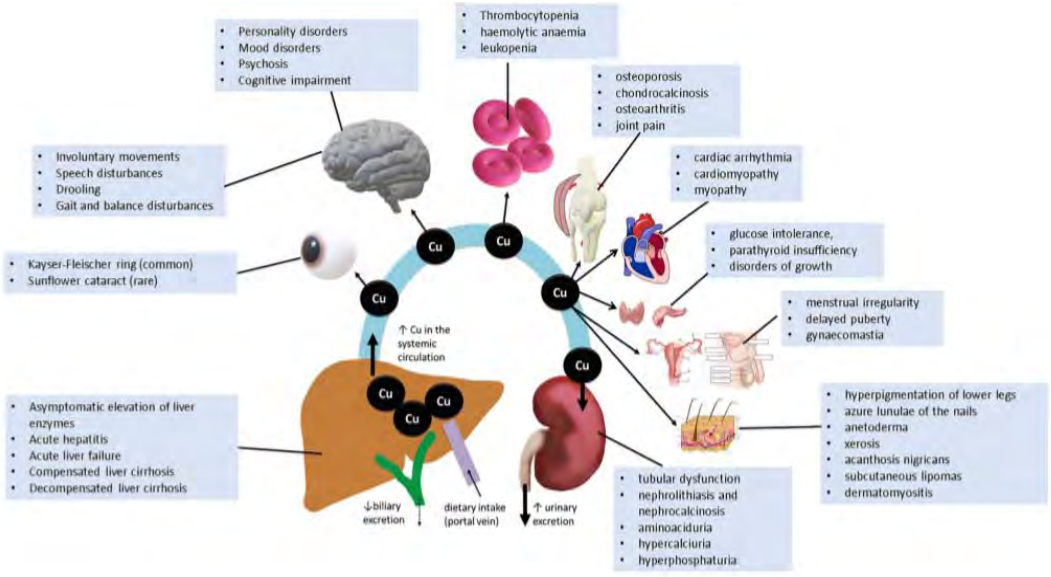


Figure 1.5. Toxicity of copper in the pathogenesis of WD (Członkowska *et al*., 2018).

1.3. Epidemiology

A sporadic autosomal recessive ailment, Wilson's disease, affects only a handful of people in the United States. Occurrence of WD is varies from 12 to 29/100000, in European population whereas in Asian countries exclusive of Pakistan and India varies between 33

to 68/ 100000. The occurrence of WD is higher in China (58.7 per 1,000,000) and Asian countries than in western countries (Yang *et al*., 2022). Studies of epidemiology in understudied communities have revealed an elevated incidence likely attributable to close family ties for example (Canary Islands: 1 per 2,600; Sardinia 1 per 7,000) (Coffey *et al*., 2013; Liver, 2012b; Lo & Bandmann, 2017).

Also, in a study from the United Kingdom (UK), the calculated occurrence of the individuals expected to carry two mutant pathogenic the *ATP7B* alleles was approximately 1/7,000 individuals (Coffey *et al*., 2013). Epidemiological data from the UK seem to be currently most reliable according to the WD genetic studies (Lo & Bandmann, 2017). Though, advance investigations are necessary. Limited sensitivity of several copper metabolism tests, unknown age-related clinical penetrance of the *ATP7B* mutations, and the diverse presentation resulting to under-, and misinterpretation may all contribute to an underestimation of the WD's prevalence. It would appear that the number of patients with WD is rising as both medical professionals' and patients' access to genetic testing improves. However, modern genetic approaches that sequence the full *ATP7B* gene are expensive and not widely available, but they are helpful when they are since they allow for a more precise definition of predominance. Death rates among asymptomatic WD patients who adhere to treatment are comparable to those of common population (K Dzieżyc *et al*., 2014). On the other hand, in the entire WD population; regardless of adherence, initial stage of disease or type of treatment, clinical symptoms, studies normally show that rates of mortality in patients with WD (5 to 6.1%) are higher than healthy controls (Beinhardt *et al*., 2014; Svetel *et al*., 2009). The presence of advanced neurological and hepatic diseases as well as lack of adherence with treatment influences the survival.

More than 800 mutations in the *ATP7B* gene are documented up till now from different countries as per WD record (http://www.wilsondisease.med.ualberta.ca/index.asp),) along with greater than 100 nonpathogenic variants (Aggarwal & Bhatt, 2013; Hedera, 2017) However, there are no epidemiological studies conducted in Pakistan's local communities.

1.4. Clinical features/ Signs and symptoms

Wilson disease is congenital, meaning it is present at birth, but symptoms don't show up until copper accumulates in the liver, brain, or eyes. Symptoms of WD commonly appear between the ages of 5 and 40. Some people, however, show signs much earlier or much later in life (Walshe, 1962). Wilson's disease has a hepato-biliary pathogenic flaw, but the repercussions of the unrelenting copper buildup were felt throughout the body. The wide range of organs and tissues that copper can damage results in a clinical picture that can be difficult to diagnose. Speech problems, swallowing, lack of physical coordination, involuntary movements, jaundice, Kayser-Fleischer rings, fluid retention in abdomen or legs, and bloating due to abdominal fluid retention are all symptoms of liver disease (Gul *et al.,* 2022). Leg or foot swelling, itchiness, and sudden weight loss are all symptoms of a potentially serious condition called fluid overload. Wilson's disease usually shows multiple symptoms but most common clinical symptoms that are presented are hepatic and neurological disturbances. Clinical features act at different stage of life in different individuals having the mutated gene. Late onset of WD may be because of with enormous diagnostic trials as symptoms may relate to the other age associated diseases (Ala *et al*., 2005). The likelihood of WD and homozygous mutations increase the in populations with high degree of consanguineous marriages (Hedera, 2017). Presence of disease without clinical appearance, proper analysis and management may lead to deterioration of disease, eventually to death.

1.4.1. Hepatic Manifestations

1.4.1.1.Liver

The *ATP7B* copper transporter is most highly expressed in the liver, which also plays a critical role in maintaining a healthy systemic copper balance. *ATP7B* deficiency causes a buildup of liver copper by decreasing rate at which copper being excreted via the biliary system. Therefore, WD typically manifests itself first as liver injury. The content of copper in the livers of WD patients is frequently raised by a factor of 5-20 compared to healthy persons. When illness is present, copper's cellular localization shifts, and it is not uniformly distributed throughout the liver. Metal ions are initially coupled to cysteine-rich proteins

(Metallothioneins) in the cytoplasm of hepatocytes, where they are stored until they can be removed during the detoxification process. Over time, copper begins to build up in lysosomes, which can be visualized with stains like orcein and Timm's, rhodanine (Figure 1.6a) (Mounajjed *et al*., 2013). Plain mitochondrial changes in hepatocytes can be noticed early in the disease course (Huster, 2014). Hepatic steatosis is caused, in part, by mitochondrial dysfunction, which leads to a decrease in hepatocytic energy metabolism (Figure 1.6b).

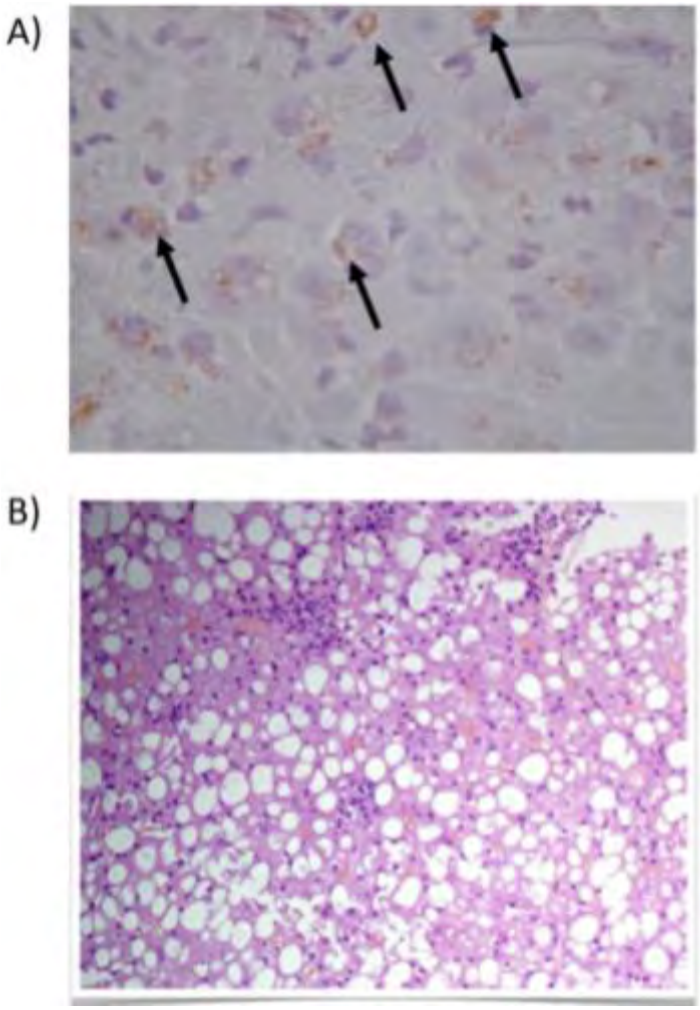


Figure 1.6. WD Liver pathology Image adapted from (Członkowska *et al*., 2018).

A-Histochemical demonstration of copper in liver detected showing rhodanine-positive granules B-which is occasionally vague from non-alcoholic fatty liver ailment. B adapted from (Stättermayer *et al*., 2015).

The type of hepatic connection in WD may vary from asymptomatic state to wide range of liver situations (Patil *et al*., 2013). In some cases, acute hepatitis is noticed while other shows liver cirrhosis. Children pretentious with disease may present with hepatomegaly or alteration in enzyme concentration such as aminotransferases and ceruloplasmin. Patients with past cases of numerous episodes of low-grade hemolysis and jaundice might be suffering from early stages of WD. If liver biopsy is done in these patients fibrosis can be found along with biochemical irregularities (Roberts & Schilsky, 2008).

Somewhere between 40 and 50 percent of people are with WD, initial clinical manifestation is hepatic dysfunctions (Brewer, 2001; Liver, 2012b). Even though WD is been detected as early as 2 years of age with persistently elevated liver enzymes, symptoms rarely emerge until the age of 5 years (Beyersdorff & Findeisen, 2006). One study found that the presentation of hepatic symptoms in patients older than 40 is uncommon (Gow *et al*., 2000). Average age for onset of hepatic symptoms is 11.4 years.

The liver symptoms that accompany WD can take a few different forms. It is possible to develop symptomless enlargement of both liver and the spleen, which is accompanied by an increase in liver enzymes. To give just one example, acute transitory hepatitis is the mode of presentation for twenty-five percent of the people in whom hepatic symptoms precede the beginning of the disease. Hemolytic anemia with hepatic dysfunction, or an increase in unconjugated bilirubin (indirect), should raise suspicions of Wilson's disease in the clinician, while the uninitiated may confuse these symptoms with those of viral hepatitis (Brewer, 2001). There is a possibility that the hepatic symptoms of WD could be confused with the symptoms of autoimmune hepatitis (Scott *et al*., 1978; STERNLIEB & SCHEINBERG, 1972). WD also makes its presence as an acute fulminant hepatitis. Actually approximately 5% of the incidents for acute failure of the liver is due to Wilson’s disease (Dayal & Kumar, 2014). In many cases, severe Coombs-negative hemolytic anemia is observed. This anemia is thought to be produced by intravascular hemolysis, which in turn is thought to be caused by the abrupt release of enormous quantities of copper in bloodstream due to liver failure (ROCHE-SICOT & BENHAMOU, 1977). On the other hand, WD is the progression of cirrhosis to a more advanced stage. The cirrhosis does not have any WD-specific symptoms; however, due to the wide-ranging modes of the hepatic

presentation that WD is capable of adopting, any individual less than age of 50 years with a strange liver condition should be examined for WD (Brewer, 2001). In case of acute liver failure the vital presentation of disease is Coomb's negative hemolytic anemia but in dangerous complaint acute renal failure is detected (Eisenbach *et al*., 2007). Conferring to European association for study of liver every year 6 to 12 percent of persons with acute liver failure admitted for liver transplant have WD. If the disorder goes untreated it leads to death in 95% individuals. Liver cirrhosis with hypertension in portal region along with splenomegaly is an significant clinical sign in patients with chronic liver failure (Roberts & Schilsky, 2008).

1.4.2. Neurological Manifestations

1.4.2.1.Brain

Brain copper levels in WD patients may reach the values 10 to 15 times higher as compared to control subjects (Horoupian *et al*., 1988; Mikol *et al*., 2005). Long-term experience to high copper levels eventually results in damaged astrocytes, dysfunction of blood-brain barrier and complaint of other brain tissues including oligodendrocytes and neurons. Pathological changes including demyelination, astrogliosis, and tissue disintegration are most often reported in the thalamus, basal ganglia, cerebellum, and upper brainstem these irregularities are depicted as T2 hyperintense lesions on magnetic resonance imaging (MRI). Demyelination predominantly affects bundles passing through basal ganglia and the pontine fibers (Figure 1.7) (Dusek *et al*., 2017; Meenakshi-Sundaram *et al*., 2008). Inflammatory changes in basal ganglia with the buildup of heavy iron-laden macrophages are commonly present. T2 hypo intense lesions on MRI are associated with increased iron deposits (Figure 1.8).

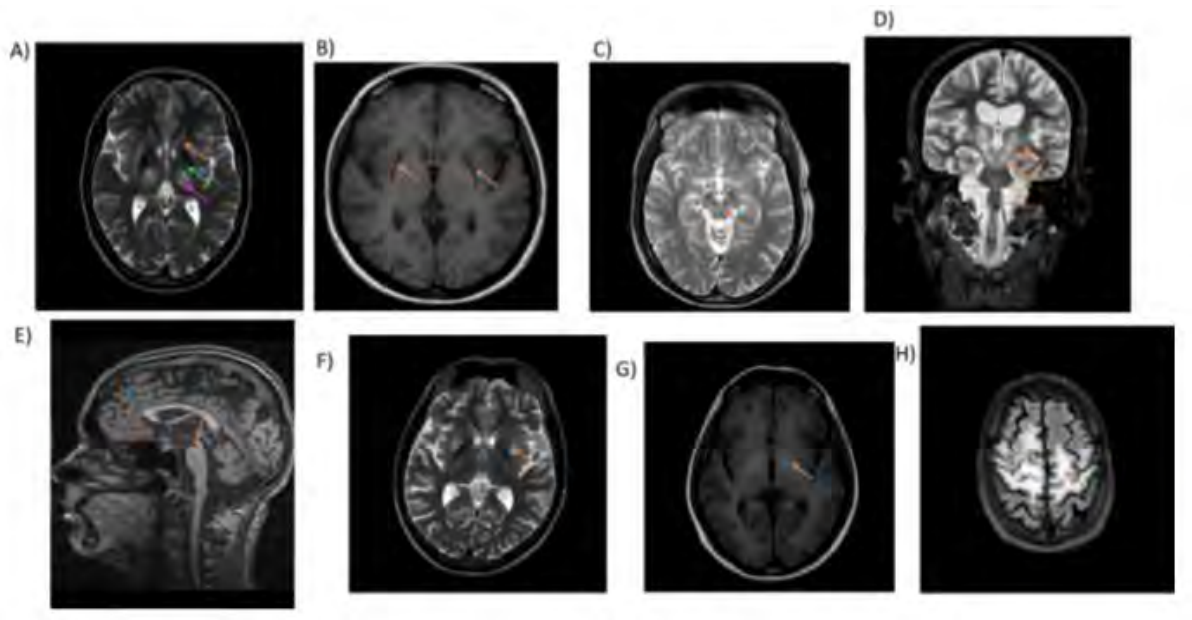


Figure 1.7. Adopted from (Członkowska *et al*., 2018) variations of Brain MRI in WD.

(a,b) Severe tissue damage can be pictured, WD deviations are explained as 'face of the giant panda' in midbrain (c) Deformity seen T2 signal beside the dentatorubrothalamic pathway (d) In severe forms of WD diffused brain atrophy (e) can be perceived in cortex and midbrain (f) Hyperintense changes in globi palidi due to manganese buildup (g) Diffused white matter fluctuations hemispheres due to myelin destruction.

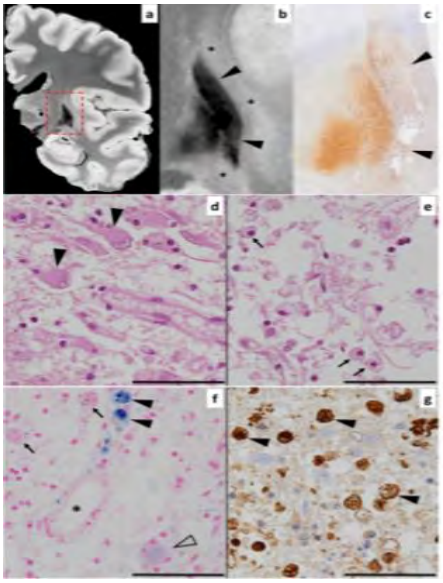


Figure 1.8. Post mortem MRI and histopathology in neurological WD. Adopted from (Członkowska *et al*., 2018)

(a) T2*-weighted post mortem MRI (b) amplification of the basal ganglia area obvious by dashed red rectangle in (c) low power magnification of Turnbull iron staining showing consistent area with MRI on image (d,e) Reactive astrocytes with pale large nuclei shown by hematoxylin-eosin staining (e) agrees to rarefied area in central putamen (f) Berlin-blue staining showing iron-negative and iron-positive macrophages (g) Ferritin staining shows frequent strongly positive macrophages

40 to 60% Individuals with Wilson's disease exhibit Neurological dysfunction (Brewer, 2001; Walshe, 1962) Neurological dysfunction in Wilson's patients are presented at average age of 19 years, though neurological symptoms may seem at age of 6 years (STRICKLAND & Leu, 1975). Onset of neurological symptoms at end of the age spectrum, has been described as late as age 72 years (Ala *et al*., 2005).

Dysarthria, having a character that is extrapyramidal or cerebellar, or both, is prevalent in people who have WD, and those people may also have cerebellar character (Hellman, 2002).

Tremor, whether it be postural, resting or can be kinetic it is neurological hallmark of WD that presents itself most of the time first. WD can cause proximal upper limit tremors to take on a course, "wing-beating" appearance, but distal WD tremors can also be fairly mild in amplitude.

The term **"whispering dysphonia"** refers to a chuckle that is caused by Wilson's illness and is described as one in which the majority of the sound is produced in response to stimulation (Cartwright, 1978; Parker, 1985).

Gait abnormalities including cerebellar and extrapyramidal patterns are a common element of neurological Wilson's disease. Tics and chorea are uncommon although severe generalized myoclonus linked with white matter lesions has lately been explained (Barbosa *et al*., 2007).

A wide range of many neurological features can appear in the WD. **Cerebellar dysfunction** appears in approximately 25% of cases with neurological WD. (Svetel *et al*., 2001). The painless variant of moving toes and painful legs syndrome has also been stated in a person with WD (Papapetropoulos & Singer, 2006).

Focal or severe dystonia, motor impairments in cranial region, drooling, open jaw and running saliva are important symptoms of neurological abnormality (Figure 1.9) (del Rosario *et al*., 1998). In Dystonic syndrome twitching or abnormal position of different parts of body occurs. It is reported in 10 to 65 % of the patients with neurologic dysfunction and may also cause involuntary head rotation, eye closure by force, difficulty in swallowing, loss of speech, failure to move, abnormal posturing of neck and the patient may become bed ridden (Svetel *et al*., 2001).

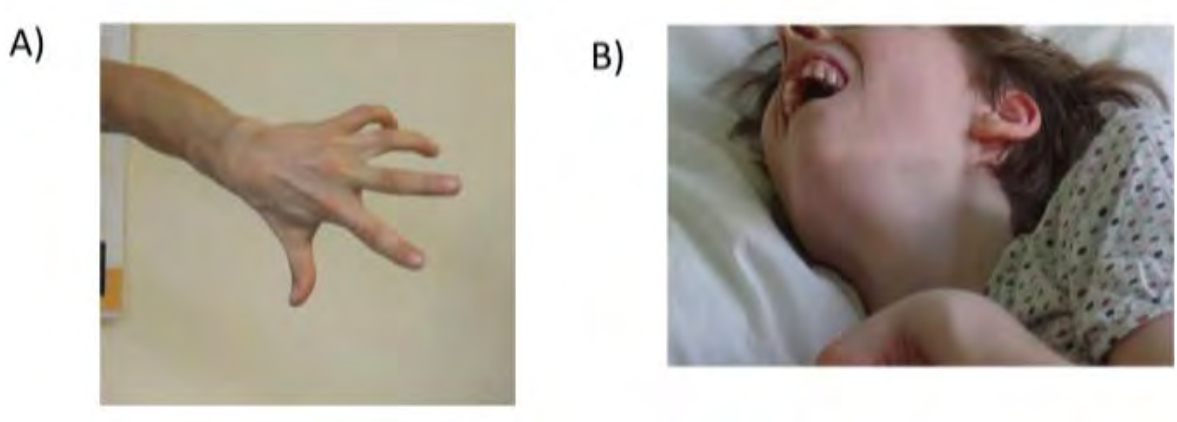


Figure1.9. Adopted from (Członkowska *et al*., 2018) A- hand dystonia. B- severe dystonia

Chorea is also detected in some cases, but it is not a very common indication. In early neurologic disease person may lose attentiveness, ability to get synchronized, headaches, sleeplessness and migraines are also seen but seizures are not that common. Patients showing neurological symptoms of disease may or may not have substantial hepatic abnormalities (Brewer, 2005b).

Approximately 26-30% of those afflicted also experience **autonomic dysfunction** (Deguchi *et al*., 2005).

Seizures are a potentially debilitating symptom of WD; however they only affect a small percentage of sufferers. The most common type of seizure is a partial one, which, when left untreated, can lead to benign epilepsy in childhood. Spikes in the centrotemporal region have been identified (Kumar, 2005). Patients who have WD may experience their first neurological symptom, which could be a headache or a seizure, depending on the severity of the disease. It has also been reported that the first sign of WD onset is a peripheral sensorimotor polyneuropathy that involves axonal and demyelinating involvement (Jung *et al*., 2005).

Olfactory impairment, it has also been reported that people with WD who are experiencing neurological dysfunction have problems with their sense of smell. There is a correlation between olfactory malfunction severity and neurological dysfunction. Individuals who have WD have also been observed to have hypersomnia, pseudobulbar emotional liability and muscle cramps.

1.4.3. Psychiatric Indicators

The worrisome frequency with which WD manifests itself in the form of psychological disorder in therapeutic settings is a cause for concern. Some researchers have noticed that psychiatric descriptions were obvious at time of the early staging in 65% cases with disease.

Most people who have WD will, at some time, experience psychiatric symptoms. These symptoms arise most frequently in people who have also demonstrated neurological impairment, which reflects the fact that both conditions originate in the central nervous system (Rajput *et al*., 2017). WD can result in wide range of psychological symptoms to manifest in the affected individual. It is characterized most frequently by changes in personality, particularly depression, as well as shifts in one's mood, and it is also one of the most common neurological disorders (Brewer, 2005a). Depression may be as severe, that a person may attempt suicide.

Although it is uncommon, **psychosis** can be a symptom of WD. Antisocial behavior has also been reported in WD, as sexual disinhibition and preoccupation (Akil *et al*., 1991).

Cognitive impairment also develops in WD. Cognitive disabilities comprising visuospatial processing and impairment of frontal-executive ability have also been reported (Seniów *et al*., 2002).

Psychiatric features of WD can present themselves in a variety of ways, some of which can be quite subtle at times. The student's poor presentation at school, particularly when combined with the stomach complaints, should trigger consideration of WD. Because the symptoms of both diseases can be quite similar, it is important to evaluate the chance that young people who are suspected of drug usage have WD.

Psychiatric and behavioral symptoms are very generic that is why they are often misdiagnosed as problems related with adolescence. In children due to disease complaint poor performance at school, mood swings, inappropriate behavior and change in personality is observed (Ferenci, 2004). These patients are often incorrectly determined as suffering from other neuropsychiatric aberrations such as schizophrenia and bipolar disorder (Hedera, 2017). Synchronized liver disease is present in neuropsychiatric patients but can only be evaluated by laboratory examination of liver as they are usually asymptomatic for hepatic dysfunction.

1.4.4. Ophthalmological Manifestations

The description of pigmented corneal rings by Kayser and Fleischer came roughly a decade before Wilson's description of Wilson's disease. Fleischer made the connection between the two in a publication he wrote in 1912, and Kayser and Fleischer are credited with having discovered WD (Fleischer, 1912; Kayser, 1902). Copper that has accumulated in Descemet's membrane is responsible for the formation of Kayser-Fleischer rings. WD is characterized by the deposition of excess copper across the cornea, however sulfur-copper developments can only take place in Descemet's membrane. These developments are responsible for the appearance of copper deposits in the cornea (Johnson & Campbell, 1982).

Kayser-Fleisher ring (KF ring) is a golden-brown ring present in 95 % of patients with raised copper concentration in body suffering from WD. It results by accumulation of

copper in the corneal Descemet's membrane of (Liver, 2012a). Bilateral ring is noticed in most cases, and it may also seem like deep blue or greenish yellow. In 85 percent patients stated to have abnormal neurological condition, KF ring is found (Birkholz & Oetting, 2009).

Prevalence of KF ring is 33% to 65% in individuals with hepatic involvement of disease. Pre symptomatic individuals may also develop KF ring in 40% cases, but as a result of of handling using chelating agents it will vanish in 85 percent cases (Birkholz & Oetting, 2009). Kayser-Fischer rings are almost often bilateral, but there have been reports of the creation of unilateral rings (Innes *et al*., 1986). The pigment first manifests itself at the limbus, which is located on the cornea's periphery. It subsequently spreads centrally (Sussman & Scheinberg, 1969).

1.4.5. Other Manifestations

Less frequent disease presentation includes gigantism, osteoarthritis, vertebral column abnormalities, hemolytic anemia osteoporosis, pancreatitis, glucose intolerance cardiomyopathy and myopathy. Repeated miscarriage, delayed puberty, menstrual irregularities and infertility is also associated with Wilson's disease although it is not a very common clinical presentation of disease.

The involvement of bone and joint is a component of Wilson's disease that receives insufficient attention. There is radiographic proof of osteoporosis in as many as 88 percent of persons with WD (Cairns *et al*., 1969; Golding & Walshe, 1977). There is a possibility of impromptu breaks occurring. Pain in the joints, particularly the knees, is another typical symptom of WD, and joint involvement is more common in patients with the condition (Golding & Walshe, 1977).

Other abnormalities, such as intolerance for glucose and parathyroid hormone insufficiency, have also been reported in addition to cardiovascular dysfunction. Examples of cardiovascular dysfunction include cardiac arrhythmia and congestive heart failure (Yarze *et al*., 1992). Skin abnormalities caused by hyperpigmentation of the anterior lower legs may develop in patients with WD. These changes in the skin have the potential to be

mistaken as Addison's disease (Leu *et al*., 1970). Gynecological abnormalities i.e. delayed puberty, menstrual irregularity, gynecomastia, and cardiovascular dysfunction i.e. cardiac arrhythmia have also been seen (Ferenci *et al*., 2005; Lau *et al*., 1990).

1.5.Diagnostic testing

Early diagnosis and treatment of Wilson's disease are essential for preventing the progressively increasing symptoms that can ultimately prove fatal if the condition is not caught in time. Early diagnosis of disease is challenging because of the wide age range of onset and the many vague symptoms.

Despite the fact that genetic testing provides irrefutable evidence of a diagnosis of Wilson's disease, commercial genetic testing is not feasible due to the high number of known mutations seen in this disorder. Future technological advances may make this more plausible, but at present still a diagnosis of WD needs to be made through the strategic use of multiple diagnostic tools. Which tests are performed, and how they are performed, largely depends on whether or not the patient's clinical presentation suggests copper has spread beyond the liver.

1.5.1. Hepatic Copper Determination

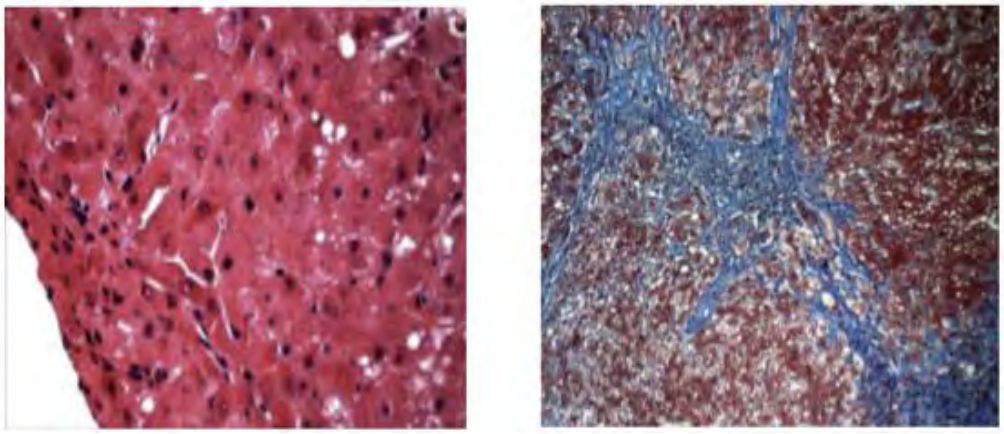
The most precise and sensitive diagnostic method now available for Wilson's illness is a liver biopsy to determine the hepatic copper level. WD is characterized by an enhanced hepatic copper level, even in patients with no outward symptoms. Most reliable biochemical evidence for diagnosis is an elevated copper concentration in WD, which can be larger than 250 g/g of dry weight. Sampling error during the measurement of hepatic parenchymal copper content can lead to a false negative result. Copper levels in the liver are higher in heterozygotes than they are in homozygotes or normal persons, but they are still consistently below 250 g/g of dry mass (Ferenci *et al*., 2003).

According to a study out of 114 liver biopsies 83.3% of individuals with WD had hepatic copper levels more than 250 mg/g, and in merely 3.5% it was less than 50 mg/g (Benson, 1979; Smallwood *et al*., 1968).

1.5.2. Liver biopsy

It is not prudent to do a liver biopsy on every individual who may have Wilson's disease because of the risks associated with the invasive operation, as well as the discomfort associated with the biopsy itself. It needs to be reserved for circumstances in which more straightforward methods have failed to produce an accurate diagnosis.

A liver biopsy is obligatory to distinguish WD from other chronic hepatic diseases and also, to allow a final diagnosis when other non-invasive measures do not give reliable results. Histological conclusions on biopsy may include necrosis, glycogen deposition, fibrosis steatosis and progressive damage to parenchymal tissues in liver (figure 1.10) (Nicastro *et al*., 2010).



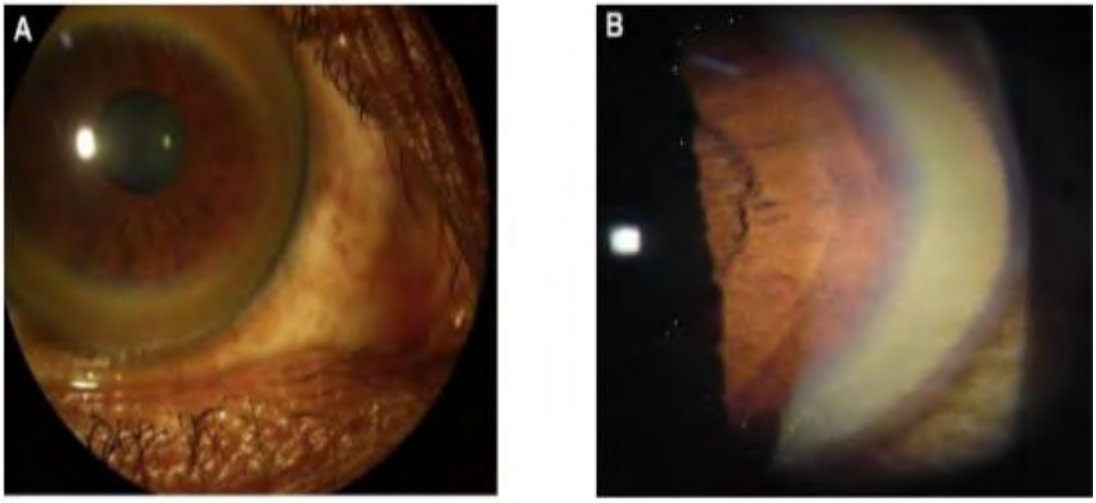
A) Mild steatosis **B)** Fibrosis

Figure 1.10. Liver biopsies presentation of fibrosis and mild steatosis Adopted from (Liver, 2012b). Amount of copper deposited in hepatocytes may differ in regions of liver like at early stage of disease it will be found attached to metallochaperones in the cytoplasm. Due to this, copper will not be noticed in histochemical examination in initial phases of disease. As the disease progress marked deterioration in tissues due to copper accumulation will be present. In steatosis mitochondrial irregularities such as increased density of matrix, dilated tips of cristae and enlarged space in intracristal region is seen (Cope-Yokoyama *et al*., 2010).

1.5.3. Slit-Lamp Examination

The existence of KF rings is compelling evidence in support of a diagnosis of WD in a patient who is also exhibiting psychiatric or neurological impairment. However, it has been established that patients with central nervous system dysfunction do not have KF rings (Oder *et al*., 1991; Ross *et al*., 1985; Willeit & Kiechl, 1991). Patients who have solely hepatic symptoms rarely have KF rings present in their liver. In a study, children ages ranging from 7-17 years old who were affected with WD, a slit-lamp examination revealed the existence of KF rings in only two out of 36 individuals (5.6%) (Oracz *et al*., 2005).

Slit lamp examination is achieved to examine KF ring (figure 1.11) formed in corneal region of the eye due to deposition of copper. A golden-greenish granular layer is usually visible with bare eye. It is typically present in patients with neurological and psychiatric manifestations of WD than in young patients with hepatic abnormalities. A skilled ophthalmologist inspects the eye in alleged patients for WD to detect KF ring for establishing diagnosis. It may also be noticed in patients suffering from other chronic hepatic conditions such as cirrhosis, hepatitis and biliary issues but on the basis of other clinical findings distinction from WD is possible (Roberts & Schilsky, 2008). On liver transplantation or other medical treatment, it may vanish in some patients, but the chances of reoccurrence are at all times there.



(A) Naked eye examination (**B)** Slit lamp examination

Figure 1.11. Kayser- Fleischer ring (KF) in patient with WD Adopted from (Patil *et al*., 2013).

1.5.4. Ceruloplasmin

The measurement of the level of ceruloplasmin in the serum is straightforward, risk-free, and used as a screening test for WD; however, it is not sufficient on its own. 5–15 percent of people with WD may have a ceruloplasmin level that is slightly less than the normal range, whereas 10–20 percent of heterozygotes may have lower levels (Brewer, 2001; GIBBS & Walshe, 1979; Scheinberg *et al*., 1987). In individuals with WD, the acute phase reactant ceruloplasmin may become momentarily elevated into the normal range as a result of inflammation or infection, as well as the consumption of steroids or the use of birth control pills (STERNLIEB & SCHEINBERG, 1972).

The major carrier of copper in blood is a 132 kDa protein identified as ceruloplasmin, which is present in form of apoceruloplasmin (no attached copper molecules); is a positive acute phase reactant. It is secreted by liver and it has the ability to bind 6 copper per molecule forming holoceruloplasmin (GIBBS & Walshe, 1979). The level of ceruloplasmin in blood can be measured by antibody dependent assay or by its enzymatic activity to different substrates. Antibody dependent assays include radioimmunoassay, nephelometry and radio immune-diffusion assay. Genetic mutations decrease the ability of apoceruloplasmin to bind with free copper molecules and make it very unstable that result in reduced total serum ceruloplasmin concentration (Rajput *et al*., 2017).

The concentration of ceruloplasmin differs in normal individuals from birth to death, like at age of six months it is very low and then at early childhood it reaches to 0.30.5 g/L after that it will drop to reach adult range. In patients suffering from WD its concentration is lower than 0.1g/L (Roberts & Schilsky, 2008). Ceruloplasmin concentration is also reduced by some other diseases like malabsorption syndrome, familial aceruloplasminemia, liver failure and renal protein loss so decreased serum ceruloplasmin value alone is not sufficient for patient undergoing evaluation for WD (Merle *et al*., 2009). In diseased individuals the concentration of serum ceruloplasmin can be normal or close to

normal due to other causative factors such as estrogen therapy, pregnancy, setting of inflammation and tissue injury (Rajput *et al*., 2017).

1.5.5. Measurement of 24-Hour Excretory Copper

The quantity of copper found in urine collected over 24 hours has the potential to be the most accurate screening test for WD, particularly in people who have psychiatric or neurological impairment (Brewer, 2001). Copper levels in the urine can often rise up to 100 mg/d in WD patients who are experiencing symptoms. On the other hand, copper levels in the urine may be abnormally high in certain other circumstances. The levels of copper in the urine of heterozygous carriers of WD are somewhat higher, although they do not exceed 100 mg/d (Brewer, 2001).

Copper concentrations in the urine are sometimes an indicator of other disorders, such as obstructive liver disease. Urine must be collected from patients in jugs that are free of copper and are provided by the laboratory in order to reduce the likelihood of obtaining inaccurate readings.

24h urinary copper concentration is measured for evaluation in WD in untreated patients, and after treating with chelating agents. For precise determination of amount of copper in urine person should not be suffering from renal failure. The quantity of excreted copper in urine should be within range of 40µg to 60µg per 24h in normal healthy individual. This amount exceeds in case of Wilson's disease i.e., 200µg per 24h. In heterozygous individual the amount of copper is more than normal people but infrequently exceed 40µg per 24h limit (Ferenci *et al*., 2007).

D-Penicillamine trial is used to identity asymptomatic relatives of patients and people having marginal 24h urinary copper. D-Penicillamine (500 mg) is administered usually in children before collection of urine and again after 12h. When the amount of copper measured is beyond 1600 mcg per 24h, it clearly distinguishes Wilson's disease from other hepatic conditions (Tu *et al*., 1967).

1.5.6. Ceruloplasmin Bound Copper and Free Copper

Amount of free copper in serum is measured using laboratory tests in patients suspected of WD and if increased concentration is detected, it is considered as an important diagnostic finding for WD. Copper is typically present in body bound to different proteins such as 65 to 90 percent is bound to ceruloplasmin and only a small fraction of Cu is bound to albumin and amino acids (Catalani *et al*., 2018).

Increased concentration of free copper in serum indicates decreased concentration of ceruloplasmin in blood. Due to some limitations of this test such as its dependency on measuring ceruloplasmin concentration and serum copper from same test and highly variable values of both, the results are not considered very reliable.

The usual amount of free copper in serum is 100-150 µg/L but in WD it reaches to m250 µg/L, mostly in unprocessed patients. As a result of lengthy treatment the amount of free copper might decrease to a level even less than normal concentration, getting 0.84 µmol /L (Frydman *et al*., 1985).

1.5.7. Neuroimaging Studies

Brain abnormalities in WD patients with neuropsychiatric symptoms can be detected with CT and MRI scans. Nearly all individuals diagnosed with WD who exhibit neurological impairment have been shown to have abnormalities on magnetic resonance imaging (MRI), according to studies that have been published (Sinha *et al*., 2006).

Mostly basal ganglia region of brain is inspected for changes but in some cases central pons and tectal-plate region has also shown abnormalities (Prashanth *et al*., 2010). Hyper intensity on T2 MRI and increased density on CT scan is detected in brain of patients with neurological participation of disease. In lesser of patients ‘giant panda face’, (figure 1.12) reported in scan which is considered a characteristic feature of WD (Patil *et al*., 2013). Neuroimaging may also be performed in patients giving only hepatic abnormalities or asymptomatic patients to assess any neurological changes prior to onset of neurological symptoms. In late phases of disease secondary brain atrophy arises if neurological abnormalities are persistent.

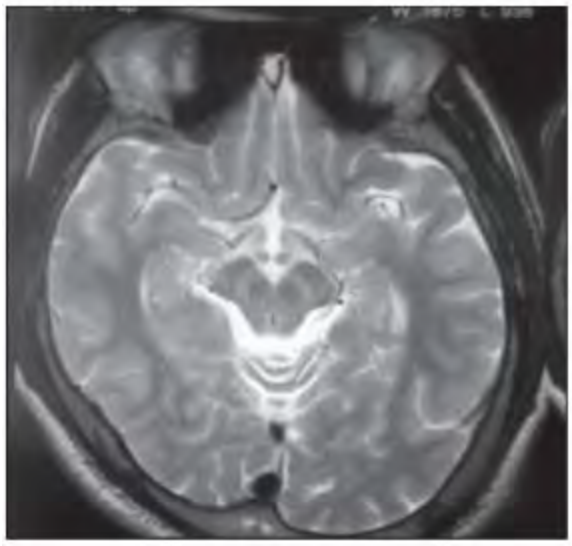


Figure 1.12. Axial T2 image of brain presenting giant panda like face that is common in neurologic Wilson's disease (Hitoshi *et al*., 1991).

Uptake of glucose also decreases in basal ganglia region of brain in WD, but this finding is very non-specific and cannot be used to distinguish WD from other neurodegenerative disorders (Hedera, 2017). Auditory evoked brainstem potentials, Single photon mission of computed tomography, and spectroscopy are also used to find out impairments caused by Wilson's disease (Tarnacka *et al*., 2008).

There was evidence of lenticular hyper-echogenicity in patients with WD who were experiencing neurological impairment as well as in two of the three people who were neurologically asymptomatic. Positron emission tomography, sometimes known as PET scanning, can identify anomalies in patients with WD; however, this test is not routinely available. In the context of WD, transcranial brain parenchyma sonography was first identified (Walter *et al*., 2005).

In the evaluation of a patient doubted of having WD, the assimilation of radioactive copper into ceruloplasmin is rarely required but has the potential to be beneficial in some situations. Although it has been hypothesized that measuring the copper levels in cerebrospinal fluid could offer precise reflection of copper amount present in the brain, this test is also not commonly used (Hartard *et al*., 1993).

A high catalog of doubt is requisite for the early age diagnosis and recovery in WD otherwise progressive deterioration of symptoms can be aggressive. Due to many nonspecific symptoms, broad range of age of on set and early diagnosis of disease is problematic. In 2001, at 8th international conference on Wilson's disease a score chart was devised for proper diagnosis of Wilson's disease. Different biochemical tests are performed in laboratory for the assessment of disease. Many non-Wilsonian hepatic instabilities such as proteinuria, progressive intrahepatic cholestasis in children, hypercalciuria and aminiaciduria can be confused with WD due to overlying symptoms. A series of diagnostic tests are required for establishment of diagnosis for WD.

Table 1.1. Wilson's disease scoring system developed at 8th International Meeting, Leipzig 2001 (Ferenci, 2003).

Common signs and clinical symptoms	Score
Kayser-Fleischer ring (KF ring)	
Present	2
Absent	0
Serum ceruloplasmin	
Normal (>0.2 g/L)	0
0.1-0.2 g/L	1
<0.1 g/L	2
Neurological symptoms	
Severe	2
Mild	1
Absent	0
Coombs negative hemolytic anemia	
Present	1
Absent	0
Other tests	

Liver copper (in lack of cholestasis)	
>5x ULN* (>4 µmol/g)	2
0.8-4 µmol/g	1
Normal (<0.8 µmol/g)	-1
Rhodanine-positive granules	1
Urinary copper (in lack of acute hepatitis)	
Normal	0
1-2x ULN	1
>2x ULN	2
Normal, but >5x ULN after D-penicillamine	2
Mutational investigation	
On both chromosomes detected	4
On 1 chromosome detected	1
No mutations detected	0
Total score	**Evaluation**
4 or more	Diagnosis established
3	Diagnosis possible, more tests needed
2 or less	Diagnosis very unlikely
*ULN, upper limit of normal.	

Different diagnostic approaches summarized in table 1.2 are used in combination to confirm WD in patients, making diagnosis complex for physicians. In 2009, a Global Assessment Scale GAS devised for diagnosis of WD was published to assess diverse disease manifestation, its progression and response to treatment (Aggarwal *et al*., 2009).

According to two tier design of this scale, tier 1 is named as Global Disability and it includes hepatic dysfunction, cognitive disabilities, motor damage and osseo-muscular malformations. Tier 2 is designed for neurological assessment of the disease and it includes

scholastic performance, facial expression, psychosis, depression, parkinsonism, KF ring and other uncommon features of WD (Aggarwal *et al*., 2009).

Table 1.2. Predictable tests used for identification of Wilson's disease (reproduced from Elsevier publications) (Liver, 2012b).

Test	**Finding**	**False negative**	**False positive**
Serum ceruloplasmin	Decreased by 50% of lower normal value	ceruloplasmin less than half the lower usual value Patients with severe hepatic inflammation have normal levels. -Pregnancy, oestrogen treatment, and immunologic assay overestimation.	Low levels in -Malabsorption -Aceruloplasminemia -Heterozygote's
Serum free copper	>20 mcg/L	Normal if ceruloplasmin overestimated by immunologic assay	
24 h urinary copper	>100 mcg/24 h >40 mcg/24 h in children	Normal -Incorrect collection -Children without liver disease	Increased -Hepatocellular necrosis -Cholestasis -contamination
Hepatic copper	>250 mcg/ g dry weight	Due to regional variation -In patients with active disease -In patients with regenerative nodules	Cholestatic syndrome
KF ring by slit lamp examination	Present	Absent -In up to 50% of patients with hepatic Wilson's disease -In most asymptomatic siblings	Primary biliary cirrhosis

1.6. Risk Factors

1.6.1. Genetics

The g6pc*ATP7B* gene mutations located on the chromosome 13 are responsible for this WD (Petrukhin *et al*., 1994). The number of mutations identified in *ATP7B* gene so far is now approaching 858 (Stenson *et al*., 2017). Available at: http://www.hgmd.cf.ac.uk/ac/index.php (Accessed: 3rd May 2018). Missense mutations are although most frequent, insertions, deletions, splice site and nonsense, mutations all play role in disease pathogenesis. Most of affected individuals are compound heterozygotes i.e., they have received various mutations from each of their parents as a result of their inheritance. The significant number of alterations observed in this gene has made viable genetic testing for WD (Loudianos *et al*., 2002). Whether the abundance of the mutation's accounts for prominent inconsistency in age of symptom onset and clinical presentation in Wilson's disease patients is uncertain.

The most recurrent mutation H1069Q in the Northern Europe and US has been known to be associated with less severe disruption of copper metabolism and later onset of the symptoms (Gromadzka *et al*., 2006). Nonsense and frame-shift mutations may associate with earlier onset of symptoms and more severe disruption of copper metabolism (Gromadzka *et al*., 2005). A wide range of age variability at which symptoms first appear and clinical presentation in WD patients with the same mutation, even in homozygotic twins, suggests that additional variables are at play. Methionine is one such example. Wilson's disease symptoms may be influenced by prion-related protein gene homozygosity at codon 129 (Merle *et al*., 2006; Senzolo *et al*., 2007).

Other mutations are common in Southern Europe, such as the missense mutation M645R in the mainland Spain. The R778L in exon 8 is found more commonly in South-eastern Asia where the mutation has an allele frequency of 14-49% (Ferenci, 2006).

Generally, Wilson's disease carriers with a single allele mutation are thought to be immune to the disease's symptoms. The discovery of a nucleotide loss in the 5'UTR of the *ATP7B* gene in three elderly sisters, the development of depression and Parkinsonism in those women was questioned (Sechi *et al*., 2007).

Toxic effects of copper on Wilson's disease are a result of its overuse, but decrease in the protein X-linked inhibitor of apoptosis (XIAP) caused by copper rise accelerates caspase 3–initiated apoptosis, which leads to cell death in Wilson's disease (Mufti *et al*., 2006). Wilson's disease is also characterized by a deficiency in apoceruloplasmin ceruloplasmin due to a failure in *ATP7B*-mediated copper incorporation. However, this deficiency is neither diagnostic nor universally present in Wilson's disease. Ceruloplasmin levels in 10–20% of heterozygotes who are clinically asymptomatic may be lower than average, while only 5%–15% of those with WD may have normal or slightly lowered levels (GIBBS & Walshe, 1979). WD can lead to liver disease, psychiatric illness or progressive neurologic disorder.

1.6.1.1.Genes involved in pathogenicity of Wilson's Disease

1.6.1.1.1. PNPLA3

Triglyceride metabolism involves PNPLA3, or patatin-like phospholipase domain-containing protein 3. Hepatic steatosis in WD patients has been linked to a form of the PNPLA3 gene that is more typically seen in those with NAFLD (Stättermayer *et al*., 2015). PNPLA3 loss of function has been linked to accumulation of triglycerides in the hepatocytes and stellate cells (Pingitore *et al*., 2014).

1.6.1.1.2. *ApoE* ε4

The *ApoE ε4* allele of the apolipoprotein E gene (ApoE) plays a role in neurodegenerative diseases and lipid metabolism, was suggested to be a modifier of WD phenotype (Schiefermeier *et al*., 2000) However, a sizable investigation found no association between the *ApoE* 4 genotype and the hepatic or neurological symptoms in WD (Litwin *et al*., 2012). Females with the *ApoE* 4 allele, especially having H1069Q mutation in homozygous form, tend to have earlier onset of illness compared to their female counterparts with the *ApoE* 3/3 genotype. Copper accumulation in Bedlington terriers is due to mutations in the copper metabolism domain containing 1 (COMMD1) gene (previously MURR1). One study found COMMD1 variations in 30% of 63 cases with copper accumulation (Stuehler *et al*., 2004). However, subsequent research involving other populations was unable to replicate similar results (Wu *et al*., 2006).

1.6.1.1.3. *ATOX1*

The copper chaperone *ATOX1* has also gathered much consideration given its interaction with *ATP7B31*, but correlational studies on the patients with WD could not recognize an important role for *ATOXI* variants (Bost *et al*., 2012; Lee *et al*., 2011).

1.6.1.1.4. *MTHFR*

Oxidative stress is supposed to be the main reason of liver damage related with copper buildup. In a group of 435 patients with WD, variations of genes related to antioxidant enzymes, including manganese superoxide dismutase and catalase, were associated to age of onset of WD (Gromadzka *et al*., 2015). Variants in the Methylenetetrahydrofolate reductase (MTHFR) gene were investigated in a sizable population of Polish patients diagnosed with WD. The authors found a link between WD MTHFR polymorphisms and phenotype, although the work was critiqued for its lack of phenotypic description (Gromadzka *et al*., 2015) because the *MTHFR* 677T allele was linked to enhance risk of liver disease in those patients. The methionine-tetrahydrofolate reductase (*MTHFR)* gene is intriguing despite the lack of data because mutations in this gene might affect methionine and folate metabolism, which may have knock-on effects on epigenetic mechanisms of gene expression and regulation.

1.6.1.1.5. ATPase/ *ATP7B* gene

ATPase is a copper-transporting intracellular P-type ATPase having molecular weight equals to 165 kDa (1411 amino acids), plays a vital role of disposition of copper in hepatocytes. It is a P-type ATPase having phosphorylation domain and cation channel with a conserved motif i.e., DKTGT. Wilson ATPase has, 8 trans-membrane domains including CPC motif in segment 6; and an ATPase region and 6 copper-binding domains (GMXCXXC). The structure of ATPase has been determined by the homology mapping using sarcoplasmic Ca^{2+} P-type ATPase Serca1as model, and structures of portions of protein have been solved (Fatemi & Sarkar, 2002; Singleton & Le Brun, 2007). Conformational changes linked with copper binding also effect Wilson ATPase function; therefore, the N-terminal region plays a regulatory role. The copper-binding domains 1–5

of the human Wilson ATPase were deleted for functional research (Portmann & Solioz, 2005). When intracellular copper concentrations are remarkably high, it travels from trans-Golgi to bile canalicular (apical) membrane of (La Fontaine & Mercer, 2007). Mechanism of the biliary excretion of copper is unclear. Regulatory functions of COMMD1 relate to that of nuclear transcription factor NF-kappa B and other transporters (De Bie *et al*., 2005). X-linked inhibitor of apoptosis interacts with COMMD1 and can also bind copper, becoming non-functional. This may also have a bearing on propensity to apoptosis in WD (Mufti *et al*., 2006).

Two transmembrane copper transporting P-type ATPase proteins, Wilson's disease protein *ATP7B* (164 kDa) containing of 1465 amino acids and ATP7A (175 kDa) Meknes's protein, are homologous (La Fontaine *et al*., 2010). Both the proteins show 59% sequence similarity of amino acid but differ in their tissue expression pattern. *ATP7B* protein is the most notably expressed in hepatocytes and in brain cells. *ATP7B* is also expressed in small amount in muscle cells, kidneys, pancreas, placenta and breast tissues where it plays key role in transport of excess copper out of cells (Zuzel, 2010).

Cytogenetic location of *ATP7B* gene is 13q14.3. It is a large gene having 21 exons and size of 80kb (Cater *et al*., 2007). *ATP7B* gene comprises different functional domains like, a nucleotide-binding domain that binds ATP, eight hydrophobic transmembrane regions, 6 copper binding domains, a phosphorylation domain and Cation channel. Six metal binding domains (MBD) present at N-terminal are also required for transport of copper, and a transduction domain that is tangled in transduction of the energy stored in ATP (Figure 1.13) (Merle *et al*., 2007).

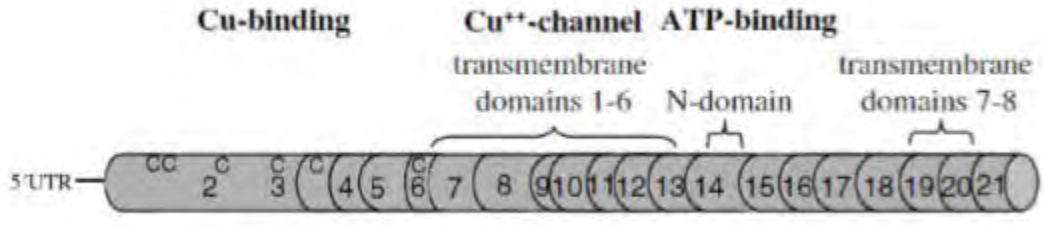


Figure 1.13. Wilson's disease gene (*ATP7B*) Adopted from (Merle *et al*., 2007)

Both the liver and the brain have been shown to express the WD gene known as *ATP7B* (adenosine triphosphatase, copper transporting, beta polypeptide). The brain contains a greater number of alternatively spliced versions of *ATP7B* than the liver does. The splice variants seen in the brain feature various different combinations of missing exons, in contrast to the most abundant version found in the liver, which has all of the exons (Cater *et al*., 2007; Wilson *et al*., 2009). The alternative splicing of *ATP7B* is regulated in the brain and liver by processes that are specific to those tissues. Splicing of exon 12 in alternative forms takes place in the brain but not in the liver (YANG *et al*., 1997). It is unknown whether these different variants of splicing maintain their biological function. Numerous therapeutic approaches have been investigated with the goal of either modifying the splicing pattern of disease-causing mutant pre-messenger RNA (pre-mRNA) or removing mRNA that contains a disease-causing mutation. For instance, the open reading frame of the *ATP7B* is maintained despite the absence of exons 6, 7, 8, 12, and 13 (Petrukhin *et al*., 1994), additionally, exons 8, 12, and 13 are considered to be hotspots for mutations in Taiwanese patients (Lee *et al*., 2000; Wan *et al*., 2006). Therefore, in order to assess whether or if splice-correction treatment may be utilized for WD, it would be important to identify the function of alternatively spliced variants.

1.6.1.1.6. Mutations in *ATP7B* gene

Typical tests for WD cannot detect heterozygous carriers or be used for presymptomatic diagnosis. Molecular diagnosis is a valuable tool to overcome these limitations (Gupta, Maulik, *et al*., 2007; Mak *et al*., 2008). About 858 mutations in *ATP7B* gene are documented up till now from various countries according to Wilson's disease database (http://www.wilsondisease.med.ualberta.ca/index.asp), along with more than 100 nonpathogenic variants (Hedera, 2017). Out of 21 exons of *ATP7B*, exons 2, 8, 13, 14, 16, 18 and 21 are considered as the mutation hotspot exons. A large fraction of mutational changes that are found in *ATP7B* gene are the missense mutations producing change only at a single amino acid (Huster *et al*., 2012). When it comes to promoter and 5' untranslated region (5' UTR) mutations, there have only been a few studies, according to the Wilson Disease Mutation Database (http://www.wilsondisease.med.ualberta.ca/index.asp) (Davies *et al*., 2008). H1069Q, a point mutation in exon 14 is most common mutation in the

European countries, but this mutation is not found in Asian countries (Ferenci, 2006). Asian countries mutational data is mostly accessible from Japan, China, South Korea, and India. R778L, a missense mutation in exon 8 is mostly found in these Far Eastern countries with 14 to 49 percent allelic frequency. Different studies achieved in India gives a highly diverse data as the mutations found in one region of the country are completely absent in other regions (Ferenci, 2006). Several research have tried to tackle the difficult challenge of linking genetics and phenotype. Different variations of *ATP7B* have been shown to exhibit varying degrees of copper-transporter activity in vitro (Huster *et al*., 2012). Acute liver failure and a younger age of illness onset have both been linked to truncating mutations in *ATP7B* (Merle *et al*., 2010). However, in spite of these and other studies in the small populations (Członkowska *et al*., 2018) other data trying genotype-to-phenotype correlations have not been conclusive. The late diagnosis, overlap of neurological, psychiatric, and hepatic symptoms, and general lack of phenotypic categorization all contribute to this. However, more research is needed to offer clear evidence for particular connections, as both genetic and environmental factors interact to influence the complex phenotype. Few mutations reported from Asian countries in *ATP7B* gene are summarized in table 1.3.

Table 1.3. Mutations reported from Asian countries in WD patients (*ATP7B* gene).

Country	**Location**	**Mutation**	**Reference**
China	Exon 1	c.121A>G	(Hua *et al*., 2016)
China	Exon 2	c.748G>A	(Hua *et al*., 2016)
China	Exon 2	525insA	(Wan *et al*., 2010)
China	Exon 2	685insA	(Hua *et al*., 2016)
China	Exon 2	p. Lys300Ter	(Hua *et al*., 2016)
China	Exon 2	p. Gly250Arg	(Hua *et al*., 2016)
China	Exon 3	Cys490ter	(Wan *et al*., 2010)
China	Exon 3	Gln511ter	(Wan *et al*., 2010)
China	Intron 4	c.1708-1G>C	(Hua *et al*., 2016)

China	Exon 8	Met729Val	(Hua *et al*., 2016)
China	Exon 8	Arg778Gln	(Wan *et al*., 2010)
China	Exon 8	Leu770Phe	(Wan *et al*., 2010)
China	Exon 10	Lys832Arg	(Wan *et al*., 2010)
China	Exon 12	Arg919Gly	(Wan *et al*., 2010)
China	Exon 12	Thr935Met	(Wan *et al*., 2010)
China	Exon 12	Gly943Asp	(Wan *et al*., 2010)
China	Exon 13	Ser986Phe	(Wan *et al*., 2010)
China	Exon 13	Pro992Leu	(Hua *et al*., 2016)
China	Exon 13	His1019Pro	(Hua *et al*., 2016)
China	Exon 14	Arg1041Trp	(Wan *et al*., 2010)
China	Exon 14	Gln1142His	(Wan *et al*., 2010)
China	Exon 16	Trp1153Cys	(Wan *et al*., 2010)
China	Exon 16	Ala1168Pro	(Wan *et al*., 2010)
China	Exon 18	Pro1273Gln	(Wan *et al*., 2010)
China	Exon 18	Asp1279Gly	(Wan *et al*., 2010)
China	Exon 20	Ile1348Asn	(Wan *et al*., 2010)
China	Exon 21	Met1392Lys	(Wan *et al*., 2010)
India	Exon 2	c.365 Ins A/G	(Rangaraju *et al*., 2017)
India	Exon 2	c.445 G > T	(Gupta *et al*., 2007)
India	Exon 2	c.855 T>A	(Gupta *et al*., 2007)
India	Exon 2	c.1041 C > A	(Gupta *et al*., 2007)
India	Exon 2	c.678delG	(Aggarwal *et al*., 2013)
India	Exon 2	c.813C>A	(Aggarwal *et al*., 2013)
India	Exon 3	c.1366 G> C	(Gupta *et al.,* 2007)
India	Exon 3	c.1543 + 51G > A	(Gupta *et al.,* 2007)

India	Exon 4	c.1544G > A	(Aggarwal *et al*., 2013)
India	Exon 4	c.1708-1G > C	(Gupta *et al.,* 2005)
India	Exon 8	p. Tyr743fs	(Gupta *et al*., 2005)
India	Exon 12	Ile913fs	(Gupta *et al*., 2005)
India	Exon 13	Thr977Met	(Gupta *et al.,* 2005)
India	Exon 14	Gly1061Glu	(Gupta *et al.,* 2005)
India	Exon 18	Leu1299Phe	(Gupta *et al.,* 2005)
India	Exon 20	Ala1357Val	(Gupta *et al*., 2005)
Vietnam	Exon 2	c.314C>A	(Nguyen *et al*., 2017)
Pakistan	Exon 2	c.815-816insT	(Naveed *et al.,* 2010)

1.6.2. Epigenetics

Epigenetic pathways are implicated in WD etiology and phenotypic presentation, as indicated by data from humans and studies in animal models. Homozygous twins with WD have been reported in some case studies, and these twins have contrasting illness characteristics (Członkowska *et al*., 2009; Kegley *et al*., 2010). Animal models of WD have also revealed the potential influence of dietary and environmental variables on epigenetic processes. Methionine metabolism mediates between the environment and the methylation processes that control gene expression. S-adenosylhomocysteine hydrolase (SAHH) is an essential enzyme because it converts S-adenosylhomocysteine (SAH) to homocysteine. With the decrease in expression or activity of SAHH, the level of SAH will increase, which acts as an inhibitor of methylation reactions, will increase. SAHH gene transcript levels and activity are reduced in the presence of hepatic copper accumulation with subsequent downstream changes in methionine metabolism parameters (Delgado *et al*., 2008).

1.7. Prevention

Screening of first-degree relatives and making a clear diagnosis of other movement and liver illnesses can help detect WD at an early stage, making it one of the few metabolic disorders amenable to pharmaceutical treatment (Liggi *et al*., 2013; Liver, 2012b; Stättermayer *et al*., 2017). Greatest threat of the disease is among the siblings of the index case where essential fast diagnosis is required despite lack of symptoms and age (Karolina Dzieżyc *et al*., 2014). Based on a the large group of 760 Polish patients, offspring are at about 4 percent risk of WD and there is greater risk of the disease among cousins; WD has even been detected in asymptomatic close relative (Karolina Dzieżyc *et al*., 2014). Having a strong foundation of WD knowledge among patients and their loved ones is crucial, and patient support groups fill a necessary niche (Graper & Schilsky, 2017).

1.8. Treatment of WD

The treatment for WD consists primarily of alleviating symptoms and is intended to restore and maintain copper balance. The only exception to this is liver transplantation. It does not correct the fundamental problem that led to the development of WD. Therefore, a commitment to treatment for the rest of one's life is necessary. In most cases, limiting the amount of copper consumed through diet is pointless; instead, pharmaceutical control is required.

A life-long management of disease is mandatory when the diagnosis is made. In the beginning the intramuscular administration of dimercaprol was used to treat diagnosed patients, but now it is substituted by therapy using chelating agents such as Penicillamine, zinc, trientine and ammonium tetrathiomolybdate (Walshe, 1956).

1.8.1. D-Penicillamine

A metabolic by product of penicillin i.e. Penicillamine, that is a chelating agent, was commenced as WD treatment in 1956 by Walshe (Walshe, 1956) and rapidly became the typical of therapy. When treatment is started, copper is quickly flushed out of the body through urine. In rare cases, functional improvement can be shown as early as 2 weeks after treatment has begun. The likelihood of improvement in psychological symptoms is

lower than in neurological symptoms, yet improvement can occur in almost every area of function (Brewer, 2005a).

Penicillamine is used to decrease the attachment of copper to proteins making its attachment with drugs more active. It is used for treatment of patients with presentation both hepatic and neurological symptoms. Medication in children is done in 2 or 3 divided doses, 1h before the meal or 2 h after it, as drug absorption decreases by 50 percent after having meal. 250 mg/ day is required for rapid removal of copper from liver among children. D-Penicillamine is also linked with many side effects such as interfering with collagen cross linking and pyridoxine action (Falkmer *et al*., 1970).

Twenty to thirty percent of patients will experience severe sensitivity reactions, which might include fever, skin rash, thrombocytopenia, eosinophilia, leukopenia, and lymphadenopathy. These reactions frequently necessitate the discontinuation of penicillamine treatment (Haggstrom *et al*., 1980; Sternlieb & Scheinberg, 1964). The skin condition known as penicillamine dermatopathy, which is characterized by a brownish coloring, is caused by chronic subcutaneous bleeding that occurs during secondary trauma (Sternlieb *et al*., 1981).

In neurologic WD the retrieval from this management is very slow and it may take up to three years. In the start the disease may become worse before getting better in 10-15 patients. Sensitivity due to drug may result in neutropenia, fever, bone marrow toxicity, proteinuria, dermatological toxicity, nephrotoxicity, anemia and immunosuppression if the treatment is sustained for a very long time (Czlonkowska *et al*., 1996).

1.8.2. Zinc

It was first anticipated by Schouwink in his doctoral thesis in 1961, that the use of zinc in treatment of the Wilson's disease has slowly assumed an increasingly important role in disease management (Hoogenraad, 2006). Administration either as acetate, gluconate, sulfate or zinc decreases intestinal absorption of copper present in diet by induction of metallothioneins formation in intestinal enterocytes. The metallothioneins bind to copper

and zinc, which traps them within intestinal mucosal cells, which are ultimately shed and emitted.

Therefore, zinc has been utilized for the most part as a preservation therapy subsequent to first treatment with more powerful decoppering agents (Brewer, 2001). 50 mg is normal dosage routine for zinc. Zinc is generally well accepted, although gastric uneasiness may occur. Zinc regulates free amount of copper by binding it to the enterocyte metallothionein preventing its entry into portal flow. When these enterocytes are shed, excess copper attached to them is also removed (Schilsky *et al*., 1989). Zinc is typically used in maintenance treatment and is also considered to be safe during pregnancy. 150 mg per day is considered active dose in adults distributed into 2 or 3 doses but in children only 75 mg per day is considered sufficient. In zinc treatment neurological worsening is not common and it is considered well tolerated with few side effects. Hepatic deterioration, gastric irritation, elevated serum lipase, immunosuppression, and amylase are reported in few cases (Roberts & Schilsky, 2008). In presymptomatic and asymptomatic patients zinc therapy is used as first-line treatment.

1.8.3. Trientine

Trientine is also a chelating agent used for elimination of copper from body in patients who are intolerant to penicillamine or have deteriorating neurological disorder. Primary dose of trientine used for treatment is 750-1500 mg/ day in 2 or 3 divided doses. In children 250 mg/ day is directed in divided doses and the efficiency of treatment is measured in 24 h copper concentration that must be about 200-500 µg/day (Roberts & Schilsky, 2008). Trientine has few side effects such as lupus like reaction, loss of taste, cutaneous reactions, and rashes. In certain patients iron overload is seen due to copper deficiency induced by this drug (Walshe, 1979).

83% of patients who experienced a worsening of their neurological condition either passed away or did not fully recover. Trientine has been linked to several adverse effects, including lupus nephritis and sideroblastic anemia.

1.8.4. Ammonium tetrathiomolybdate (TM)

Tetrathiomolybdate has been investigated for the first time as a possible treatment for WD in 1984. Since then, Brewer and his colleagues have been principally responsible for advancing its availability as a treatment for WD (Brewer, 2001; Brewer *et al*., 2006). Ammonium tetrathiomolybdate acts by interfering with the uptake of copper from intestine and binding copper from plasma. At high dose, insoluble copper complex is formed that is deposited in liver. This treatment is still not accessible commercially in several countries. Typically, it is used in patients with neuropsychiatric expression that are not bearing other drugs. Bone marrow depression, hepatotoxicity and antiangiogenic effect are considered potential opposing effects of this drug (Ogra & Suzuki, 1998).

Tetra thiomolybdate not only inhibits the copper absorption by the gastrointestinal tract, but it also forms the complex within the bloodstream, preventing the uptake of non-ceruloplasmin bound copper by cells. This dual mechanism of action gives tetra thiomolybdate its distinctive property (Brewer, 2001; Brewer *et al*., 2006). On the other hand, taking benefit of this dual ability needs a somewhat complex dosing system. When taken in conjunction with food, tetra thiomolybdate is able to bind copper in the gut, but when taken on its own, the molecule is absorbed directly into the bloodstream. Therefore, a dose of 20 milligrams is administered six times a day (Brewer *et al*., 2006; Cox *et al*., 2005). Tetra thiomolybdate is not proposed as a treatment for the long term; rather, it is only intended to be used for the first eight weeks of treatment, after which zinc therapy for the long-term preservation will take place.

Table 1.4. Drugs used in the management of WD.

Drug	Mode of action	Interactions	Frequency of adverse events leading to discontinuation of treatment	Assessment of effectiveness of treatment and adherence
D-penicillamine (DPA)	Promotes urinary excretion of copper	- Do not combine with myelosuppressive agents, cytostatic, antimalarials, gold therapy, oxyphenbutazone, phenylbutazone	20–30% during treatment168,170 Can be divided into: - 'early' AEs (first 3 weeks) hypersensitivity: fever, cutaneous manifestations	- Copper urinary excretion 200–500 µg/24 h (at the beginning of the treatment >1000 µg/24 h) - Serum NCC 5–15 µg/dl - Normalization of

		- DPA interacts with heavy metals (iron salts if needed should be given after 2 h break)	including generalized pruritus, rashes, urticarial eruptions and exfoliative dermatitis, accompanied by lymphadenopathy, arthralgia, leukopenia thrombocytopenia, and proteinuria - 'late' AEs (3 weeks to few years) include: paradoxical neurological worsening, renal insufficiency in the course of Goodpasture syndrome with fatal glomerulonephritis and intra-alveolar hemorrhage, myasthenia-like syndrome; lupus-like syndrome, or fatal bone marrow aplasia to mild symptoms, such as gastric symptoms, hair loss or hypogeusia	copper urinary excretion 2 days after stopping the treatment with DPA
Trientine (TN)	Promotes urinary excretion of copper	- Mineral supplements should be avoided (iron chelation)	7.1%; AE frequency is 4-fold less than with DPA170 - Gastritis - Sideroblastic anemia - Lupus like reactions - Loss of taste	- Copper urinary excretion 200–500 µg/24 h (at the beginning of the treatment >1000 µg/24 h) - Serum NCC * 5–15 µg/dl - Normalization of copper urinary excretion 2 days after stopping the treatment with trientine
Zinc salts (ZS)	Blocks intestinal absorption of copper	- ZS diminish absorption of tetracyclines and chinolones	3–7%168 - Gastritis - Biochemical pancreatitis	- Copper urinary excretion <75 µg/24 h - Serum NCC 5–15 µg/dl; (>12 months of treatment)

		- Diuretics increase urinary zinc excretion - Iron salts, milk, milk products, wholegrain bread, products containing phytates, high -fiber products and chelating agents diminish absorption of ZS	Immunosuppression - Bone marrow depression	

NCC, non-ceruloplasmin-bound copper. NCC is not routinely used. * NCC <5 μg/dL shows over treatment and adjustment of therapy required. Reduction of NCC during conduct is currently used as an efficacy measure (Kozić *et al*., 2003).

1.8.5. Recommended diet in WD

Disease progression can be controlled by dietary control in WD patients. A diet low in copper content is recommended by the physicians. Food products with high copper concentration like organ meats, chocolate, shellfish, and mushrooms and nuts should be avoided (Hassan & Masood, 2004). Copper content in water should be tested, its concentration is usually found high in water coming from copper pipes and well water. Water purification system is suggested if amount of copper is high in water. Copper vessels and utensils should not be used for cooking or food storage (Roberts & Schilsky, 2008).

1.8.6. Liver transplantation

Patients diagnosed with WD who experience full hepatic failure have a death rate that is close to one hundred percent despite receiving medical treatment (Schafer & Shaw, 1989; Schilsky *et al*., 1994). The dreaded complication can be surgically treated with an orthotopic liver transplant. There was an 89.1% 12-month survival rate, an 82.9% 3-year survival rate, a 75.6% 5-year survival rate, and a 58.8% 10-year survival rate, according to a research synthesising the expertise of an association of Italian clinics. (Medici *et al*., 2005).

In case of fulminant WD or cirrhosis, orthotropic liver transplant (OLT), is considered as encouraging treatment but it is costly. After liver transplantation biochemical tests show progress in metabolic condition resulting in improved chances of survival. 79-87 percent of patients persist after transplantation in the first year but after this time the likelihood of survival is greater than before (Eghtesad *et al*., 1999). In neurological Wilson's disease generally, the symptoms are not eradicated after transplantation. For liver transplantation finding a suitable donor and immune suppression after surgery is a very difficult procedure. In case of chronic liver disease 90 percent chances of survival were found as compared to 73 % in patients with the severe liver failure (Yoshitoshi *et al*., 2009).

WD patients who suffered from hepatic dysfunction as well as neuropsychiatric disfunction had a poorer survival rate than who suffered solely from hepatic dysfunction. The transplantation of organs from related living donors has also been successfully utilized in WD (Wang *et al*., 2003). Despite this, there is a possibility that copper metabolism would continue to be subpar if the donor carried WD. In patients with Wilson's disease, the most common reason for undergoing orthotopic liver transplantation is due to hepatic failure; nevertheless, the efficacy of this procedure in halting the disease's progression in neurological symptoms remains debatable.

1.9. Glycogen storage disease (GSD)

Human brains rely heavily on glucose for their primary source of energy. Therefore, keeping glucose levels steady is essential for meeting cellular energy needs under both normal physiological conditions and stress. The liver and skeletal muscle are the primary sites for glucose storage as glycogen, with some glucose also being stored in the brain. Glycogen is stored in the liver, skeletal muscles, and the brain, with the latter serving as an emergency source of energy for the brain. Through the processes of glycogen synthesis and breakdown, glucose and glycogen are converted back and forth. Thus, defects in enzymes alongside these pathways are linked to abnormalities in glucose metabolism and breakdown, resulting in symptoms like hypoglycemia, hepatomegaly, and possibly liver disease in hepatic forms of glycogen storage disorder (GSD), and skeletal myopathy and cardiomyopathy, respectively. GSDs are the most common form of presentation for a

diverse range of inherited abnormalities of carbohydrate metabolism that are impairments in glycogen metabolism. Focusing on current treatment and future directions, we discuss the genetics, epidemiological, clinical, and metabolic findings of different forms of GSD and glycolysis abnormalities in this comprehensive review (Kanungo *et al*., 2018). Glycogen is a branching polymer of glucose that is mostly stored in the liver and muscle during times of plenty and then broken down and released as glucose during times of need. This glycosyltransferase enzyme, Glycogen, takes the shape of a 3-dimensional snowflake with many branches. Glucose residues from UDP glucose are attached to start the glycogen synthesis process, with as many as 10 glucose molecules serving as the core unit. Glycogen debranching enzyme and glycogen synthase, which add alpha 1,6 branch points every 12–13 glucose residues to elongate and create a globular granule of 30,000 units of glucose, respectively, attach subsequent glucose to this core unit (Kanungo *et al*., 2018; Roach *et al*., 2012).

1.10. Liver Glycogenesis and the Glycogen storage disease

Glycogen is broken down into glucose by the enzymes glycogen phosphorylase, glycogen debranching enzyme, and phosphoglucomutase. Only small amounts of glucose are kept in the brain; the majority of glucose in the body is stored as glycogen in the cytoplasm of the liver and the muscle cells. Skeletal muscle glycogen supplies energy to muscles during strenuous exercise, whereas liver glycogen acts as the primary storage source that maintains blood glucose homeostasis.

On-demand glucose maintenance in the circulation requires the breakdown of liver glycogen. In contrast, insulin secretion, glycogen synthesis, and storage in the liver and muscles are all prompted by postprandial excess blood glucose. Glycogen reserves are broken down by the liver and converted into glucose in response to the hormone glucagon during times of stress or brief periods of fasting (glycogenolysis). During periods of high energy demand or fasting, glucose is released into the circulation to help maintain stable blood glucose levels. Glycogen is used by skeletal muscles in a similar fashion, although usually only after several minutes of intense activity. The glycogen metabolism pathways

are schematically shown in Figure 1.14, and they show the many steps involved in the production or degradation of glucose and glycogen into one another.

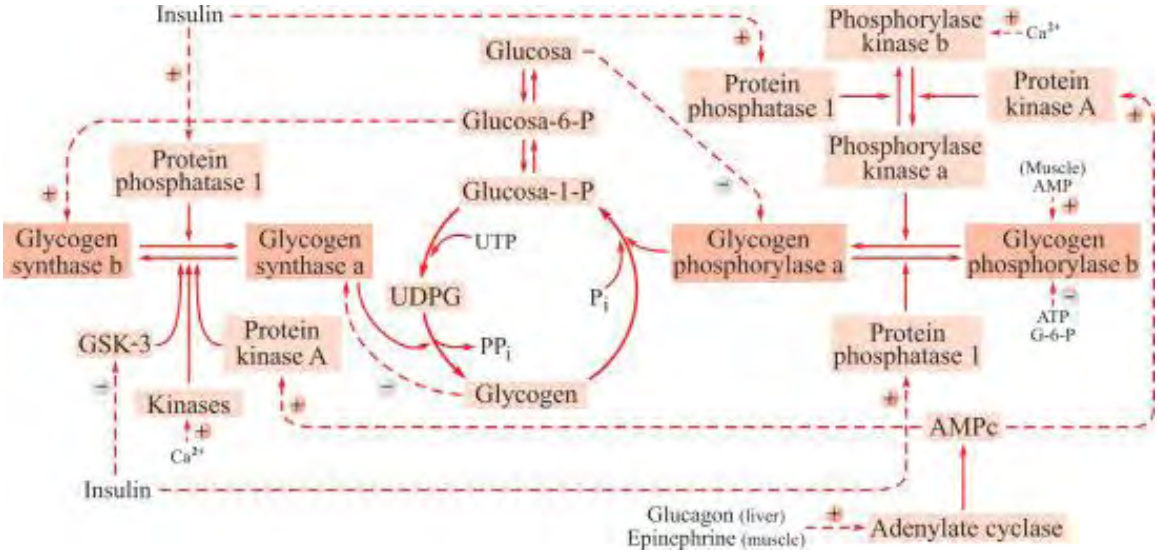


Figure 1.14. captured from J. Preiss, in Encyclopedia of Microbiology (Third Edition), 2009.

Glycogen storage disorders (GSDs) are caused by mutations in genes encoding particular enzymes in the glycogen metabolism pathway, whereas errors in glucose oxidation are known as glycolysis defects. The clinical signs of GSDs differ from one condition to the next because of the differences in enzyme defects and the degree to which each is expressed in the liver, kidneys, skeletal muscles, and heart. Fasting hypoglycemia and hepatomegaly are classic symptoms of a Liver GSD. Both exercise intolerance and rhabdomyolysis, as well as permanent muscular weakness without rhabdomyolysis, can be symptoms of muscle GSDs (Nagappa & Narayanappa, 2022). McArdle (GSD5) and Tarui (GSD7) diseases are examples of dynamic disorders characterized by exercise intolerance and rhabdomyolysis, while debrancher defect (GSD3a) and lysosomal glycogen breakdown defect (Pompe disease) are examples of cytoplasmic disorders characterized by fixed muscle weakness without rhabdomyolysis (GSD2) (DiMauro & Spiegel, 2011).

1.11. Pathophysiology of Glycogen Storage Diseases

Glycogen storage diseases, also known as glycogenosis, are a set of inherited metabolic conditions that are characterized by abnormalities in enzymes that govern glycogen breakdown and (ironically) glycogen synthesis. GSD stands for glycogen storage disease. The liver and/or the muscle is the primary organ affected by these conditions; however, on rare occasions, neurological abnormalities have also been recorded. Roman numerals, another synonym that is based on the defective enzyme, or the name of the author of the first description may be used to denote GSD (Laforêt *et al*., 2012). Glucose is kept in a huge molecule called glycogen. In the middle of the 19th century, Claude Bernard made its chemical and physiological isolation and characterization (Bernard, 1877). The molecular weight of polysaccharide glycogen ranges from the millions to the hundreds of millions. It is round and made up of straight chains of D-glucose residues connected by 1-4 links. 1-6 connections cause the straight chains to branch every four to ten residues (Smit, 1987).

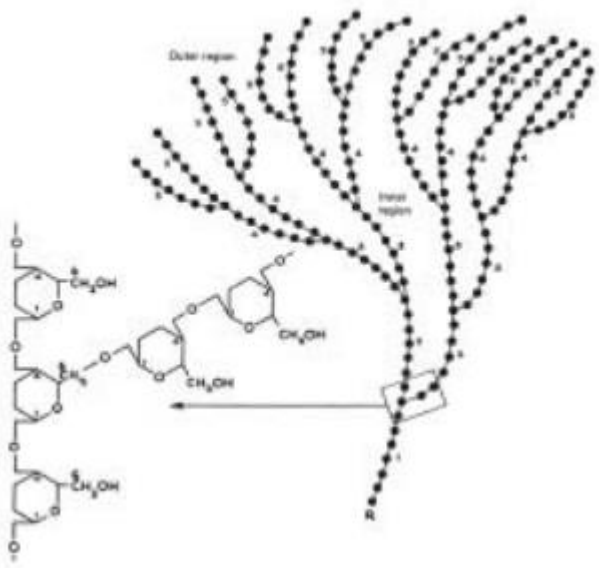


Fig 1.15. The glycogen molecule with enlargement of the structure at a branch point (Rake *et al*., 2000).

Almost every cell in the human body can store some glycogen, with muscle and liver cells storing the majority of it. Glycogen can be stored in muscle cells up to 1-3 percent of their weight and in liver cells up to 5-8 percent (Scriver, 2001). Role of glycogen in the muscles is to provide substrates for the generation of ATP for muscle contraction, while glucose derived from glycogen in the liver is mainly used to maintain the normal blood glucose concentration throughout fasting. Glycogen storage diseases (GSDs) are the inherited disorders that affect glycogen metabolism. Synthesis and degradation of the glycogen are catalyzed by enzymes that are inactivated by hormones (Saudubray *et al*., 2012). Nowadays, defects in virtually all proteins involved in synthesis or degradation of glycogen and its regulation have been found to cause some type of GSD (Scriver, 2001). Due to glycogen storage and (features of) hypoglycemia, GSDs that disrupt glycogen breakdown in the liver result in hepatomegaly. Exercise intolerance, muscle cramps, susceptibility to exhaustion, increasing weakening, and other characteristics of (cardio) myopathy are caused by GSDs that impact the glycogen breakdown in muscle. If it is kept in mind that there is some overlap and that some other tissues and organs are also affected, this classification is clinically beneficial. As they were identified, the disorders were given numbers.

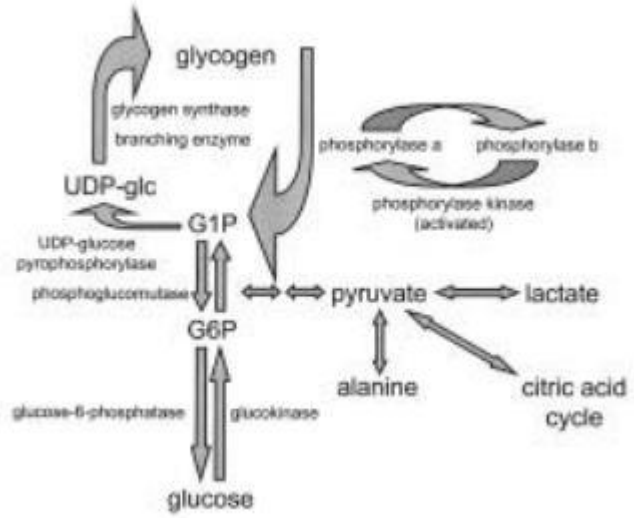


Figure 1.16. Liver glycogen metabolism (Rake *et al*., 2000).

Table 1.5. Classification of Glycogen Storage Diseases affecting the liver

Type	Synonym	Subtypes	defective enzyme/ transporter	tissue	chromosome	(main) symptoms
I	Von Gierke	Ia I-non-a	glucose-6-phosphatase glucose-6-phosphate translocase (Ib) phosphate translocase (Ic)	L, K L, K, NG L, K, NG	17q21 11q23 11q23	• HM, GR, HG, LA, HL • as Ia, + or – NP, I, IBD • as Ia, + or – NP, I, IBD
III	Cori / Forbes	IIIa IIIb IIId	Debranching enzyme (transferase + Glucosidase) debranching enzyme (transferase + Glucosidase) debranching enzyme (transferase)	L, M L L, M	1p21 1p21 1p21	HM, GR, HG, HT, (C)MP HM, GR, HG as IIIa
IV	Andersen		branching enzyme	L, (M)	3p12	HM, C, (HT, (C)MP)
VI	Hers		phosphorylase	L	14q21-22	HM, GR, (HG), HL
IX		IXa - XLG I IXa - XLG II IXa IXb	phosphorylase-b-kinase phosphorylase-b-kinase phosphorylase-b-kinase phosphorylase-b-kinase	L, E, LC L L L, M	Xp22 Xp22 ? 16q12-13?	HM, GR, (HG), HL HM, GR, (HG), HL HM, GR, (HG), HL HM, GR, (HG), HT, MP
XI	Fanconi-Bickel		GLUT 2	L, K, I	3q26	HM, GR, HG, RPTD, OP
0				L	12p12	GR, HG

GSD glycogen storage disease; XLG x-linked glycogenosis; GLUT glucose transporter; L liver; K kidney; NG neutrophil granulocyte; M muscle; E erythrocyte; LC leucocyte; I intestine; HM hepatomegaly; GR growth retardation; HG hypoglycemia; LA lactic acidosis; HL hyperlipidemia; NP neutropenia; I recurrent infections; IBD inflammatory bowel disease; HT hypotonia; (C)MP (cardio)myopathy; RPTD renal proximal tubular dysfunction; OP osteopenia (Rake, 2003).

1.12. Signs and symptoms

While its degradation is essential for supplying energy during exercise, glycogen synthesis demonstrates a critical avenue for the elimination of extra glucose. Human genetic illnesses brought on by changes in the mutated enzymes involved in glycogen metabolism serve as

a reminder of the significance of this process. The heart, kidney, liver, muscle, and/or brain typically exhibit some degree of malfunction as a result of disturbances in glycogen metabolism (Andersen *et al*., 2008). Weak muscles (low muscle tone), a big abdomen, an enlarged liver, low blood sugar (hypoglycemia), delayed growth, and liver cirrhosis are all possible general signs of GSD. While aberrant glycogen storage is a hallmark of these illnesses, there is a wide range of phenotypes connected to them, and their onset can occur at any age from infancy to maturity.

1.13. Types of GSD

1.13.1. GSD type 0 (GSD0a/b)

1.13.1.1. Pathophysiology and Clinicals

A decrease in glycogen storage in the liver is the direct consequence of GS activity that is impaired (Buschiazzo *et al*., 2004) Studies have characterized the wide-ranging phenotypic spectrum of GSD 0a, which includes postprandial hyperglycemia with hyperlactatemia, postprandial ketotic hypoglycemia, development delay, growth failure, and no hepatomegaly (Iijima *et al*., 2021). Lack of the enzyme responsible for incorporating UDP-glucose onto glycogen strands and extending them within the liver. This results in a hallmark presentation of ketotic hypoglycemia following an overnight fast in the absence of hepatomegaly and hypoglycemic seizures, as well as a lack of hepatic glycogen storage, the absence of hepatomegaly, and a liver's inability to maintain glucose homeostasis during fasting (Aynsley-Green *et al*., 1977; Rutledge *et al*., 2001).

1.13.1.2. Genetics

Differentially expressed in tissues are two genes (GYS1 and GYS2) that produce various isoforms of the GS (Browner et al., 1989). GYS2 is mostly expressed in the liver, whereas GYS1 is mainly expressed in the cardiac and skeletal muscles. Therefore, depending on which gene is impacted, the symptoms will change. Cardiomyopathy and exercise intolerance are potential consequences of the GYS1 gene's deficiency in glycogen storage (Suba et al., 2006). A rare autosomal recessive metabolic ailment known as glycogen storage disease type 0a is brought on by mutations in the GYS2 gene, which has 16 exons and is found on chromosome 12p12 (Orho *et al*., 1998).

1.13.1.3. Treatment

In order to stimulate gluconeogenesis and prevent hypoglycemia, the current course of treatment for GSD0 is avoiding fasting and eating often, high-protein meals. Uncooked cornstarch (UCCS) is also used as needed during the day or at night to treat hypoglycemia (Heller *et al*., 2008).

1.13.2. GSD type Ia (von Gierke)

1.13.2.1. Pathophysiology and Clinicals

The most prevalent and severe form of glycogen storage disease in children, type I (GSD I), usually manifests in the first few months of life. Von Gierke provided the first account of patients in 1929, describing enlarged kidneys and a liver that contained an excessive amount of glycogen (Von Gierke, 1929). Although there are no precise incidence figures for GSD I, it affects about one in 20,000 newborns worldwide and is inherited as an autosomal recessive disorder.

GSD1a, also known as Von Gierke disease or Glucose6-phosphatase (G6Pase) deficiency results from impaired ability of the hydrolase subunit of G6Pase, also known as G6Pase-α to hydrolyze G6P, leading to impaired function of G6Pase in removing the phosphate group from glucose-6- phosphate (G6P), thus, impairing free glucose availability in the last step of gluconeogenesis, resulting in impaired glucose homeostasis and hypoglycemia. Fructose and galactose must be converted into glucose by the enzyme G6Pase, which is present in the liver, kidney, and intestines.

The first GSD I patients had deficiencies in the hepatic glucose-6-phosphatase enzyme, which catalyses the final step of both gluconeogenesis and glycogen breakdown and functions inside the lumen of the endoplasmic reticulum and needs to cross the endoplasmic membrane to be efficient (MIM232200) (Bream, 2019). To establish the first metabolic disorder in which an enzyme defect was recognized, Cori and Cori2 described six comparable patients in 1952 and found that the lack of the enzyme glucose-6-phosphatase (G6Pase) caused von Gierke disease. Among the six individuals, only two showed a significantly low level of hepatic G6Pase, whereas the other four had normal

enzyme activity. In 1959, a subgroup of patients who did not have the standard glucose-6-phosphatase problem was described, and a few years later, a defect in the transport of gucose-6-phosphate was shown to exist. Therefore, the genuine enzyme malfunction is designated by the designation glycogen storage disease type Ia (GSD Ia), which describes the disorder. Thus, glycogen storage disease type Ia (GSD Ia) designates the real enzyme problem, while glycogen storage disease type Ib (GSD Ib) designates the transport defect. Deficient G6Pase activity in intact and disturbed microsomes indicates a defect in the catalytic unit, the actual G6Pase. However, most of the previously described patients were later discovered to have mutations in the gene encoding the glucose-6-phosphate translocase and therefore also belong to the GSD Ib group. The GSD Ic and GSD Id disease subtypes had also been proposed to be caused by an abnormal inorganic phosphate transport (MIM232220). When hypoglycemia causes neuro-endocrine stimulation, the liver in GSD I is unable to produce free glucose in response. The flaw causes a buildup of glucose-6-phosphate to enter glycolysis, which raises the formation of lactate (Bream, 2019).

Although hypoglycemia and lactic acidosis in newborns with GSD I may be detected, it is more typical for patients to first present at 3 to 4 months of age with hepatomegaly and/or hypoglycemic seizures (Bream, 2019).

Hypoglycemia, hyperlactacidaemia, hyperlipidemia and hyperuricemia are the most characteristic metabolic derangements of GSD I (Saudubray *et al*., 2012). GSD Although final height is frequently normal, I patients frequently have doll-like faces (because to fat deposits in the cheeks), small stature, and protuberant abdomens due to enlarged livers. The size of other organs does not expand, only the size of the kidneys does. When exogenous sources of glucose are used up during a fast, hypoglycemia sets in because the liver's lack of G6Pase activity prevents the completion of both the glycogenolytic and gluconeogenic pathways. There is evidence that GSD I patients can produce some endogenous glucose even when both endogenous glucose synthesis mechanisms are impaired (Collins *et al*., 1990). The mechanism behind this endogenous glucose production is still unclear. Fasting lactic acidosis and short fasting tolerance, which may be less than 2 hours, are defining characteristics of GSD Ia, however the latter improves with maturity.

The presence of hyperuricemia is caused by both decreased renal clearance and increased production of urate. Triglyceride, VLDL, and LDL production are all enhanced in hyperlipidemia, and peripheral lipolysis is diminished. Hypertriglyceridemia puts patients at an elevated risk for pancreatitis. Neutropenia and neutrophil malfunction in GSD Ib patients cause recurrent bacterial infections. Patients with GSD Ia and Ib have improper platelet aggregation and a propensity for excessive bleeding, respectively (Blundell *et al*., 2018). Untreated patients display short stature, a rounded 'doll face', protruding abdomen (marked hepatomegaly), truncal obesity, and wasted muscles (Saudubray *et al*., 2012) Symptoms of hypoglycemia (paleness, sweating, abnormal behavior, decreased consciousness, coma, convulsions) are usually accompanied by hyperventilation, a symptom of lactic acidosis. Long-term cerebral function seems to be normal if hypoglycemic damage is prevented. Other symptoms directly related to metabolic derangements are skin xanthomas, which are positively correlated with the degree of hyperlipidemia, and gout, which is associated with hyperuricemia but rarely develops before puberty (Mayatepek *et al*., 2010). Furthermore, they may suffer from episodes of chronic diarrhea (Milla *et al*., 1978). Patients with GSD Ib may have neutropenia and neutrophil dysfunction that predispose to frequent infections and IBD (Visser *et al*., 2000). In most individuals, the size of the kidneys is only slightly increased. Even in prepubertal patients, it is possible to see a condition known as "silent" glomerular hyperfiltration. There is a possibility that microalbuminuria and then proteinuria will develop, which will eventually lead to hypertension and end-stage renal disease. Also renal proximal and distal tubular functions may be impaired, especially in metabolically poorly controlled patients (Chen *et al*., 1988). Other complications that may develop are anemia, osteopenia, ovarian cysts, pancreatitis and pulmonary hypertension (Saudubray *et al*., 2012).Most problems' frequency, severity, and causation, as well as their connection to metabolic regulation, are still unknowns. Additionally, it's unclear how the majority of the difficulties will be handled.

Most of the problems' incidence, severity, and causation, as well as their relationship to metabolic regulation, remain obscure. Furthermore, it is unclear how to best treat the majority of these issues (Rake *et al*., 2002). There is evidence that apolipoprotein E (apoE) levels are elevated in patients with GSD1a who are hyperlipidemic, and this may provide

some protection against atherosclerosis (Trioche *et al*., 2000). Due to the overlap of presentations found in GSD1a and GSD1b, enzyme activity assay and/or mutation analysis of both G6PC and SLC37A4 can validate the first clinical presentation of fasting hypoglycemia, lactic acidosis, hyperuricemia, and hypertriglyceridemia (15,22). In a family with a known familial mutation, prenatal genetic diagnosis using chorionic villus sample was reported (QU *et al*., 1996).

1.13.2.2. Genetics and epidemiology

Mutations in the -glucose-6-phosphatase (G6Pase-) encoding the G6PC gene on chromosome 17p21.31 cause GSD1a, an autosomal recessive disorder. About one in every 100,000 people has GSD1, and GSD1a accounts for 80% of all cases. GSD1a has an estimated 1 in 20,000 occurrence rate among Ashkenazi Jews (Ekstein *et al*., 2004) No clear-cut genotype-phenotype correlation has been identified (Kishnani *et al*., 2014). Among the enzymes involved in glycogen synthesis and breakdown, G6Pase is unique since its active hydrolyzing site is situated inside the lumen of the endoplasmic reticulum (ER), whereas the other enzymes involved in glycogen metabolism are in the cytoplasm (Pan *et al*., 1998).

So, glucose-6-phosphate (G6P) is a substrate, and the end products are glucose and phosphate, all of which need translocation across the endoplasmic reticulum (ER). Arion proposed a model for the G6Pase system's role in cellular metabolism in 1975. The G6Pase system in this transport model includes the G6Pase catalytic subunit localised on the ER luminal surface and at least one membrane transporter (Pan *et al*., 1998; Waddell & Burchell, 1993).

While much research has been achieved, many concerns remain unsolved about the G6Pase system's precise working mechanism, including the amount of proteins involved, the stoichiometry of those proteins, the precise topology of the system, and whether or not its components form a complex (Scriver, 2001).

It wasn't until 1993 that researchers discovered the gene that codes for the catalytic unit of the G6Pase complex. It can be found in the region designated as band q21 on chromosome

17. It spans a genomic distance of 12.5 kb and is composed of 5 exons in total. It is estimated that the protein's molecular mass is between 35 and 40 kilodaltons, and it contains 357 amino acids (Lei *et al*., 1993).

More recently also the gene encoding the G6P transporter has been identified. It is in band q23 of chromosome 11. It consists of 9 exons (Annabi *et al*., 1998). In the *G6PC* gene (GSD Ia), 86 disease-causing mutations have been identified, while in the *SLC37A4* gene, 82 disease-causing mutations have been recorded (GSD Ib). The identified changes occur in several different regions of these genes. Missense and nonsense mutations, as well as frameshifting alterations caused by insertions and deletions of a few bases, splice-site alterations, and extremely rare instances of gene rearrangement, are among the mutation types found (Chou *et al*., 2002).

1.13.2.3. Diagnosis

The clinical presentation, a curated set of biochemical abnormalities, molecular genetic analysis, or enzymology in liver biopsy tissue all contribute to the diagnosis (Chen, 1995). Symptoms of hypoglycemia might emerge soon after birth, but most patients have no symptoms as long as they are fed often and the feedings contain enough glucose to prevent hypoglycemia (Cassiman *et al*., 2010).

Sometimes lactic acidosis can cause hyperpnea that looks like pneumonia. An enlarged liver and protuberant abdomen may be the first signs of the illness, which may not be diagnosed until the baby is several months old. Ultrasound imaging of liver is quite similar in GSDI and GSD III patients, however presence of nephromegaly and characteristic biochemical abnormalities provide clues of diagnostics (Lee *et al*., 1994). In females with GSD I, polycystic ovaries have been reported after the age of 4; however, this does not appear to have any effect on fertility (Martens *et al*., 2008). Pulmonic hypertension has also been described in GSD I cases (Humbert *et al*., 2002). Patients who are not being treated typically have murky or milky serum due to high triglyceride concentrations, moderately elevated phospholipids, total lipoprotein cholesterol, and low density lipoprotein cholesterol, and low amounts of high density lipoprotein (Goulart *et al*., 2010). Some patients with severe hyperlipidemia, particularly those with severe chronic

hypertriglyceridemia (>1,000mg/dl), are at risk for developing acute pancreatitis (Chen, 1995). Crohn disease-like enterocolitis is more common in patients with GSD Ib, and can occur in patients with Ia. Nephromegaly, easily seen on ultrasound, is a hallmark of GSD I. Untreated or improperly treated individuals may have proximal tubular dysfunction (glucosuria, phosphaturia, hypokalemia, and a widespread aminoaciduria). Reversal of proximal tubular dysfunction is possible through better metabolic disease management (Lee *et al*., 1995). Nephrocalcinosis and renal calculi are more likely to develop in patients with a distal renal tubular acidification deficiency coupled with hypocitraturia and hypercalciuria. Microalbuminuria, or increased albumin in the urine as a result of hyperfiltration, is a condition seen in diabetic individuals and may develop in adolescents and young adults with GSD I (Restaino *et al*., 1993). Hepatomegaly, hypoglycemia, and hyperlipidemia are among shared symptoms between GSD types I and III. Although GSD I and GSD III have many similarities, there are important distinctions between them. The symptoms of GSD I, severe fasting hypoglycemia within 3-4 hours after feeding, generally manifest in the first few months of life. Since gluconeogenesis is unaffected and the peripheral branches of the glycogen molecule can be mobilized by the action of hepatic phosphorylase, hypoglycemia in patients with GSD III is typically milder (Clayton, 2003).

The lipid profile of people with GSD I, which includes elevated triglycerides, is similar to that of people with hyperlipidemia type II. Those without obvious hepatomegaly should have their insulin, growth hormone, and cortisol levels checked, as well as their blood for lactate, uric acid, triglycerides, and cholesterol. Neonates and children with GSD I who have mild hepatomegaly may be mistakenly diagnosed and treated for growth hormone deficiency, infant screening should be tested since fatty acid oxidation disorders and galactosemia must be considered in the differential diagnosis.

1.13.2.4. Treatment

Better glycemic control has been shown to ameliorate metabolic abnormalities in almost all cases. Lipid-lowering medications, such as fibrates and HMG-CoA reductase inhibitors, are effective in treating hyperlipidemia. Since fructose and galactose cannot be broken down into free glucose, they are typically avoided in the diet. Allopurinol is taken to lower

uric acid levels. It is possible to treat microalbuminuria with low dosages of angiotensin converting enzyme inhibitors, and this condition serves as an early indicator of renal impairment. For patients with type Ib glycogen storage disorder, granulocyte colony-stimulating factor is used to improve neutropenia and neutrophil activity.

1.13.3. Glycogen Storage Disease type II (Pompe)

Type II glycogen storage disorder (GSD2), also known as Pompe disease or acid maltase deficiency, is caused by an inability to produce acid alpha glycosidase (GAA) and is located on chromosome 17. Glycogen accumulates intra-lysosomal in all tissues, making this a rare autosomal recessive multi-system condition (Strothotte *et al*., 2010). A separate infantile type exists from the adult-onset form of the disease, which was first characterized by Pompe in 1932. Infantile onset is quite rare (about 1 in 138,000 cases), but late-onset is nearly 1 in 57,000.

1.13.3.1. Pathophysiology

It is characterized by intra lysosomal accumulation of the normal glycogen due to abnormality of hydrolase exporting glycogen from the lysosomes. Because lysosomes only contribute about 3% to energy metabolism, hypoglycemia is not a hallmark of this disease. Instead, the destruction and accumulation within lysosomes causes cell injury and loss of the normal function, primarily affecting muscle metabolism and, in the infantile form, cardiac muscle.

1.13.3.2. Clinical features

GSD2 manifests clinically as a wide spectrum of phenotypes, from the severe fast advancing infantile form to the slowly progressive late-onset variant. Infants with the most severe form of cardiomegaly typically don't make it past the first two years of life if they aren't treated, and they usually die from heart failure if they have symptoms including frequent respiratory infections, weakness, and missed motor milestones. Testing for Pompe disorders is recommended for all cases of hypertrophic cardiomyopathy in infants and children (Strothotte *et al*., 2010). Motor delays, stumbles, clumsiness, waddling gait, and obstructive sleep apnea are all signs of diaphragmatic weakness in older children who are

unable to leap or climb stairs. Truncal weakness, sleep disruption, and nocturnal hypercapnia are the key symptoms that set this proximal myopathy apart from others in adult patients with progressive skeletal muscle weakness without heart involvement, but eventually leading to respiratory failure and scoliosis (Strothotte *et al*., 2010). The great adaptability depends to some range on the managing ability of the enzyme to degrade and store the excessive glycogen.

1.13.3.3. Genetics

In GSD2, a recessively inherited disorder due to the deficiency of acid α-glucosidase (GAA) enzyme encoded by *GAA* gene that results in impaired glycogen degradation and its accumulation in the lysosomes, the cell surface receptor is bound by the recombinant human enzyme (rhGAA), a massive 110-kDa precursor containing mannose-6-phosphate (M6P) groups. (Montalvo *et al*., 2006). Once inside, rhGAA acts like the endogenous precursor and is cleaved to give intermediate forms, which are then converted to the fully mature lysosomal species (Montalvo *et al*., 2006).

1.13.3.4. Diagnosis and Treatment

Acid -glucosidase activity in muscle samples, cultured fibroblasts, or purified lymphocytes is currently used to diagnose glycogen storage disorder II (Chamoles *et al*., 2004). Early treatment with enzyme replacement therapy yields the best results, although this approach does not result in a complete recovery. Early enzyme replacement therapy has been shown to reduce mortality as well as morbidity, and it has also been linked to significant increases in both survival rates and quality of life. Since 2006, enzyme replacement therapy, also known as alglucosidase alpha, has been on the market. It is recommended that patients with uncommon illnesses receive treatment at specialized centers. In the absence of treatment, the infantile form is linked to an increased risk of passing away, while the later onset type is related with an increased risk of morbidity and mortality. In both cases, there is an increased likelihood of passing away while under anesthesia; however, this is especially true for the infantile variant, which commonly reveals arrhythmias during medical procedures. In senior patients, it is usual for them to require supportive care for infections

as well as assisted ventilation and wheelchair use. Other innovative medicines are now being researched and developed, although they are not yet accessible on the market.

1.13.4. Type III Glycogen Storage Disease (Cori/Forbes)

Patients with GSD III have an accumulation of aberrant glycogen in their liver and muscles that has very short outer chain lengths. This is the disease's defining characteristic. The mode of inheritance for this disorder is autosomal recessive (MIM232400). Most individuals have insufficient levels of the debranching enzyme in both their liver and their muscles (GSD IIIa). Only the liver is affected in 15% of patients (GSD IIIb). Van Creveld, who described two patients in 1928, was the first person to record a case of glycogen storage disorder type III (GSDIII). He did so in 1928 (Snapper & Van Creveld, 1928). In following years, Van Creveld used enzymatic testing to demonstrate that the patients in question did, in fact, have GSDIII (Chamoles *et al*., 2004).

1.13.4.1. Pathophysiology

Because of this, glycogen was classed as phosphorylase-limited dextrin, and hence a lack of amylo-1,6-glucosidase was expected, was confirmed in 1956 (Illingworth *et al*., 1956). The last step in the transition of glycogen to glucose is catalyzed by the debranching enzyme, which has two active centers. A phosphorylase enzyme separates four glucose molecules from the glycogen molecule to generate limit dextrin before the debranching enzyme begins to act (Newgard *et al*., 1989). 1,4-glucan-4-D-glucosyltransferase, the first active centre of the debranching enzyme, moves the limit dextrin's outer three glucose molecules to another chain in undamaged cells. The final glucose molecule is released by amylo-1,6-glucosidase, the second active centre (Lei *et al*., 1995).

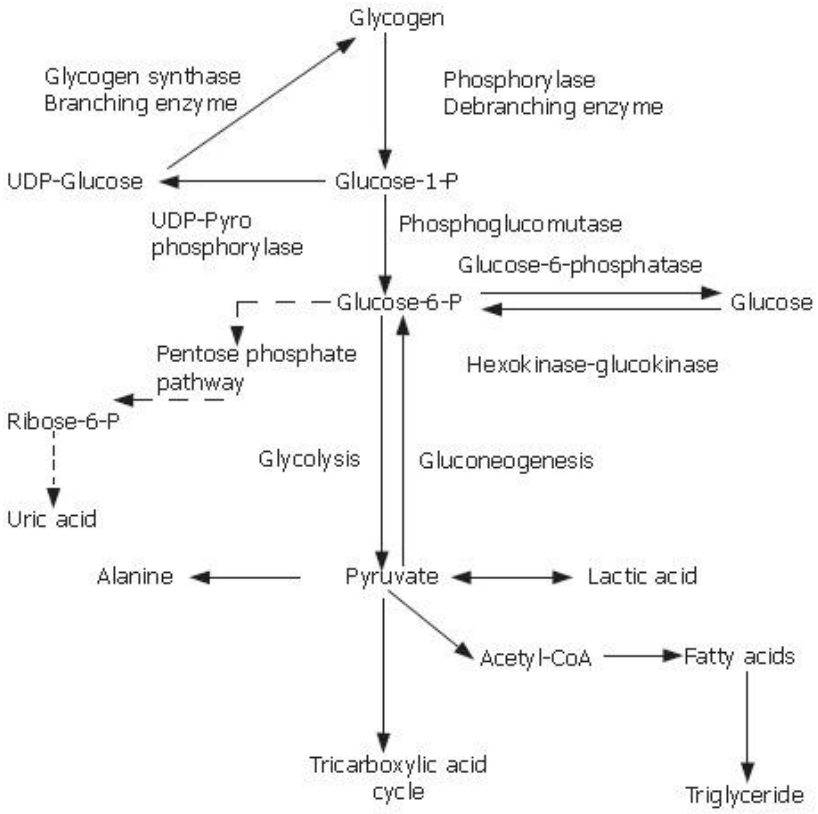


Figure 1.17. Adapted from (Özen, 2007).

1.13.4.2. Types of GSD III

There are four subtypes of GSDIII, but the most prevalent kind is type IIIa, which accounts for eighty percent of all GSDIII patients. This subtype of GSDIIIa is often referred to as the hepatic-myopathic subtype since it can affect liver tissue in addition to cardiac and skeletal muscle tissue. The other prevalent subtype of GSDIII is called GSDIIIb, and it affects about 15% of patients with GSDIII. Because in these cases only the liver is afflicted by GSDIII, this form of the disease is sometimes referred to as the hepatic subtype. It is extremely unusual for a *GDE* gene to suffer selective loss of either of its two functions, glucosidase, which causes GSDIIIc, or transferase, which causes GSDIIId.

1.13.4.3. Clinical features

Hepatomegaly, hypoglycemia, delayed growth, and hyperlipidemia are all features of GSD III in infancy and childhood that are essentially comparable to those of GSD I. Although

lactate and uric acid levels in the fasting state are normal during childhood, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) concentrations are elevated (Laforêt *et al*., 2012). Usually, liver transaminases are elevated. The kidneys may not be enlarged, but splenomegaly may be present. Most GSD III individuals' hepatomegaly and hepatic symptoms go better with age, but it's possible to develop progressive liver cirrhosis and failure. Severe muscle weakness is often first noticed in GSD IIIa patients during their third and fourth decade of life. Although ventricular hypertrophy that manifests itself as cardiomyopathy is a common finding, cardiac failure is quite uncommon. Adult liver biopsy samples have been revealed to have (periportal) hepatic fibrosis, and 10–25 percent of patients have hepatic adenomas (Labrune *et al*., 1997). There have been reports of severe cases of GSDIII patients who developed liver cirrhosis as well as complications connected to the condition including ascites, esophageal varices, and even hepatocellular cancer (Labrune *et al*., 1997; Okuda *et al*., 1998).

1.13.4.4. Genetics

This debranching enzyme deficiency is the underlying cause of GSDIII, an autosomal recessive genetic condition. The *AGL* gene, which is found on chromosome 1p21, is mutated in people with this impairment. This research by Bao *et al*. from 1996 states that the human *AGL* gene is 85 kb in size and contains 35 exons. In 1996, Bao *et al*. identified six isoforms with 5'-end differences due to the use of numerous cryptic splice sites upstream of the translation initiation site (Bao *et al*., 1996). The liver, (both skeletal and cardiac) muscle, and the kidneys all express isoform 1, but it is the most ubiquitous. Skeletal and cardiac muscle are the only tissues that have isoforms 2, 3, and 4. These isoforms originate from alternative splicing events or from transcripts that began at different transcription start sites. Exons 1 and 3 are found in isoform 1, while exon 2 marks the beginning of isoforms 2, 3, and 4. Exon 3 is shared by isoforms 1-4, and it contains the translation start codon expected in a protein synthesis process. Each isoform contains exons 4–35 (Shen *et al*., 1996). The active site is encoded by exons 6, 13, 14, and 15, while the glycogen binding site is encoded by exons 31 and 32 (Elpeleg, 1999). Multiple mutations have been reported in patients with GSD III. Patients with GSD III from the Faroe Islands have the c.1222C>T mutation (Santer *et al*., 2001), and c.4455delT in North African Jewish

patients (Parvari *et al*., 1997). Many truncated proteins result from missense and splice site mutations, minor deletions and insertions, and massive intragenic deletions and insertions. The c.4455delT mutation in the North African Jewish community generates a truncated protein that is about 97% of its length. These results demonstrate the importance of the carboxy terminus, which is located downstream of the glycogen binding site, for proper functioning of enzyme (Parvari *et al*., 1997). Mutations in exon 3 of one's AGL allele are responsible for GSDIIIb in affected individuals. Normal enzyme function is maintained in the muscles of people with GSDIIIb who lack exon 3, likely because alternate exons or translation start in muscle isoforms do not require exon 3 (Elpeleg, 1999).

1.13.4.5. Diagnosis and treatment

Diagnosing GSDIII requires either a mutation analysis or a measurement of debranching enzyme action in WBCs, muscle, and the liver cells. In order to identify the genetic condition, this can be done. During a liver pathology analysis, the enlarged hepatocytes rich in limit dextrin are visible beneath the microscope. Fibrosis and fibrous septa around the ports are also common, especially in mature specimens. Specimens taken from elderly individuals are especially valuable for this reason. In addition, a diagnosis can be made by showing aberrant glycogen in liver and/or muscle as well as a defective debranching-enzyme activity in the skin fibroblasts or lymphocytes (Jeyaraj *et al*., 2021). To prevent hypoglycemia episodes and reduce cholesterol and triglyceride concentrations, a diet that effectively cures GSDIII can improve metabolic balance (Gremse *et al*., 1990; Weinstein & Wolfsdorf, 2002). Since protein can serve as a substrate for gluconeogenesis, eating a high-protein diet may also help prevent hypoglycemia (Jeyaraj *et al*., 2021). The severity of hypertrophic cardiomyopathy in GSDIIIa patients has been shown to lessen, and in some cases reverse, with the use of a protein-rich diet, according to case studies (Dagli *et al*., 2009; Valayannopoulos *et al*., 2011). Consumption of galactose and fructose-containing products is unrestricted in GSDIII because these short glucose polymers are digested normally (Weinstein & Wolfsdorf, 2002). Liver transplant recipients have historically included those with terminal cases of cirrhosis and/or malignancy. Patients with progressive myopathy often require the use of wheelchairs because of the disease's inability to be reversed (Jeyaraj *et al*., 2021).

1.13.5. Glycogen Storage Disease Type IV (Andersen)

Due to a lack of branching-enzyme activity, GSD4 was first identified in 1956 as an autosomal recessive condition (MIM232500) (Geberhiwot *et al*., 2018). Glycogen storage disease type IV is characterized by the rapid development of liver cirrhosis and the eventual mortality of the affected kid. There's an early-onset cardiopathic form, a middle-aged myopathic form, and a late-onset neurodegenerative form (adult polyglucosan body disease) (Tay *et al*., 2004). Liver tissue from people with GSD type IV has an abnormal glycogen structure that resembles amylopectin because of its reduced number of branch points. There is some evidence for a genotype phenotype association between the hepatic and neuromuscular variants of GSD IV, both of which are caused by mutations in the same GBE gene. This occurs so infrequently that there is no recorded incidence.

1.13.5.1. Pathophysiology

During the synthesis of glycogen, GBE [1, 4-D-glucan: 1, 4-D-(1, 4-glucano-transferase] transfers a chain of at least six -1, 4-linked glycosyl units into a -1, 6 position in collaboration with glycogen synthase (Bannayan *et al*., 1976). Buildup of a structurally aberrant, less branching form of glycogen (like amylopectin) occurs in tissues where GBE is deficient (Bannayan *et al*., 1976).

1.13.5.2. Clinical features

GSD type IV is described by a high degree of clinical heterogeneity about the age at which symptoms first appear, as well as by variety in the pattern and degree to which organs and tissues are affected. The condition often manifests itself in infancy, with symptoms including failure to grow, hepatosplenomegaly, and progressive liver cirrhosis that ultimately results in mortality in early childhood. Patients are not likely to develop fasting hypoglycemia since this condition only manifests itself in the context of severe cirrhosis. There are several neuromuscular types of GSD IV, and they can be categorized as perinatal, congenital, childhood, or adult forms, according on the age at which symptoms first appear and the degree to which the disease has progressed. Fetal akinesia deformation sequence (FADS) is the most severe manifestation of this trait, manifesting prenatally and leading to

early death from cardiac or pulmonary failure, arthrogryposis, hydrops, polyhydramnios, and pulmonary hypoplasia. Myopathy or APBD (adult polyglucosan body disease) affecting the central and peripheral nerve systems is related with later-onset (juvenile/adult) phenotypes (Shin, 2006; Sindern *et al*., 2003).

1.13.5.3. Genetics

Lack of glycogen-branching enzyme is the underlying cause of the disorder (GBE, EC 2.4.1.18) (Brown & Brown, 1989) It is coded for by *GBE1* gene on 3p14 (Thon *et al*., 1993).

1.13.5.4. Diagnosis and Treatment

Clinical symptoms are used to diagnose GSD IV, together with histology and electron microscopy outcomes in muscle or liver, and in vitro evidence of *GBE* deficiency in liver, muscle, or skin fibroblasts (Brown & Brown, 1989). DNA mutational analysis can be used as a supplement to enzyme activity tests, particularly in prenatal foetal samples with inconclusive outcomes, and in those whose residual enzyme activity is higher than the average, which overlaps with heterozygote levels (Akman *et al*., 2006). An abnormality in glycogen and a lack of branching enzyme in liver, muscle, leukocytes, erythrocytes, or fibroblasts must be shown via biopsy to confirm a diagnosis of type IV illness. The glycogen-branching-enzyme gene is amenable to mutation study.

Although there is currently no cure for GSD IV, some individuals may see improvements in liver function and muscle strength with maintaining normoglycemia and receiving an adequate diet (Geberhiwot *et al*., 2018). People with progressive hepatic variant of GSD IV who develop liver failure have just one therapy option: liver transplantation. Most patients die as children, mostly from complications related to the liver failing or to cardiomyopathy and neurological dysfunction. Prevent nutritional defects (e.g., of fat-soluble vitamins) by ensuring adequate dietary intake (Magoulas & El-Hattab, 2019).

1.13.6. Glycogen Storage Disease Type V (McArdle)

A myopathy first identified by McArdle in 1951, In most cases, mutations in the *PYGM* gene (which codes for the muscle isoform of glycogen phosphorylase) are to blame for this disorder (MIM 232600). Those afflicted have almost minimal enzyme activity and are hence unable to convert glycogen to glucose in muscle.

1.13.6.1. Clinical features

Muscle soreness in young children is often disregarded since it is difficult to establish a definitive family history of myoglobinuria. Due to the lack of discomfort, parents rarely mention their child's black urine in the list of worrying symptoms in the clinical history. Second wind phenomena, in which a patient begins an activity but is forced to stop owing to pain, is also commonly connected with this illness. This is a condition whose symptoms get more pronounced with time. Mild discomfort in the muscles progresses to more severe symptoms including cramping and myoglobinuria. acute onset of rhabdomyolysis and renal failure. In the elderly, the entire picture includes a gradual but steady decline in strength and an increase in handicap. Increased muscle irritability or nonspecific myopathic alterations may be detected by electromyography. In most cases, creatine kinase levels are just slightly increased, and the condition is diagnosed based on incidental signs. Disability weakness and in older patients share many similarities with the other muscular disorders and rhabdomyolysis is linked with certain viral infections or drugs.

1.13.6.2. Genetics

One in every 100,000 people will get this autosomal recessive disorder (Santalla *et al*., 2017). Chromosome 11 is home to the myophosphorylase gene. A lack of myophosphorylase or its inactivity caused "a severe failure of the breakdown of glycogen to lactic acid," which defined the condition (McArdle, 1951). Even though many mutations have been described, As of yet, not a single mutation in any given genotype has been linked to a defined phenotype (Santalla *et al*., 2017).

1.13.6.3. Pathophysiology

About 90 percent of patients lack immunologically reactive myophosphorylase protein in muscle (McConchie *et al*., 1990). Muscle phosphorylase defect (McArdle's disease) has

normally been considered a disorder of glycogenolysis (Lewis & Haller, 1986). This leads to leads to exercise intolerance and exercise-induced myalgia (De Stefano *et al*., 1996). Myophosphorylase is an enzyme that breaks glycogen to lactic acid is isoform of Glycogen phosphorylase enzyme. Glycogen phosphorylase is a large family that includes 3 different isoforms: PYGB in the brain, PYGL in the liver, and PYGM in the muscle (Johnson, 1992). At exercise intensities requiring greater than 75–80% of maximum O2 absorption, muscle glycogen is typically the major oxidative fuel. McArdle patients with their oxidative impairment and indicate that a metabolic common denominator in these abnormal responses may be a pronounced decline in the muscle phosphorylation potential. Excessive release of potassium, phosphate, adenosine, or all three during exercise likely mediates the hyperkinetic circulation through local effects on metabolically sensitive afferents and vascular smooth muscle. McArdle patients does not appear to be due to depletion of ATP but is linked with an increased buildup of Pi and probably ADP in skeletal muscle. Deposits of Pi and ADP are known to inhibit the myofibrillar, Ca2+, and Na+-K+-ATPase reactions (Chasiotis, 1983). On the other hand, the liver isoform hydrolyzes glycogen from internal liver storage to free G1P, which helps to keep glucose levels in the blood at a healthy level. The activation methods also vary; whilst reversible phosphorylation of S15 is required for activation of the liver isozyme, both of the other isoforms can also be controlled via allosteric processes (Hue *et al*., 1975).

1.13.6.4. Diagnosis and Treatment

The majority of diagnoses are made in the twenties and thirties, while a thorough history may reveal symptoms as early as the early childhood years. Myophosphorylase deficiency can be detected in muscle biopsy samples or by molecular analysis to provide a diagnosis. Many diseases are linked to alterations in the vacuoles of muscle fibers, and these stains are necessary to tell them apart. Muscle soreness, weakness, and an increased creatine kinase level can indicate several different conditions, including common ones like muscular dystrophy and less common ones like Danon's disease.

Unfortunately, there is currently no cure, although some food and lifestyle changes may help. Even mild exercise has a positive effect on the aerobic threshold, which is lowered

by excessive weight gain. This condition has a rather benign long-term outcome as significant illness and mortality is correlated with rhabdomyolysis and acute multi organ failure and an uncommon neonatal form causing the respiratory failure, but both these two problems are rarely seen. More frequently indications and loss of function over time is restricted by ability. Certain anesthetic agents provide a higher risk profile than others; if the anesthetist is aware of this, he or she can adjust the medication accordingly. There is no additional danger during pregnancy, and genetic counselling is an option in most circumstances (Geberhiwot *et al*., 2018).

1.13.7. GSD Type VI (Hers)

GSD VI also called Hers disease is caused by the lack of glycogen phosphorylase (MIM232700) and was identified in 1959, has an incidence of 1/60,000-85,000.

1.13.7.1. Clinical features

This condition usually presents along with the hepatomegaly and growth retardation early in childhood. Ketotic hypoglycemia and hyperlipidemia are usually mild if present. Lactic acid and uric acid are usually normal. The heart and skeletal muscles are not affected, and the hepatomegaly progresses with age and usually disappears around puberty.

1.13.7.2. Genetics

Heritable deficiencies of phosphorylase cause glycogen-storage (Lei *et al*., 1995). Xp22.1-22.2 is associated with the X-linked variant of GSD6 (Willems *et al*., 1991).

1.13.7.3. Pathophysiology

For glycogen to be broken down into glucose 1-phosphate, glycogen phosphorylase must first phosphorylate the -1,4-glycosidic bonds that hold it together. Using glycogen, a form of glucose storage, necessitates the presence of the enzyme phosphorylase, which is present in nearly all mammalian cells. Glycogen is found in the highest concentrations in the liver and the muscle. Glycogen functions as a buffer reserve of glucose units that helps keep blood glucose levels stable in the liver. Glycogen in the muscle, on the other hand, may be swiftly broken down to supply energy for contractions. The activity of phosphorylase is

controlled by several different allosteric ligands in addition to being regulated by phosphorylation by phosphorylase kinase, which is in turn controlled by neurological and hormonal signals.

1.13.7.4. Diagnosis and Treatment

It is possible to diagnose through mutation analysis of the gene for liver phosphorylase. Preventing hypoglycemia with a high-carbohydrate diet and frequent feeding is useful, but most individuals do not need any special therapy, and asymptomatic hepatomegaly is the most prevalent result.

Patients affected by this condition will show signs of hepatomegaly and abdominal distension if they are not treated within the first year of life. Negative emotions and behaviors such as anger, irregular sweating, difficulty waking up in the morning, and slow linear growth are further symptoms and indicators. Results from the laboratory indicate abnormalities, such as ketosis with mild hypoglycemia, increased AST and triglycerides, and abnormal cholesterol levels. Serum glucose does not rise in response to glucagon (Lei *et al*., 1995).

1.14. Intrahepatic Cholestasis OF Pregnancy

1.14.1. Cholestasis

Cholestasis is a pathological condition in which the liver's ability to produce, secrete, or excrete bile is compromised, resulting in an overabundance of bile entering the duodenum and blood. Patients affected by this condition often exhibit symptoms such as pruritus, weariness, darker urine, and jaundice. Patients in the early stages of cholestasis are often asymptomatic and only present with abnormally high levels of serum alkaline phosphatase (ALP) and gamma- glutamyl transferase (GGT). Liver cirrhosis, liver failure, and even mortality can result from hyperbilirubinemia, which can occur as the disease advances (Kuntz, 2006). Cholestasis itself exacerbates liver damage in people with cholestatic liver disorders, which are hepatobiliary illnesses with cholestasis from diverse origins.

1.14.2. Etiology and classification

Bile is secreted by the liver cells called hepatocytes and choanocytes at a rate of roughly 600 mL per day in a healthy adult. Choanocytes also make bile, albeit at a lower rate (about 150 mL/day) than the liver does (approximately 225 mL/day). Cholestasis is a condition in which bile production or production of bile is impaired; this impairment may have intrahepatic or extrahepatic causes (Lu, 2022).

Intrahepatic cholestasis, caused by a problem with the hepatocytes, bile canaliculus, canals of Hering, bile ductule (15 m), or choanocytes of the interlobular bile duct, is characterized by a lack of imaging evidence for bile duct obstruction (15 to 100 m). Major causes include things like drug and alcohol abuse, contagious infections, compromised immune systems, and other comparable things.

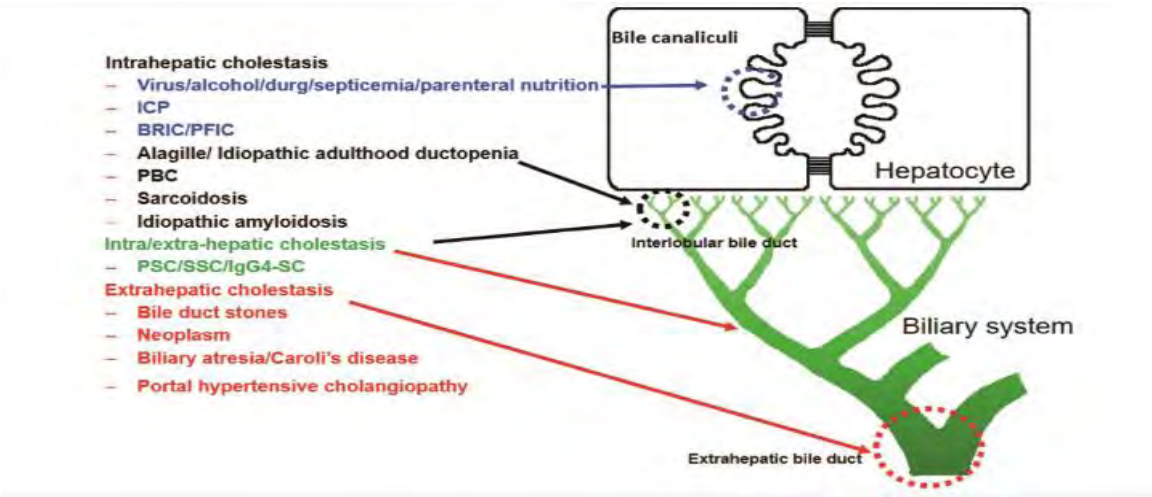


Figure 1.18. Causes and locations of cholestatic liver damage.

PFIC: progressive familial intrahepatic cholestasis; PBC: primary biliary cholangitis; IgG4-SC: IgG4-related sclerosing cholangitis; PSC: primary sclerosing cholangitis; and SSC: secondary sclerosing cholangitis.

Injury or blockage of any of the bile ducts outside of the liver, including the common bile duct to the ampulla, the left or right hepatic ducts, or the septal bile duct (>100 m), the regional bile duct (300 to 400 m), or the segmental bile duct (400 to 800 m), causes extrahepatic cholestasis (Wu *et al*., 2021). Even while extrahepatic factors have a larger role, biliary malignancy that spreads into the intrahepatic bile duct or the hilar bile duct can

also trigger this condition. Acute cholestasis is typically caused by bile duct stones, pancreatic cancer, or a benign biliary stricture (Hilscher *et al*., 2020). A diagnosis of chronic cholestasis is made when the condition has persisted for longer than six months. Clinicians need to be able to tell the difference between extrahepatic and intrahepatic cholestasis, but doing so based on symptoms, signs, and biochemical data alone can be challenging. Instead, a thorough diagnostic technique is required to differentiate between these disorders. Some patients with PSC can have intrahepatic or extrahepatic lesions, as this disorder can affect both small and big intrahepatic bile ducts (Liver, 2009).

1.14.3. Epidemiology

There is currently a lack of statistics regarding the incidence of cholestatic liver disorders (Bortolini *et al*., 1992). Cholestasis is more common in individuals with PBC and PSC and was present in 882 (35% of 2,520) patients with newly diagnosed chronic liver disorders. Five hundred and sixty-six percent of the patients in a study of a thousand with chronic viral hepatitis had raised GGT or ALP at discharge, and this elevation was linked to an increased risk for and severity of liver fibrosis and cirrhosis. The total incidence of cholestasis was found to be 10.26% by Cao *et al*., who surveyed 4,660 patients hospitalised for chronic liver illnesses in Shanghai. The incidence of cholestasis rose with patient age (Cao *et al*., 2015).

1.14.4. Clinical manifestations

Clinical symptoms from cholestasis might occur independently of those brought on by the underlying disease, and bile abnormalities can bring about additional complications. In the earliest stages of the disease, a patient may experience either no symptoms at all or vague signs including weakness, loss of appetite, nausea, and abdominal pain. Jaundice, pruritus, weariness, steatorrhea, xanthoma, and hepatic osteodystrophy are some of the most common symptoms of cholestasis (Cao *et al*., 2015).

1.14.5. Biomarkers

ALP, GGT, bile acid, bilirubin, and many molecular markers are the most widely used indicators for cholestasis. Symptoms of early cholestasis include an increase in ALP and

GGT levels. The accumulation of bile salts in cholestasis is hypothesized to stimulate the production of ALP and GGT, which in turn causes the proliferation of tiny bile ductule. It is not well understood how ALP and GGT get access to the bloodstream and why their levels rise in cholestasis. Abnormally high levels of bile excretion are caused by the increased internal pressure of the bile canaliculus and ductule, which in turn stimulates the formation of ALP. Serum ALP is already elevated, and bile acid's surface activity dissolving ALP from lipid membranes may contribute to this (Siddique & Kowdley, 2012). In addition to being elevated in individuals with bone disorders and certain cancers, ALP can also be elevated during pregnancy, during normal childhood growth, and during puberty. GGT increases before and for a longer period of time than other blood enzymes in these patients. The GGT enzyme in the liver has the highest diagnostic sensitivity for cholestasis, but it also has a low specificity. GGT has comparable or higher sensitivity and specificity to ALP for the diagnosis of cholestasis. When other potential causes of liver injury (alcohol, infection, etc.) have been ruled out, damage to hepatocytes and choanocytes can be deduced from an elevation in both ALP and GGT. The bile canaliculus and choanocytes have been damaged if GGT is increased but ALP is not. An high ALP without a corresponding increase in GGT suggests that liver damage are unlikely. However, in certain cholestatic liver illnesses such familial intrahepatic cholestasis (FIC types 1, 2, 4, 5, and 6) and USP53 deficient disease, the levels of combined bilirubin or bile acid are elevated but GGT is normal or often lowered (Vitale *et al*., 2019).

1.14.5.1. Bile acids

For the diagnosis of bile secretion dysfunction, bile acid is more sensitive than bilirubin but less sensitive than ALP. Elevated serum bile acid is seen in patients with a wide variety of liver disorders. Serum bile acid levels should be between 1.0 and 6.0 mol/L while fasting and 6.0 and 9.0 mol/L during the postprandial phase (2 hours after eating). Patients with cholestasis may have bile acid levels higher than 10 mmol/L; mild elevation is defined as 20–40 mmol/L, moderate elevation as 10–20 mmol/L, and severe elevation as >40 mmol/L (Kuntz, 2006).

1.14.5.2. Bilirubin

Serum bilirubin, especially direct bilirubin, can rise due to cholestasis. Direct bilirubin elevations are typically more noticeable than indirect bilirubin elevations due to hepatocellular damage-induced anomalies in bilirubin up nutrients, their storage, and supply of nutrients to the other organs of body (Ramadori & Cameron, 2010). Apart these, liver act as metabolic synthesis, conjugation, and excretion. Hereditary disorders like Gilbert's syndrome or hemolytic anaemia can cause an elevation of bilirubin without also affecting liver enzyme levels (Siddique & Kowdley, 2012). The liver is largest organ of body, positioned between the heart and gastrointestinal tract, between the portal and the general circulation. The major function of liver is taking site for drugs and target for toxicity resulting from drugs, that helps in regulation of homeostasis and pathology. For these reasons, liver can be easily susceptible to diseases (Beckwitt *et al*., 2018).

In past decades, Bile acid disorder (BASD), a form of genetic disorder is considered to be major cause of cholestasis (1%-2%) diseases and the cause can be intrahepatic or extra hepatic. Cholestasis is a disease mainly caused due to accumulation of bile acids resulting from impair bile flow or stoppage or slowing of bile secretion and cause liver injury. The major cause of cholestasis is formation of gall stones or tumors scientifically referred as obstruction of extrahepatic bile duct. These types of diseases have visible effect on human genetics and possess multifactorial etiology (Henkel *et al*., 2021).

Obstetric cholestasis which ensues during third term of pregnancy is unusual liver disorder generally characterized by onset of itching and impaired bile flow (Lammert *et al*., 2000). The first case of disease was reported in 1883. The condition resolves immediately or within weeks after pregnancy (Kreek, 1987). Several Scandinavian clinicians reported few cases of pruritus and associated jaundice in obstetric patients, but the clinical details of disease were not described until mid 1950's. Sixty cases were presented in reports from different medical centers of Sweden between 1957-1959. These cases were categorized as “Recurrent jaundice of pregnancy”, “Benign Cholestasis of Pregnancy” and “Jaundice in late pregnancy” due to appearance of symptoms during third trimester and in successive pregnancies (Kreek, 1987).

1.15. Epidemiology and Etiology of ICP

One of the most frequent liver disease linked with gravidity is intrahepatic cholestasis. Although symptoms subside on their own after birth, between 45 and 90 percent of women have a worsening of their illness in successive pregnancies. The prevalence of ICP varies by both ethnic group and location (Lee & Brady, 2009).

The prevalence rate among Poles is predicted to be between 1% and 4%. Among indigenous Andeans, the prevalence is up to 25%. Approximately 2%-4% of pregnant women in Europe, 1%-2% in North America, and 1%-2% in Australia are diagnosed with ICP. Multiple pregnancies and advanced maternal age are both associated with an elevated risk of morbidity (Floreani & Gervasi, 2016). Incomplete understanding of the causes of ICP has led researchers to focus on genetic, hormonal, and environmental variables. Mutations in the multidrug resistance protein 3 (MDR3), a hepatobiliary transport protein involved in biliary production of phospholipids, are thought to have a significant role in the etiology of ICP (Jacquemin & Cresteil, 1999). On average, MDR3 mutations are found in 16% of ICP cases; they are also associated with more severe illness and TBA concentrations of 40 mol/L or higher (Jacquemin & Cresteil, 1999).

Multidrug resistance-related protein 2 is another transport protein that contributes to ICP development (MRP2). The association between MRP2 and disease risk was identified in a population of South American women but not in a Caucasian population (Meier *et al*., 2008). The gene encoding the protein BSEP may also be mutated. Drug-induced hepatitis or ICP has been linked to a decrease in bile salt export pump BSEP (bile salt export pump) protein levels, which in turn is caused by a change in the amino acid at position 444 (Lang et al., 2007). ICT is strongly linked to the BSEP gene mutation, as shown in recent large-scale investigations of 563 women with ICP (Dixon *et al*., 2014). Rare mutations within the *FIC1* gene (*ATP8B1*) occurring within the bile duct membrane and the *FXR* gene (*NR1H4*) were also detected in Caucasian patients diagnosed with intrahepatic cholestasis of pregnancy, in addition to those in MDR3 and BSEP (Van Mil *et al*., 2007). Hormonal steroid metabolites may be responsible for mutations in these genes. The higher rates of intrahepatic cholestasis of pregnancy (ICP) seen in pregnancies with more than one baby

or in women taking oral contraceptives provide further evidence that sex steroids have a role in the etiopathogenesis of ICP. Corresponding with the natural history of ICP, oestrogen, progesterone, and their metabolite concentrations rise throughout pregnancy, peak in the third trimester, and decline after birth (Geier *et al*., 2003).

ICP pregnant ladies have significantly greater amounts of progesterone sulphate and disulfate compared to healthy pregnant women. The liver is responsible for breaking down steroid hormones used in sex. A number of research established that pregnancy hormones affect bile acid metabolism, but many of these experiments were unethically conducted on animals (mostly mice) (Abu-Hayyeh *et al*., 2013). Damaging effects of glucuronates, estrogens, and progesterone on the function of hepatobiliary transport proteins involved in the excretion of bile acids into the hepatic bile ducts were observed in vitro. The bile acid ratio between hydrophilic and hydrophobic may shift as a result, with the hydrophobic acids becoming more prevalent. In turn, this hinders the placental transport of bile acids that are water-soluble and their subsequent excretion by the mother's kidneys. The maintenance of equilibrium within the maternal-fetal pool of bile acids likely plays a particularly important role in the pathogenesis of ICP. In a normal pregnancy, bile acids are transported from the fetus to the mother, whereas in a In a high-risk pregnancy for ICP, transplacental transport happens in the reverse way. As a result, both the mother and the developing child have elevated bile acid levels. Damage to the liver and other tissues can be attributed to apoptosis and oxidative stress, both of which are triggered by high amounts of TBA (McIlvride *et al*., 2017).

1.16. Diagnosis of ICP

In case of unidentified reasons, Intrahepatic Cholestasis of pregnancy shows symptoms of pruritus and increased values of liver function tests.

1.16.1. Clinical outcomes

1.16.1.1. Liver Test

Liver functions do not alter in normal pregnancy. While in ICP liver function tests are performed to trace any liver dysfunction. Alanine Transaminase (ALT) and Aspartate

Aminase ASP levels are elevated. Transaminases are intrahepatic enzymes. Elevated serum levels indicate hepatic injury, while ALT is more sensitive biomarker. Gamma-glutamyl transferase (GGT) levels also increase in some cases. Cholesterol, triglycerides, alkaline phosphatases, nucleosidase, and lipoprotein-X become detectable. In 10% cases, Bilirubin is increased (HEIKKINEN, 1983). Alkaline phosphatase level decline within a week or a month after delivery.

1.16.1.2. Pruritus

There is onset of pruritus is in third trimester of pregnancy in 80% ICP cases (Kenyon *et al*., 2002). While rest 20 % cases might have early onset of pruritus. Pruritus initiates on palms and soles, and spreads to limbs and trunk. Among other dermatological problem, it is indicative of the obstetric cholestasis. However, due to severity of pruritus rashes may occur. Pruritus exacerbates as pregnancy advances. Lesion and rashes are not uncommon, Women have reported increase in itching at night, resulting in insomnia (Geenes & Williamson, 2009). In in vitro studies of animal model, individual bile acids or lysophosphatidic acids (LPA) has caused itching, while ICP patients also have elevated level of these pruritogen (Kremer *et al*., 2014). LPA and autotoxin are major potential therapeutic target for pruritus (Kremer *et al*., 2010).

1.16.1.3. Serum bile acid

The most specific biomarker of ICP is Increase in total serum bile acid (HEIKKINEN, 1983). In every one out of six patients of ICP, mild jaundice is observed along with excretion of bile acid in urine. The less severe form of ICP is onset of pruritus without any lesions and normal level of serum bile salt level, i.e. ‘pruritus gravidarum’ (Kreek, 1987) The normal reference range for total serum bile acids in pregnancy is between 10 and 14 μmol/L (Geenes & Williamson, 2009). The level of serum bile acids is reported 100 times high than the upper limit of normal in some cases (HEIKKINEN, 1983). Primary diagnosis of ICP is characterized by exclusion of other causes of pruritus and liver abnormalities. Absence of acute liver infection further supports the diagnosis of ICP.

1.17. Prevalence

The prevalence of ICP varies between 0.2%- 2%, showing variations among different populations indicating involvement of genetic, nutritional and environmental factors. (Wikström Shemer & Marschall, 2010). In Pakistan, a recent study conducted at CMH Kharyan in 2016, showed 3.1% incident rate of ICP (Hafeez *et al*., 2016). In another study, among obstetric patients affected with liver abnormalities, 6% had ICP (Hussein, 2013). It varies not only in different populations but differ in cases of same population also.

Baltic States, Chile, Scandinavia, and Bolivia shows highest incidents with up to 15%. Study of Indian population,0.79% prevalence was reported (Dang *et al*., 2010).

1.18. Causes of Intrahepatic Cholestasis of Pregnancy

1.18.1. Environmental Factors

Environmental factors have an impact on ICP. Low Selenium plasma levels, caused by a lack of selenium in the diet, were linked to ICP in Chile, whereas increasing selenium levels reduced the incidence of ICP. There was also a seasonal change in the sickness. In comparison to other seasons, ICP incidence was lower in the summer when selenium levels were relatively greater (Reyes & Sjövall, 2000). During the winter, there is a higher incidence of ICP, which is also associated with a deficiency in vitamin D. Vitamin D deficiency in pregnant women leads to meconium staining and, as a result, fetal discomfort (Wikström Shemer & Marschall, 2010). Vitamin D modulates bile acid detoxification by attaching to its receptor.

1.18.2. Genetic basis

In many cases, six obstetric cholestasis-affected girls have been reported in pedigrees of large consanguineous families (Jacquemin & Hadchouel, 1999). According to the study, 9 percent of 65 index cases of ICP had parous sisters, and 11% had moms with ICP history. In a family of five generations, three sisters were impacted by the disease. Mendelian dominant mode of inheritance is demonstrated by parent to offspring transmission and total penetrance (Hirvioja & Kivinen, 1993).

Variations in disease among ethnic groups point to the role of population-specific genetic risk factors. For example, in the United Kingdom, prevalence is 0.7 percent, while in Sweden, prevalence is 2–3 percent (Dixon & Williamson, 2008).

1.18.2.1. Genetic variations

Progressive familial intrahepatic cholestasis (PFIC), familial cholestasis syndromes, and benign recurring intrahepatic cholestasis are some of the genetic components in the pathogenicity of ICP that have been studied (BRIC). Different forms of progressive familial intrahepatic cholestasis have been linked to variations in the genes encoding the farnesoid X receptor, the bile acid receptor, ATP8B1 (FIC1), the bile salt export pump (BSEP), and *ABCB4* phospholipid translocator (MDR3) (Dixon & Williamson, 2008).

1.18.2.2. *ABCB4*

Phosphatidylcholine floppase, also known as multidrug resistance protein 3, is encoded by this gene. Phosphatidylcholine is exported over the canalicular membrane. Low phospholipid-associated cholelithiasis (LPAC) and progressive familial intrahepatic cholestasis type 3 (PFIC3) (Rosmorduc & Poupon, 2007) and intrahepatic cholestasis of pregnancy (ICP) (Müllenbach *et al*., 2005) are two cholestatic liver illnesses with onset in childhood or middle age. Homozygous mutations in *ABCB4* genes cause PFIC3 (Dixon & Williamson, 2008). One patient with PFIC and high levels of gamma-glutamyl had a 7-bp deletion at codon 132, while the other had a nonsense mutation at codon 957 (C> T), with both mutations downstream of the stop codon (De Vree *et al*., 1998). In another investigation, 16 distinct mutations in MDR3 were discovered in 17 PFIC patients (Jacquemin & Hadchouel, 1999).

ABCB4 mutations are responsible for 15% of all ICP cases. A shortened protein was found in an ICP-affected mother of a kid with a PFIC heterozygous missense mutation in MDR3 (1712delT) (Jacquemin & Hadchouel, 1999). In a large cohort of 227 cases, the synonymous variation rs2109505 (c.711 A > T, p.I237I) revealed a significant correlation with ICP (Dixon & Williamson, 2008). *ABCB4* mutations that cause ICP have been researched extensively.

Table 1.6. Showing reported mutations in *ABCB4* gene worldwide**.**

Exon of *ABCB4*	Mutation	Reference
Exon 8 **Exon 6**	c.711A>T c.504 T>C	(Wasmuth *et al.,* 2007)
Exon6 **exon 8**	481G>A (R150K). (743A>T	(Müllenbach *et al.,* 2003)
Exon14	1712delT	(Jacquemin *et al.,* 1999)
Exon 14	E528D G536R R549H	(Floreani *et al.,* 2008)
Exon 6 **Exon 8** **Exon 15**	c.504 T>C c.711A>T). c.1769 G>A R590Q	(Bacq *et al.,* 2009)
Exon 6 **Exon15** **exon 14** **Exon 16**	c. 523A>G T175A c.1769 G>A R590Q c. 1615G>A A539T c. 1633A>G R545G c. 1576G>T V526F R652G	(Ziol *et al.,* 2008)

1.18.2.3. ATP8B1

The ATPase phospholipid transporter 8B1 (*ATP8b1*) gene has 28 coding exons and is found on chromosome 18q21. FIC-1, a 140kD P-type ATPase, is encoded by this gene. The pancreas, various epithelial tissues, the small intestine, and the liver all express it. It was discovered in children with PFIC for the first time (Bull *et al*., 1998). *ATP8B1* mutations have been linked to a variety of illnesses, including PFIC, BRIC, and ICP.

Clinical severity is related to the kind of mutation or its position whether they are frameshift deletion or point mutations (Klomp *et al*., 2004). Mutations in the *ATP8B1* gene have also been found in patients with intrahepatic cholestasis (Müllenbach *et al*., 2005).

1.18.2.4. *ABCB11*

The ATPase phospholipid transporting 8B1 (*ATP8b1*) gene is found on chromosome 18q21 and contains 28 coding exons that encode FIC-1, a 140kD P-type ATPase that is expressed in the pancreas, several epithelial tissues, the small intestine, and the liver. It was

first discovered in children with PFIC (Bull *et al*., 1998). *ATP8B1* mutations have been linked to a variety of diseases, including PFIC, BRIC, and ICP.

The severity of a mutation is determined by its kind and location, missense mutations are reported more common in BRIC (Klomp *et al*., 2004). Mutations in the *ATP8B1* gene have also been found in patients with intrahepatic cholestasis (Müllenbach *et al*., 2005).

1.19. Disease Mechanism

1.19.1. Synthesis of Bile and transport

Intrahepatic cholestasis is a liver condition that occurs only during pregnancy, and it is a complex illness. In genetically predisposed women, a high dose of female sex hormone during pregnancy disrupts the bile production and flow cascade. These hormones are processed in the liver, and their metabolites interfere with the activity of canalicular transporter proteins, resulting in bile acid buildup in the blood. The following is a description of the bile synthesis and flow mechanism:

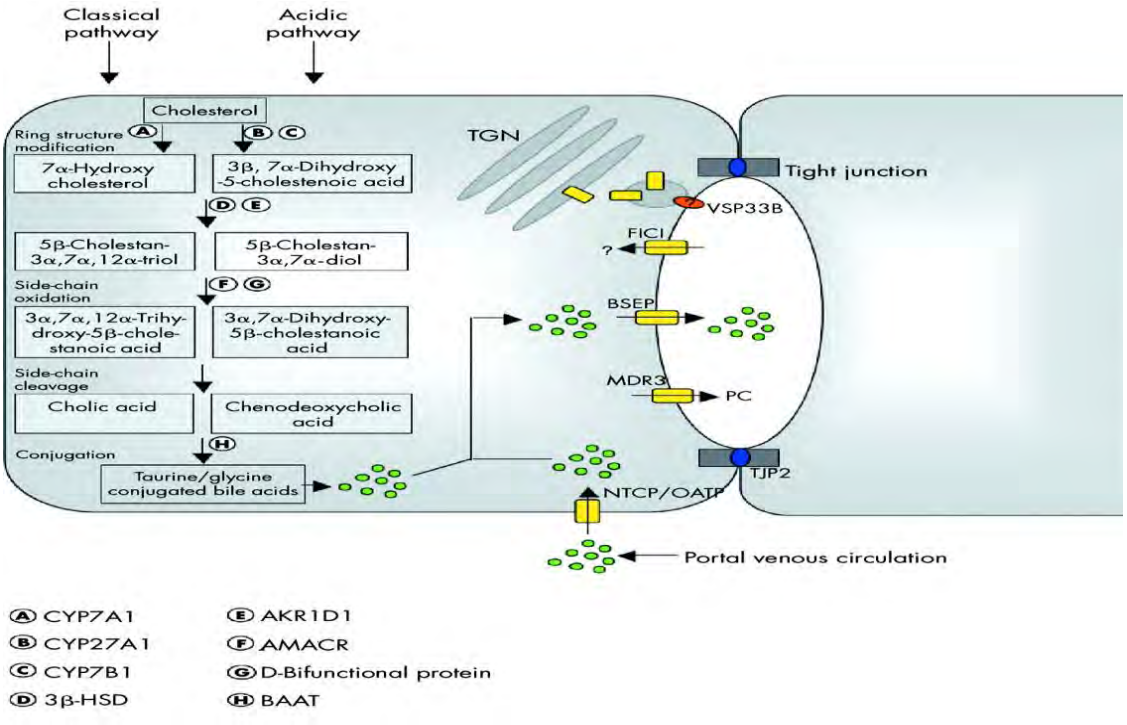


Figure 1.19. shows proteins involved in bile flow in hepatocyte (Van Mil *et al*., 2005).

Bile is made up of three different substances: bile salts, phosphatidylcholine and various organic anions. Bile salts are bile acid salts that have been conjugated with either glycine or taurine. Bile acids are the byproducts of cholesterol metabolism in the liver. Bile is formed by the secretion of bile salts, phospholipids, and cholesterol in the canalicular lumen. Bile is inherently cytotoxic, so its metabolism is tightly controlled. At the canalicular membrane, several members of the adenosine triphosphate-binding-cassette superfamily actively produce bile chemicals from hepatocytes (Ziol *et al*., 2008). The bile salt export pump (BSEP), which is expressed by the *ABCB11* gene, transports bile salts intracellularly. PC forms micelles in the lumen with bile salts to protect the luminal membrane from the poisonous and detergent effects of bile salt. The simultaneous production of PC and bile salt guarantees that bile flows continuously.

The adenosine triphosphate-binding cassette subfamily B, member 4 (*ABCB4*) gene encode the multidrug-resistance P-glycoprotein 3 (MDR3), which is a phospholipid floppase and one of the bile salts transporters. Hepatocytes release bilirubin and other organic anion via Multidrug resistance-associated protein 2 (MDR2), which is encoded by the *ABCC2* gene. Bile is produced and stored in the gallbladder before being released into the small intestine during the digesting process, when it emulsifies fats. The sodium-dependent taurocholate cotransporter peptide secretes 5% of bile, while the other 95% is reabsorbed and imported back to the liver (NTCP). A mutation in the *ABCB4* gene prevents phospholipids, primarily phosphatidylcholine, from being transported into the bile. Increased levels of free bile salts in the bile cause liver disease (Hedera, 2017; Narkewicz *et al*., 1998).

In all cholestatic diseases, the flow of bile salts from the liver to the gallbladder is blocked, leading to a compensatory route from the hepatocytes into the circulation (Williamson & Geenes, 2014).

1.19.2. Hormonal influence

Oral contraceptives and other oestrogen steroids have been linked to the etiology of obstetric cholestasis in the past. Natural steroids such estriol, estradiol, and synthetic oestrogen ethynil estradiol were given to female patients in recent studies from 1964 to 1967, and the symptoms were identical to those seen during pregnancy cholestasis.

Progesterone therapy, on the other hand, has no such side effects. When compared to singleton pregnancies (4.7%), twin pregnancies with elevated hormone levels have a higher rate of ICP (-20.9%) (Gonzalez *et al*., 1989). When oestrogen levels are high in the third trimester of pregnancy, ICP occurs. Hormone metabolism takes place in the liver, and the increased quantity of their metabolites during pregnancy influences biliary canalicular transporter activity (Kondrackiene & Kupcinskas, 2008). The cholestatic potential of glucuronides such estradiol-17-d-glucuronide and disulfated or mono-progesterone metabolites, primarily 3 and 5-isomers, is supported by experimental and clinical studies. 'Kreek, 1987,' says. Saturation of hepatic transport systems is caused by the production of large amounts of sulfated progesterone metabolites in genetically predisposed women, which is possibly associated to higher 5- and 3-reduction. These systems are used to excrete these chemicals through the bile (Meng *et al*., 1997; Reyes & Sjövall, 2000). In vitro research revealed that hormone metabolites impair the function of hepatocellular transporters such as *ABCB4* and *ABCB11* at the posttranscriptional level (Stieger *et al*., 2000). Estrogens disrupt the expression of canalicular and basolateral bile acid transporters in liver cells in an in vitro investigation through regulating transcription (Simon *et al*., 1996).

1.19.3. Reproductive Hormones and ICP

ICP susceptibility is influenced by reproductive hormones. The increase in oestrogen hormones during pregnancy, combined with genetic variations, overburdens the transporters, reducing bile salt transfer. In a mouse model, reproductive hormones and their metabolites lowered canalicular transporter expression, while 17b-oestradiol treatment reduced BSEP expression as measured by mRNA analysis. By binding to Mrp2, Estradiol-17beta-D-glucuronide (E217G) causes BSEP internalization in rats, affecting bile salt transport and flow (Crocenzi *et al*., 2003). Progesterone metabolites can cause BSEP trans-inhibition, resulting in bile acid buildup (Vallejo *et al*., 2006).

Despite the fact that ICP has no effect on mother health, it does create prenatal difficulties. Premature births have been observed in 20% of ICP patients. Meconium staining of amniotic fluid has been found in 60% of the instances analyzed, whereas foetal distress has

been detected in 35% of the cases. Increased serum bile acid levels are a risk factor for prenatal complications. Williamson and Victoria (Williamson & Geenes, 2014).

At 12 weeks, the fetus begins to produce bile acid. A trans-placental gradient exists throughout normal pregnancy, which enables the passage of bile acid and toxin across it. However, in ICP, this gradient is reversed, and increased bile acid transfer from mother to fetus through the placenta resulting in bile acid buildup in the foetal compartment, endangering the fetus's health (Brites, 2002). The addition of primary conjugate of bile acid taurocholate (TC) to primary culture of newborn cardiomyocytes produced a decrease in contraction rate. Furthermore, it harmed the cellular network's integrity and synchrony. As a result, increased bile acid levels are also linked to intrauterine death (WILLIAMSON *et al*., 2001). There is apparently a link between ICP and infant respiratory distress (Zecca *et al*., 2006). In normal term pregnancies, meconium staining of amniotic fluid (MSAF) occurs about 15% of the time and is considered a symptom of foetal distress. MSAF has been reported in 16 percent to 58 percent of instances affected by intrauterine death (IUD) in ICP (Geenes & Williamson, 2009). Women with intrahepatic cholestasis of pregnancy have been found to have higher rates of both gestational diabetes and pre-eclampsia (Wikström Shemer & Marschall, 2010).

1.20. Maternal complications

1.20.1. Pruritus

Pregnant women who have sudden itching in their second or third trimester and are discovered to have abnormal serum bile acids are typically diagnosed with ICP. In most cases, pruritus develops three weeks before a diagnosis is made, well before the bile acid rise ever becomes noticeable (Kenyon *et al*., 2002) About one third of patients indicate that pruritis is severe on their palms and soles before spreading elsewhere. A decrease in body temperature, as well as a few days after giving birth, alleviates pruritus. (Kondrackiene *et al*., 2005). Despite the presence of excoriation marks on a physical examination, cholestasis can be distinguished from pregnancy-related dermatoses by the absence of primary skin lesions.

Some bile acids are thought to have a direct pruritogenic impact on the skin, which may explain why cholestatic pruritus occurs. Traditional experimental research demonstrated that injecting or topically applying bile acids caused reproducible pruritus in human subjects (Varadi, 1974). It is true that some people with high bile acid levels don't have pruritus, and even among those who do, there's no correlation between bile acid levels and the intensity of their itch. Some researchers think that the pruritogen in cholestasis is a bile substrate that is not well understood and is released into the bloodstream following hepatocyte injury caused by bile acid buildup (Ghent *et al*., 1977). There is mounting proof that the mu receptor has a role in pruritus that is mediated by the central nervous system. Itching is a common side effect of intrathecal opioid analgesia, which is used during medical procedures such as spinal anesthesia for caesarean section. In addition, it has been observed that the use of opioid antagonists can alleviate cholestatic pruritus.

1.20.2. Other (maternal complications)

Pain in the upper right abdominal area, sickness, loss of appetite, and inability to sleep are also possible manifestations of ICP. In 10%-15% of instances, mild jaundice develops within 4 weeks of the commencement of itching (Ghent *et al*., 1977; Kondrackiene & Kupcinskas, 2008). It is possible to experience minor steatorrhea as a result of fat malabsorption. Vitamin K insufficiency with a prolonged partial thromboplastin time can occur in extremely unusual situations of severe steatorrhea. Some people advocate for giving patients with ICP vitamin K supplements on a "empirical basis" due to the theoretical danger of obstetric hemorrhage (Geenes & Williamson, 2009). Pregnancies complicated by ICP have an increased risk of developing both gestational diabetes and preeclampsia. Wikström *et al*. found a 2.8-fold greater risk of gestational diabetes and a 2.6-fold increased risk of preeclampsia among women with ICP compared to controls without ICP in their population-based cohort research published in 2013 (Wikström Shemer *et al*., 2013). In most cases, ICP resolves on its own without causing any lasting health problems. In almost all cases, hepatocellular damage heals after pregnancy, while recurrence rates are substantial (up to 90 percent in some cases)(Bicocca *et al*., 2018). In addition, subsequent pregnancies with ICP are typically more severe and occur at a younger gestational age. An correlation with biliary disease in old age has been found, however this

link is not believed to be causal. Patients with ICP have been shown to be at a higher risk for a wide range of adverse health outcomes, including gallstone disease, hepatitis C, fibrosis, cholangitis, hepatobiliary malignancy, immune-mediated disease, and cardiovascular disease. An higher risk of liver or biliary tract cancer, diabetes, thyroid illness, and Crohn's disease was reported in a Swedish registry-based research of nearly 11,000 women with ICP compared to controls without ICP (Marschall *et al*., 2013).

1.21. Obstetric complications

1.21.1. Preterm birth

There is conclusive evidence that ICP raises the chance of spontaneous preterm delivery (Wikström Shemer *et al*., 2013). Experimental evidence from animal studies suggests a relationship between high bile acid levels and spontaneous preterm delivery. Perez *et al*. found that high-dose bile acid injected once into pregnant sheep induced premature delivery in 20% of cases, and that continuous bile acid infusion caused uterine contractions and preterm delivery in 100% of cases (Perez *et al*., 1994). In a similar rodent model, Campos *et al* showed that treatment with bile acids increased myometrial response to oxytocin (Campos *et al*., 1988). Human myometrium samples from patients with ICP showed an amplified contractile response to oxytocin compared to control samples from healthy patients (Israel *et al*., 1986). Recent investigations with human myometrium samples, inspired by these studies, showed that bile acids boosted oxytocin receptor expression and sensitivity. These studies showed that after being treated with bile acids, the levels of mRNA for the oxytocin receptor as well as the proteins it encodes increased. In addition, bile acid-treated myometrial strips required significantly less oxytocin to trigger uterine contractions. The researchers found that the increased risk of spontaneous preterm birth in patients with cholestasis may be explained by an increase in bile acid-mediated increase in oxytocin-receptor expression and sensitivity. It's interesting that just cholic acid, but not deoxycholic acid, caused this reaction (Germain *et al*., 2003).

1.22. Fetal complications

1.22.1. Fetal demise

Death of the fetus is the most alarming complication of cholestasis during pregnancy. There is significant evidence in the literature linking high bile acid levels to stillbirth, but to date, most investigations have lacked sufficient sample sizes to draw any firm conclusions. As far as we know, the first to report on a cohort big enough to show a link between cholestasis and unfavorable outcomes was the team led by Glantz and colleagues in 2004. Results from a study of 505 Swedish women with mild to severe cholestasis showed that for every 1 mmol/L of blood bile acids above 40 mmol/L, the risk of spontaneous preterm delivery, foetal hypoxia episodes, and meconium staining rose by 1% to 2% (Heinonen & Kirkinen, 1999).

First to report on a big enough cohort to show a link between cholestasis and stillbirth, Geenes and colleagues did so in 2014 (Ovadia *et al*., 2019). The cause of foetal death from ICP is poorly understood. Increased measures of bile acids in amniotic fluid, cord serum, and meconium in cholestasis-affected pregnancies are assumed to be the underlying cause of worse foetal outcomes. Fetal death is thought to be directly related to bile acid effects on the fetal heart and not to chronic placental insufficiency. Clinical studies in support of the arrhythmogenic effect of bile acids on the fetal heart have reported fetal atrial flutter and supraventricular tachycardia in cholestatic pregnancies (Williamson *et al*., 2011). In addition, animal and human stem cell cardiomyocytes models in vitro demonstrate that bile acid treatment reduces contractile performance and promotes arrhythmia. This impact was suppressed when UDCA was given in those same studies (Geenes, Chappell, *et al*., 2014).

1.22.2. Respiratory distress

Pregnancies complicated by ICP have been shown to have higher risks of respiratory distress syndrome (RDS) (Geenes & Williamson, 2009). Animal studies have shown a causal link between preterm birth and RDS, which is likely exacerbated by both natural and induced preterm birth. Atelectasis, eosinophilic infiltration, and the formation of a hyaline membrane were induced by direct tracheal injection of bile acids in a rabbit model; these effects were reversed by the administration of surfactant. Severe chemical pneumonitis and pulmonary edema were observed in a porcine model of bile acid exposure (Geenes & Williamson, 2009).

Human neonates with cholestatic pregnancies had a 2.5-fold increased risk of RDS, according to research by Zecca *et al*. in 2006. They reasoned that since RDS is caused by a lack of surfactant, high bile acid levels must be affecting the action of alveolar enzymes.(Zecca *et al*., 2006).

1.22.3. Meconium staining

One of the complications of ICP is amniotic fluid discoloration from meconium. According to a new meta-analysis of perinatal outcomes for women with intrahepatic cholestasis, the incidence of meconium staining increased from 11% to 19% in pregnancies complicated by ICP. This increase had a pooled odds ratio of 2.60 (Ovadia *et al*., 2019). Meconium transit was recorded in 44% of patients with cholestasis in a research conducted on women in Southern California in (Alsulyman *et al*., 1996).

Vagal stimulation of the developed foetal gastrointestinal tract is widely accepted as a secondary cause of meconium staining. Peristalsis of the intestines and relaxation of the anal sphincter are two of the mechanisms through which hypoxic stress might facilitate meconium expulsion. It is beyond the scope of this review to discuss the impact of meconium on perinatal outcomes. Less is known about the mechanism that causes meconium staining in ICP, but it is not related to foetal hypoxic stress. Studies on animals have revealed that bile acids can enhance intestinal motility in a direct fashion. Increases in bile acid concentration were correlated with increases in colonic smooth muscle contractility in a rabbit model (Snape Jr *et al*., 1980). Although evidence is few, an increase in intestinal motility due to bile acids may be a more plausible explanation for meconium transit in cholestasis than neuronal activation due to foetal hypoxia.

1.23. Treatment

No recognized treatment exists for ICP because its cause is unknown. Pruritus is one of many pregnancy-related symptoms that can be treated with medications including dexamethasone, S-adenosyl-L-methionine (SAMe), and ursodeoxycholic acid. Recent research has revealed that the tertiary bile acid ursodeoxycholic acid, which is present in human serum at a concentration of 3-8%, can considerably reduce pruritus, a common

symptom of pregnancy (Chappell *et al*., 2012). Another study found that UDCA helped foetal outcomes by decreasing foetal distress and itching, as well as lowering ALT levels (Bacq *et al*., 2012). Additionally, UDCA decreases bile acid levels in colostrum, which is beneficial to the fetus (Brites, 2002). In ICP instances, Geene *et al*. (2014) found higher levels of bile acid in both the maternal and foetal compartments as compared to controls. With UDCA, total bile acid was reduced in the mother and the developing child, and the bile acid profile was altered in both (Geenes, Lövgren-Sandblom, *et al*., 2014). Cardiomyocytes have been demonstrated to be harmed by TC (WILLIAMSON *et al*., 2001). However, UDCA blocks both the depolarization and hyperpolarization of cardiomyocytes in response to TC, resulting in less fibroblast development into myofibroblasts (Adeyemi *et al*., 2017). Therefore, UDCA can be employed for the care of the fetus in ICP as an antiarrhythmic and antifibrotic medication. Patients with *ABCB4* mutations benefit from treatment with ursodeoxycholic acid (Dixon *et al*., 2014).

USCD is helpful for infants experiencing respiratory distress. The beneficial effects of UDCA include normalizing foetal bile acid levels, decreasing oxidative stress, and preventing cell death (Kong *et al*., 2016). Considering the facts and figures, it's clear that ursodeoxycholic acid should be used as part of the active therapy for people with ICP. Rifampicin increases hepatic microsomal enzymes and blocks bile acid uptake by hepatocytes. The suggested daily dose is 10 mg/kg. For this reason, hepatotoxicity should be monitored while it is being used for the treatment of pruritus (Venigalla & Gourley, 2004)

1.23.1. UDCA

Treatment for ICP typically involves UDCA, also known as Ursodiol. Naturally occurring UDCA is produced when microorganisms in the gut convert primary bile acids into tertiary bile acids. To slow down the absorption of cholesterol, UDCA works in the intestine to break up micelles. Some research suggests that when UDCA acid is given exogenously, it accumulates in hepatocytes and bile, leading to a reduction in hepatic cholesterol synthesis, secretion, and intestinal cholesterol reabsorption. Anticholelithic (reduces cholesterol) and choleretic (increases bile flow) describe the effects of UDCA, which is used to boost the

total volume of bile released. UDCA's putative action at the BSEP and MRP3 receptors may facilitate its cholerectic effects.

This is why UCDA is utilized for the non-invasive removal of gallstones. Decreased bile cholesterol concentration causes cholesterol in gallstones to dissolve over time (Lazaridis *et al*., 2001). To recap, the enterohepatic recycling of bile acids is highly effective. Chronic administration causes UDCA to concentrate in the liver and bile where it is actively recycled. Clearance of exogenous UCDA is poor because enterohepatic recycling is so efficient. Changes in bile composition occur as UDCA gradually replaces BHA as the predominant bile acid in circulation and the biliary system. Though just 3% of the body's total bile acid stores are made up by endogenous UDCA, prolonged exogenous UDCA can increase this percentage to as much as 50%. To put it simply, UDCA is a benign, hydrophilic bile acid that can be used in place of cholic acid, a toxic, hydrophobic bile acid that has been linked to negative results. The ratio of cholic acid to deoxycholic acid is normalized, and the overall levels of main bile acids are lowered, thanks to UDCA. It has been established that bile acid levels in amniotic fluid and cord blood can be lowered by UDCA. Because of the time lag between the peak of bile acid content in meconium and the start of treatment, this method is ineffective (Geenes & Williamson, 2009). Pruritus is alleviated with UDCA in around 60% of women and goes away entirely in about 40%. Improvement in symptoms and lower levels of serum bile acids are typically seen during the first two weeks of treatment. In most cases, a maximum daily dose of 21 mg/kg/d (about 1500 mg for a 72 kg patient) of UCDA is used (Bacq *et al*., 2012).

1.23.2. Dexamethasone

In addition to corticosteroids, dexamethasone has been studied for its potential use in ICP therapy. Results from a small, early observational trial of Finnish women indicated that dexamethasone reduced symptoms and total bile levels. Inhibition of placental oestrogen synthesis, as shown by reduced levels of circulating estriol and estradiol, was postulated as the underlying mechanism. Yet, follow-up research has cast doubt on these conclusions, and repeated prenatal exposure to corticosteroids is a cause for concern (Geenes & Williamson, 2009).

1.23.3. Cholestyramine

Cholestyramine is a potent ion exchange resin that binds to bile acids in the gut, blocking them from being recycled back into the liver and instead committing them to excretion in the feces. There is no proof that cholestyramine lowers total bile acid levels, however it has been shown to alleviate pruritis symptoms. There are worries about the reduction in vitamin K levels that can occur while using cholestyramine to treat ICP, thus it isn't typically used as a first-line treatment (Geenes & Williamson, 2009).

1.23.4. Rifampin

Rifampin is a macrocyclic antibiotic that is a semisynthetic derivative of rifamycin, a class of antibiotics generated by Streptomyces mediterranei. It is often used to treat tuberculosis because of rifampin's ability to decrease bacterial RNA synthesis. Rifampicin is a promising second-line treatment for primary biliary cirrhosis, although its application in ICP has not yet been investigated (Geenes & Williamson, 2009). Total bile acids and other liver function enzymes were found to be considerably reduced in these studies when rifampin medication was compared to placebo. It has been hypothesized that rifampin boosts UDCA performance by inhibiting bile acid absorption by hepatocytes and increasing bile acid detoxification. Case reports are all that exist to describe its use to the obstetric population, although the results are promising (Liu *et al*., 2018).

1.23.5. Other (treatment)

Potential for Bleeding due to vitamin K insufficiency after steatorrhea can prompt prophylactic vitamin K use. Insufficient evidence supports this method (Geenes & Williamson, 2009). Pruritus can be alleviated with menthol, lotions, and antihistamines (both oral and topical), all of which have been found to be effective. Research into guar gum activated charcoal and S-Adenosyl-L-methionine for the treatment of ICP has shown mixed results, and a 2013 Cochrane Review stated that the evidence was insufficient to draw any firm conclusions about their effectiveness (Bicocca *et al*., 2018).

1.24. Management

1.24.1. Antenatal testing

There is still debate on whether antenatal testing should be done for ICP. Increased foetal mortality rates have been well-documented in the literature, yet placental insufficiency is not regarded to be a contributing factor in stillbirth. Furthermore, reduced variability, tachycardia, and bradycardia in foetal heart tracings have been documented in the context of ICP, although they were not associated with foetal mortality (Geenes & Williamson, 2009). Therefore, it is not known whether antenatal testing can detect babies at risk of not making it. Two of 83 individuals with ICP in early research by Fisk and Storey had ICP exacerbated by foetal death, but nonstress testing showed no abnormalities in either of these cases. Therefore, it is not known whether antenatal testing can detect babies at risk of not making it. Additionally, there are several accounts of foetal deaths in the literature despite recent normal prenatal testing (Alsulyman *et al*., 1996; FISK & Bruce Storey, 1988).

1.24.2. Delivery timing

Elective early delivery of ICP has also become widely used in the management of ICP because to the growing foetal death rates with increasing gestational age, especially after the 36th week. Puljic and colleagues undertook the most complete evaluation of foetal death risk to date, comparing each extra week of expectant care against early delivery in cholestasis-affected pregnancies. They assessed the composite risk of expectant management for one extra week, foetal death, and infant death for each week of gestation from 34 to 40. Their 2015 analysis covered approximately 1.6 million pregnancies. At 36 weeks gestation, delivery had lower perinatal mortality than expectant management (delivery risk: 4.7 vs. 19.2 for expectant management), and the risk of expectant management increased afterward (delivery risk: 18.3 vs. 33.6 for expectant management at 39 weeks) (Puljic *et al*., 2015). These results are consistent with the published literature showing an increase in foetal mortality after 36 weeks (Lo *et al*., 2015).

OBJECTIVE OF STUDY

The main objectives of present study are as following:

1- Study of clinical presentation and genetics of Wilson's disease cases from local population.

2- Mutational Analysis and Clinical investigations of patients affected with Glycogen storage disease type 1a (GSD 1a) in Pakistan.

3- Enrolment of Intrahepatic cases of pregnancy cases, their clinical features and molecular analysis.

CHAPTER 2

2. METHODS

This research was given approval by the Quaid-i-Azam University Bioethics Committee. The study was carried out at Molecular Biology Lab, Quaid-i-Azam University (QAU), Islamabad, Pakistan. Families affected with metabolic disorders of liver including Wilson's disease (WD), Glycogen Storage Disease Type 1a (GSD 1a or von Gierke) and Obstetric Cholestasis (Intrahepatic cholestasis of pregnancy, ICP) were recruited by conducting extensive surveys from Pakistan Institute of Medical Sciences PIMS (January 2018 to January 2020), Children Hospital Lahore CHL (January 2018 to January 2020) and Wah General Hospital, Wah Cantt (June 2019-November 2019).

2.1. Ethics statement/ Study design and participants

This study was approved by the Institutional Review Board of Children Hospital Lahore CHL, Pakistan Institute of Medical Sciences PIMS and Wah General Hospital, Wah Cantt. In accordance with the principles outlined in the Declaration of Helsinki, all participants provided written informed consent. All patients with WD or GSD 1a were included (male and female, with and without a history of cousin marriage). Women who were pregnant at the time of the study were included whether or not they had a previous diagnosis of intrahepatic cholestasis during pregnancy (ICP).

Eighty WD patients along with available family members were identified from the Neurology and Gastroenterology departments of Children Hospital Lahore (CHL) and Pakistan Institute of Medical Sciences (PIMS).

40 Glycogen storage disease type 1a patients along with available family members were identified from the Neurology and Gastroenterology departments of Children Hospital Lahore, CHL and Pakistan Institute of Medical Sciences, PIMS. Clinical profiles were recorded; Blood of affected patients and their relatives was collected along with the genetic history and clinical data.

Pregnant females affected with ICP were involved in this study. Clinical data was collected from females with obstetric Cholestasis who visited Wah General Hospital, Wah Cantt during 6 months from June 2019 to November 2019.

The signs of ICP include pruritus, nausea, dark urine, jaundice and accumulation of bile acids in hepatocytes. Affected female were diagnosed by local gynaecologist and clinical phenotypes were recorded.

2.2. Pedigree Design

For Glycogen storage disease and Wilson's disease, a questionnaire was used to interview the individuals and collect data on a variety of criteria, including risk factors. Normal elders or guardians were interviewed to obtain family history and to clarify genetic relationship. Pedigrees of families were drawn according to method described by Bennet *et al*. (1995) (Bennett *et al*., 1995) using Haplopainer (software) http://haplopainter.sourceforge.net/. Traditionally, a square in the pedigree represents males whereas a circle represents females. Affected individuals are depicted with filled symbols, while unaffected persons are depicted by hollow symbols. Double marriage line between the partners corresponds to consanguineous marriage while deceased individuals are indicated with diagonal line over squares and circles. The generations are represented by Roman numerals, whereas the individuals within those generations are indicated by Arabic numerals. Mode of inheritance of a disease was observed to deduce the segregation pattern within the family.

2.3. Clinical Evaluation of Patients

Detailed clinical assessment and blood collection of patients was done by visiting PIMS, CHL and Wah General Hospital, Wah Cantt by the help of expert physicians. The clinical manifestations of Wilson disease are predominantly hepatic, neurologic, and psychiatric, with many patients having a combination of symptoms. Kayser-Fleischer rings (KF rings), 24-hour urinary copper elevation, low plasma ceruloplasmin, and increased liver copper concentrations were used to establish a diagnosis of Wilson's disease. All the patients included had Global Assessment scale for Wilson Disease (GAS) score of 4 or more. Affected individuals were clinically accessed for neurological and hepatic abnormalities.

For sampling of GSD 1a, affected individuals were clinically accessed for enlarged liver and kidneys and growth retardation and short stature. GSD 1a was diagnosed by severe hypoglycemia, growth retardation, hepatomegaly, bleeding diathesis, lactic acidemia,

hyperlipidemia, Hyperuricemia, abnormal levels of glucose, lactate, uric acid, triglycerides and cholesterol. Some chronic conditions that can arise include gout, hepatic adenomas, osteoporosis, and kidney disease.

All pregnant females diagnosed with signs and symptoms of ICP and controls with no previous history of ICP were included in the study. Symptoms of ICP can range from mild to severe and may begin in second or third trimester. The signs of ICP include severe itching, loss of appetite, pruritus, nausea, dark urine, jaundice and accumulation of bile acids in hepatocytes. Affected female of each family were diagnosed by local gynaecologist and clinical phenotypes were recorded.

2.4. Statistical Analysis of the clinicals of the patients via SPSS

For statistical analysis of risk factors of WD, the prospective cross sectional study design is used in present study. Data were collected through survey method via verbal interviews. The questionnaires were designed to inquire into and provide information about symptoms, causes and effect of WD, GSD and ICP. Data was recorded in excel sheet. Participants of the study were approached from hospitals including PIMS, CHL Lahore,Wah General Hospital. The study consists of 80 WD, 40 GSD, and 50 ICP participants with same number of controls. For data collection, participants were informed about study purpose. Consent to participate in study was acquired from them after providing verbal instruction. Participants were asked to fill demographic information along with instrument measuring the symptoms, causes and effect of the diseases. Participants were assured that data provided by them will be kept confidential. The inclusion criteria were the participants suffering from said diseases. At the end participants were thanked for their cooperation. After collection of data, data processing and analysis was made using SPSS 20.0. The frequency and percentages of demographic profile of the samples was computed and were shown in graphical representation. For WD, the significance of different risk factors among persistent and non-persistent group were checked. To check the significance of qualitative variables, we used chi-square test. For the variables whose cell frequencies is <5, we used Irwin Fisher's Exact test. For quantitative variables, we used Shapiro-Wilk test to test the normality of the data, the non-significant (at $\alpha = 0.05$) result showed that age at diagnosis

(in years), treatment duration (months), and urinary copper (ug/24h) are normal; to check the significance of these variables we used independent sample t- test and Mann Whitney-U test is used for rest of the quantitative variables.

For GSD and ICP, descriptive analysis is performed. For qualitative variables, the frequency and percentages were computed, and the quantitative variables are recorded by using mean and standard deviation. The graphical representation also showed for the prevalence (in %ages) of risk factors among different age groups.

2.5. Sample Collection

Peripheral blood samples were taken from affected and normal members of families with Glycogen storage disorder, Wilson's disease, and Intrahepatic Cholestasis. Sterile 10 mL syringes were used for blood collection and blood was transferred to potassium EDTA vacutainer tubes (BD, USA). Blood samples were kept at 4°C in laboratory at Quaid-i-Azam University, Islamabad for future use.

2.6. Genomic DNA Extraction

Genomic DNA was isolated from whole blood using an organic approach, also known as the phenol/chloroform isolation method.(Sambrook *et al*., 1989) (Table 2.1). For genomic DNA extraction, 750 µL of whole blood from each individual was transferred in an autoclaved eppendorf tube. Later, 750 µL of solution A was added to each tube and tubes were inverted 4-5 times and placed at room temperature for 8-10 minutes. After incubation period, tubes were provided with 2 minutes centrifugation at 13,000 rpm (revolution per min) in a microcentrifuge (Hittich Mikro 120, Germany). The supernatant obtained was decanted and the pellet was re-dissolved in 400 µL of solution A. There was another round of two minutes of 13,000 rpm spinning with the tubes. After removing the supernatant once again, pellet was added to tubes containing 400 µL of solution B, 25 µL of 10% SDS, and 5 µL of proteinase K. After the pellet was resuspended, the tubes were placed in the incubator to incubate for the night (Binder, Germany) set at 37°C.

On following day, 500 µL of freshly prepared equi-volume mixture of solution C and D was added into eppendorf tubes. The tubes were centrifuged at 13,000 rpm for 10 minutes while being inverted many times to ensure proper mixing. Three distinct phases formed as a result of centrifugation (upper, middle and lower). The upper aqueous phase was transferred in a new 1.5 mL eppendorf tube, mixed with equal volume of solution D and spun at 13,000 rpm for 10 minutes. After spinning, the aqueous phase was again separated from organic layer and transferred in a new 1.5 mL eppendorf tube. For genomic DNA precipitation, 60 µL of sodium acetate (3 M, pH 4.5-6) and 500 µL of chilled isopropanol were added into the collected aqueous phase. The tubes were inverted few times and again centrifuged for 10 minutes at 13,000 rpm to pellet out genomic DNA. The supernatant was carefully decanted, 200 µL of 70% ethanol was added to each tube and tubes were spun at 13,000 rpm for 7 minutes to wash pellet. The supernatant was decanted and pellet was dried in incubator set at 37˚C. After drying the pellet, DNA was dissolved in suitable volume (80-150 µL) of TE (Tris-EDTA) and incubated overnight at 37˚C.

2.1. Composition of Genomic DNA Extraction Solutions

Solution A	0.32 M sucrose, 10 mM Tris (pH 7.5), 5 mM MgCl2, 1% (v/v) Trition X-100
Solution B	10 mM Tris (pH 7.5), 400 mM NaCl, 2 mM EDTA (pH 8.0)
Solution C	Phenol (pH 8.0)
Solution D	Chloroform 24 volumes, Isoamyl alcohol 1 volume
T.E buffer	10 mM Tris (pH 8.0), 0.1 mM EDTA

2.7. Quantification of DNA

We measured the absorbance of samples at 260nm using spectrophotometric techniques to get an idea of the nucleic acid content. During DNA extraction, the 260/280, 260/230, and 260/325 absorbance ratios are used to evaluate DNA purity and identify any contaminants

present in the sample material. Using a spectrophotometer modelled after the Nano Drop, we measured the absorbance at 260 and 280 nm to ascertain the DNA's content and purity (Thermo Scientific). For blanking the instrument with 2µL of TE buffer select "Blank" to confirm that the measure is 0, after that place DNA sample 2µL that the measures the concentration of DNA sample.

2.8. Molecular Analysis of Wilson's disease cases

2.8.1. Exon amplification conditions for *ATP7B* gene

The exon amplification was done using PCR carried out in 200 µL PCR tubes (Axygen, USA). PCR reaction mixture comprises undermentioned constituents.

- PCR buffer (2.5 µL)
- Sample DNA (2.5 µL) (40 ng)
- dNTPs (2.5 µL) (10 mM, Fermentas, UK)
- Taq DNA Polymerase (0.3 µL) (5 U/ µL, Fermentas, UK)
- $MgCl_2$(2.5 µL) (25 mM)
- Forward and reverse primer (0.5 µL each) (0.1 µM)
- PCR water (13.7 µL)

A 2.5 µl DNA sample of each proband was amplified in 25 µl of PCR reaction containing 0.3 µl of Taq DNA Polymerase (5 U/µL Thermo Scientific Inc.), 2.5 µl of 2.5 mM deoxynucleotide. Triphosphate mixture, 0.5 µl of each forward and reverse primer (10 pmol/µl)(Table 2.2), 2.5 µl of 10 × reaction buffer and 2.5 µl of MgCl2 (25 mM). After the PCR reaction mixture was prepared, it was centrifuged and agitated in a vortexer before being transferred to a T1 Thermocycler (Biometra, Germany) for amplification. The PCR thermo-cycling conditions were as follows:

Initial denaturation at 95°C for 5 minutes was followed by 10 cycles at 95°C for 45 seconds, 69°C-64°C (according to melting temperature of each primer pair) for 45 seconds at an increment of -1 in each subsequent cycle, 72°C for 45 seconds, and finally 30 cycles at 95°C for 45 seconds, 59°C-54°C (according to melting temperature of each primer pair) for 45 seconds, 72°C for 45 seconds. After being amplified and purified with a PCR kit

(Wiz Bio Solutions, Seongnam, Korea), the resulting PCR products were evaluated for size on an agarose gel with a 1 kb size ladder.

An agarose gel concentration of 2% was used to verify the amplification (2 p% agarose gel, in 40 mL of 1X TBE buffer). The polymerized gel was then loaded with 5 µL of PCR product and 3 µL of loading dye. After 25 minutes of exposure to 120 volts, the data were recorded using a gel documentation system (SYNGENE, UK). Before Sanger sequencing, we further purified the size-specific PCR products for each exon.

Table 2.2 Exon-specific amplification primers for *ATP7B* gene (exons 1, 2, 3, 4, 5, 8, and 13)

Exon No.	**Primer Sequence (5'→3')**		**Product Size (bp)**	**Annealing Temp (˚C)**
1	F	GCAACTTTGAATCATCCGTGT	326bp	59
	R	AAAATCCTCCTGGTGGGAGT		
2A	F	TAGATGCTGCCTTTAGCTTGC	766 bp	60
	R	TAAGGGAGCCACTTTGCTCTT		
2B	F	TCTCACTCAGCAACCAAGAGG	847 bp	60
	R	AGGGCTCACCTATACCACCAT		
3	F	CTCACCAAGAGCCCTGAAAC	394	59
	R	CGAGGTCTATACGCAGCATTC		
4	F	GGGTAAGAGACCAGACATCGT	473	60
	R	AACAAACCAGACACGTCCAAG		
5	F	CTTGGCTGCCTGTTACCTAGA	382	60
	R	TCCATGGGAAAAGTTGAAGAA		
8	F	GCCCATCACAGAGGAAGAAGT	573	59
	R	ACCAGATTAGCTGGGATTTCA		
13	F	CCCCCTGAAATGTCCTTATGT	370	60
	R	GTGGCTCTCAGGCTTTTCTCT		

2.8.2. Candidate gene sequencing approach

The *ATP7B* (ENST00000242839) sequence was retrieved from the Ensemble Genome Browser (http://asia.ensembl.org/index.html) under accession number. Primers were

developed with the use of the primer3 programme (http://bioinfo.ut.ee/primer3-0.4.0/). Primers with a single hit were chosen using the Blast like alignment tool (BLAT) (http://genome.ucsc.edu/cgi-bin/hgBlat) to determine their specificity. Primers' product sizes and melting temperatures were verified in silico using PCR (https://genome.ucsc.edu/cgi-bin/hgPcr).

2.9. Molecular analysis for GSD 1a

The *G6PC* gene has been found to be associated with Glycogen storage Disease type 1a. Glucose-6-phosphatase (G6Pase; EC 3.1.3.9) catalyses the hydrolysis of glucose-6-phosphate to glucose and phosphate at the end of gluconeogenesis and glycogenolysis, causing a deficiency in which leads to glycogen storage disease, an autosomal recessive condition. The GSD1a disease gene, *G6PC* (ENSG00000141349.10), mapped to chromosome 17: 44,070,620-44,082,151 (Yang Chou, 2001). we investigated genetic variations in the G6PC gene in all exons 1-5, and the intronic boundaries in 20 Pakistani patients with Glycogen storage disease 1a, and the identification of hotspot mutations in 20 patients and their families for segregation analysis. The *G6PC* sequence (ENSG00000141349.10) was downloaded from the Ensemble Genome Browser (http://asia.ensembl.org/index.html). Five sets of primers, spanning intron-exon junctions and flanking regions, were constructed using primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). To determine the specificity of each primer, we utilized the Blast like alignment tool (BLAT) (http://genome.ucsc.edu/cgi-bin/hgBat) to find the best matches. Primers' product sizes and melting temperatures were verified with in silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr).

2.9.1. Candidate Gene Sequencing Approach

Identification of new disease-causing genes is possible by complementing linkage analysis with candidate gene sequencing approach. Suitable candidate gene is selected on basis of known biological, physiological and functional relevance of the gene to the disease in question. The *G6PC gene* that spans chromosome 17 was selected as a candidate gene to be associated with GSD 1a. Genomic sequence of *G6PC* (ENSG00000141349.10) derived from Ensemble Genome Browser, Primers were designed from flanking regions of all the

exons including exon-intron junction by using Primer 3 online primer design tool (Table 2.3), The specificity of each primer was determined using a tool called Blast like alignment tool (BLAT), and those that only produced a single hit were chosen.

Table 2.3. Primers for amplification of exons 1, 2, 3, 4, 5a and 5b.

Exon No.	Primer Sequence(5' 3') →		Product Size (bp)	Annealing Temp (˚C)
1	F	TTGAGTCCAAAGATCAGGGC	483bp	57 59
	R	TGAATAGCCTGGGGAAAGCA		
2	F	CCACCCAGTTCTCCCTTCTA	519bp	58 60
	R	CTTTCTCAGGACACAGCGCT		
3	F	GGTAGATGGGTGGATAGGGG	289bp	58.34 57.92
	R	AGAATACGTGGTGTGTCAGC		
4	F	AAAATTCCACTGAGAGCACCT	358bp	57.48 57.48
	R	ACCCACAGAAATGCTAACAGT		
5a	F	GCAGAACGGATGGCATGTCA	385bp	61 59
	R	AGCTCTCCCTGTACATGCTG		
5b	F	GTGGACTCTGGAGAAAGCCC	524bp	60 60
	R	GACCCTCCAATCTGCCATCC		

2.9.2. Exon amplification conditions for *G6PC* gene

The exon amplification for *G6PC* gene was made by PCR of was carried out in 200 µL PCR tubes (Axygen, USA) using primers shown in table 2.3. PCR reaction mixture comprises following components;

- PCR buffer (2.5 µL)
- Sample DNA (2.5 µL) (40 ng)
- dNTPs (2.5 µL) (10 mM, Fermentas, UK)
- Taq DNA Polymerase (0.3 µL) (5 U/ µL, Fermentas, UK)

- $MgCl_2$ (2.5 µL) (25 mM)
- Forward and reverse primer (0.5 µL each) (0.1 µM)
- PCR water (13.7 µL)

Agarose gel at 2% concentration was used to verify the amplification (2 percent agarose gel, in 40 mL of 1X TBE buffer). PCR product (5 µL) combined with loading dye (3 µL) was used to load the polymerized gel.

2.10. Purification of PCR products

Wizbio solutions PCR clean up kit was used for purification of amplified product for both WD and GSD 1a. Purification step removes left over primers, dNTPs and enzyme from the PCR product. It consists of the following steps;

1) Transfer the PCR product to 1.5ml tube.

2) Add 5 volume of GP Buffer to 1 volume of the sample and mix thoroughly.

3) Transfer the mixture to a Spin Column

4) Centrifuge at 13,000 rpm for 1 min. and discard the filtrate.

5) Add 700µl of Wash Buffer (ethanol added) to the Spin Column.

6) Centrifuge at 13,000 rpm for 30 sec. and discard the filtrate.

7) Centrifuge for an additional 1 min. and transfer the Spin Column to a new 1.5 ml tube.

8) Apply 50µl of Elution Buffer into the center of the column matrix.

The samples were than subjected to Dideoxy sanger sequencing method.

2.11. Sanger Sequencing

Amplified PCR products of *ATP7B* gene and *G6PC* gene were then sequenced by DNA Core Facility, Centre for Applied Molecular Biology, Lahore Pakistan. Sanger sequencing/chain termination sequencing was done in order to target the specific exons and to identify disease causing genetic variants. Dideoxy sequencing uses a template DNA

strand to create a complementary DNA strand. Deoxyribonucleoside triphosphates (dNTPs) and modified dideoxy ribonucleoside triphosphates (ddNTPs) are used to extend DNA strands in the sequencing reaction. The ddNTPs are modified chemically to cause DNA polymerase to halt DNA extension at the site of incorporation by adding a fluorescent label and a chemical group that inhibits phosphodiester bond formation. Capillary electrophoresis separates the DNA fragments by size by moving them through a gel-like matrix at varying rates. There is a unique fluorescent label on each of the four modified ddNTPs. The nucleotide in the DNA template can be identified by the fluorescence signal provided by each activated fluorescent dye.

2.12. Sequencing Data Analysis

The sequenced data was analysed by using Sequencher 5.4.6 software. Pathogenicity prediction for each variant was done by various bioinformatics tools named MutationTaster (http://www.mutationtaster.org/) , Mutalyser (https://mutalyzer.nl/), polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) , PROVEAN (http://provean.jcvi.org/index.php) , Mutation assessor (http://mutationassessor.org/r3/) , and SIFT (http://sift.jcvi.org/) and public database frequency was also determined. HSF (Human Splice Site Finder) software version 3.0 (www.umd.be/ HSF3/) was used to determine effects of sequence variations on exonic splicing signals". Public databases including ExAC (http://exac.broadinstitute.org/), 1000 Genomes Browser (http://browser.1000genomes.org/index.html) and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) were consulted to determine if the variants were rare.

2.13. Molecular analysis of Obstetric Cholestasis patients by ARMS PCR

An extremely hazardous obstetrical complication is ICP that can result in intrauterine foetal death (Stoilov and others 1997). To link the gene's variation, a thorough literature review was done. The *ABCB4* gene, which includes 28 exons, is found on chromosome 7.

2.13.1. Candidate gene sequencing approach

Intrahepatic Cholestasis of Pregnancy (ICP) is a rare pregnancy specific disorder. Genetic variants of *ABCB4* gene increase ICP risk. This study was conducted to determine

frequency of ICP cases presented at a tertiary care hospital in Rawalpindi, Pakistan and to screen for genetic variants of exon 6 and 14 of *ABCB4* gene in ICP cases. The ensemble genome browser (http://asia.ensembl.org/index.html) was used to find the *ABCB4* sequence. For exons 6 and 14 through the flanking regions of the exons, including intron-exon junctions, primers were made by Primer 1 tool for ARMS PCR (Table 2.4). The specificity of each primer was evaluated using the Blast like alignment tool (BLAT) (http://genome.ucsc.edu/cgi-bin/hgBlat), and primers that produced a single hit were chosen. Additionally, in-silico PCR was performed (https://genome.ucsc.edu/cgi-bin/hgPcr).

Table 2.4a. Primers used in tetra arms PCR amplification of c.504 C>T exon 6

No.	Primer Name	5'-3' Sequence	Bps	Tm	GC%	Product size
1	*ABCB4*-F inner-C	CGACAGGAAATAGGATGGTTTGACATCCAC	30	62	46	209
2	*ABCB4*-F-outer	TCAGGATTGGGTGCTGGAGTTCTTGTTG	28	61	50	322
3	*ABCB4*-R-inner-T	TAGCCGCGTATTGAGTTCAGTGGTGGCA	28	63	53	171
4	*ABCB4*-R-outer-	TGCTAGACATGGCTGCCAGATGATCGAT	28	61	50	322

Table 2.4b. Primers used in tetra arms PCR amplification of c.1686 A>G exon 14

No.	Primer Name	5'-3' Sequence	Bps	Tm	GC%	Product size
1	*ABCB4*-F inner-A	CTTCTGCTGGATGAGGCCACGTCATCA	27	55	63	166
2	*ABCB4*-F-outer	CTGAGTGGTGGGCAGAAGCAGAGGATC	27	62	59	228
3	*ABCB4*-R-inner-G	TGTACCTCAGCTTCACTTTCTGTGTCCCAC	30	63	50	119
4	*ABCB4*-R-outer-	TTTCTGTTTCTCAGCCCAGACTCCGGAA	28	61	50	228

2.14. Amplification of DNA fragments using ARMS PCR (Intrahepatic Cholestasis)

DNA amplification was done using PCR tubes 200µl (Axygen, USA). PCR reaction mix includes following constitute.

Table 2.5. Ingredients used in arms PCR amplification

Chemical Used	Concentration of Chemicals	Required Volume
PCR Buffer	10-X(200mM(NH4)2SO4, Tris-HCL 750mM (pH8.8), 0.1% Tween20	2.5µl
MgCl2	25mM	2.5µl
dNTPs	10mM	0.5µl
Outer Primers F/R	20ng/µl	1µl
Inner Primers F/R	20ng/µl	2µl
Taq Polymerase	0.5U/µl	0.5µl
Genomic DNA	-	2.5µl
PCR Water	-	13.5µl
Total Volume	-	25µl

Pouring of PCR water at the end was done to adjust the final volume to 25µl. After adding PCR mixture in each tube, tubes were put in the Thermo-cycler (Biometra, Germany) for amplification of DNA fragments. Following were thermos cycling conditions for PCR:

DNA Template denaturation for 10 min at 95°C

Amplification cycle, (39 cycles)

i. Denaturation for 40 sec at 95°C

ii. Primer annealing for 60 sec at 68°C with decrement of 1°C each cycle followed by 58°C annealing (68-58 touchdown).

iii. Primer extension for 60 sec at 72°C

- Final extension for 10 min at 72°C
- Holding at 25°C

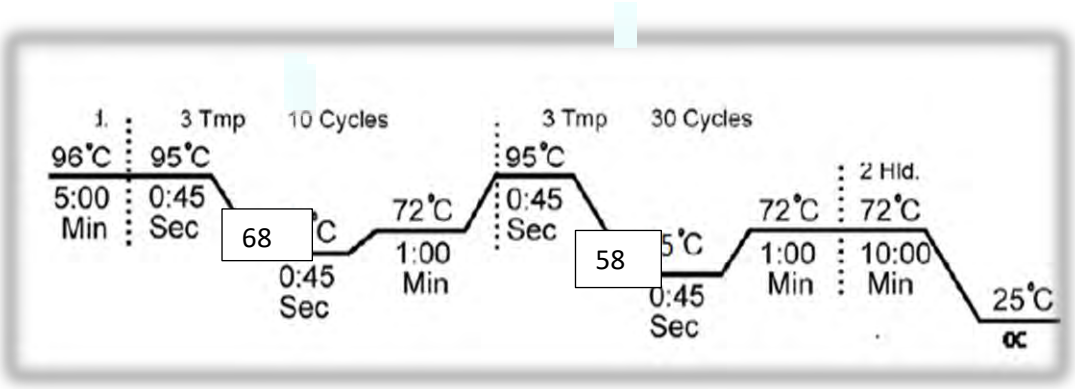


Figure 2.1 Thermo-cycler programs for ARMS PCR (ICP)

2.15. Agarose gel electrophoresis for ARMS PCR results interpretation

For the analysis in a qualitative manner 2% agarose gel was used. For preparation of 2% agarose gel 0.75g of powdered agarose was added to make a mix in 50 mL of 1X TBE buffer using conical flask.

- For 1X TBE preparation, 900 ml of the distilled water and 100mlof 10X TBE were thoroughly. Mixture was further heated for 60 seconds in a microwave oven and then cooled down.
- 5µL of Ethidium-bromide (EtBr) dye was mixed in gel mixture for staining of the DNA.
- Pouring of mixture was done in the gel casting tray and reserved then for 20 to 30 minutes for solidification.
- After the gel was loaded with 4 µL of DNA sample and 2 µL of loading dye (0.25 % bromophenol blue with 40 % sucrose), it was electrophoresed in a gel tank (Biometra, Germany). After the gel tank was filled with TBE buffer (1X), a current of 120 volts was applied for 25 minutes.
- Gel documentation system (Biometra, Gottingen, Germany) was used to visualize arms PCR bands.

CHAPTER 3

3. RESULTS

The aim of the study was the genetic screening and clinical analysis of selected metabolic liver disorders i.e., Wilson's disease, Glycogen storage disease and intrahepatic cholestasis, cases among local population. To achieve study objectives patients were enrolled from i.e., Pakistan Institute of Medical Sciences PIMS, Children hospital Lahore CHL and Wah General Hospital. A total of 80, 40 and 50 samples were collected for Wilson's disease, Glycogen storage disease and Intrahepatic Cholestasis respectively. Blood samples were collected for the molecular analysis, clinical data and demographic details were recorded via designed questionnaire for statistical analysis. For molecular analysis of Wilson's disease gene specific primers were designed for Sanger sequencing of selected exons of copper-activated P-type ATPases *ATP7B* gene. 20 patients were sequenced for the selected hotspot exons of *ATP7B* gene, depending upon the availability of funds, availability of blood samples and consent of the patients for participating in the molecular analysis. Glucose-6-phosphatase *G6PC* gene was completely sequenced in 20 Glycogen storage disease 1a cases by exon wise Sanger sequencing method. For Intrahepatic Cholestasis of pregnancy, the presence of reported polymorphisms in the *ABCB4* gene were checked by Tetra ARMS PCR method. Clinical and demographic data processing and analysis were made using SPSS 20.0.

3.1. CLINICAL PRESENTATION AND GENETICS OF WILSON'S DISEASE CASES FROM LOCAL POPULATION

3.1.1. Baseline characteristics of study participants

80 patients with Wilson's disease diagnosed by expert physicians were enrolled in the study. Clinical data was recorded by predesigned questionnaire (Annexure II) investigation was done in cases and statistical analysis was performed. Direct sequencing of both the forward and reverse directions of the exon 1, 2, 3, 4, 5, 8 & 13 in selected patients with WD was performed to screen for disease causing variants.

3.1.2. Analysis of clinical and demographic data

Wilson's disease is difficult to diagnose at first glance due to the lack of specificity of its early symptoms, and thus, patients often go without penicillamine treatment for too long. When symptoms of hepatic failure have already appeared, the response to chelation therapy

is frequently poor (Nazer *et al*., 1983). Indeed, infectious hepatitis is typically blamed for early-onset jaundice in children, but chronic liver disease is largely disregarded as a possible cause (Silverberg & Gellis, 1962).

Serum ceruloplasmin 0.2 g/l (normal range 0-2 to 0-6 g/l) and/or urine copper excretion >1.25 umol/24 h with an elevated hepatic copper (>300 ug/g dry liver) were used to make a diagnosis. WD was detected based on characteristic clinical features including high urinary copper levels, Steatosis was observed in 68 patients (85%), Cirrhosis was presented in 31 cases (i.e. 38%), and Jaundice was seen in 70 cases (i.e. 87.5). Elevated copper in the liver, aberrant MRI findings, and a low ceruloplasmin serum level.

Eighty patients with Wilson's disease were seen in the Paediatric Department and Liver Unit of Pakistan institute of medical sciences (PIMS), and Children Hospital Lahore (CHL). All enrolled patients showed autosomal recessive inheritance of the WD, as evidenced by the generation skip seen in both affected people with unaffected parents.

Of the eighty cases 48 had only hepatic involvement (i.e. 62 %), twelve had only neurological (15%) symptoms, four were asymptomatic (5%) and sixteen had both hepatic and -neurological manifestations (i.e. 20 %). Seizures were seen in 17 cases (i.e. 21.2%), Paralysis in 2 patients (i.e. 2.5), Decreased alertness in 28 cases (i.e. 35%), dysarthria in 13 cases (i.e. 16.2%), ataxic gait in 6 cases (i.e. 7.5%), Abnormal saliva flow in 6 cases (i.e. 7.5%), blood vomiting in 16 cases (i.e. 20%), depression in 28 cases (i.e. 35%), hepatic necrosis in 71 cases (i.e. 88.8%), psychosis in 12 cases (i.e. 15%), irritability in 29 cases (i.e. 36.2%), parkinsonism in 20 cases (i.e. 25%). The Table 3.1.1 provides a summary of the demographic and clinical characteristics of each proband. Out of 80 enrolled cases, 40 (50%) were males and 40 (50%) were females i.e., the male to female ratio was 1:1.

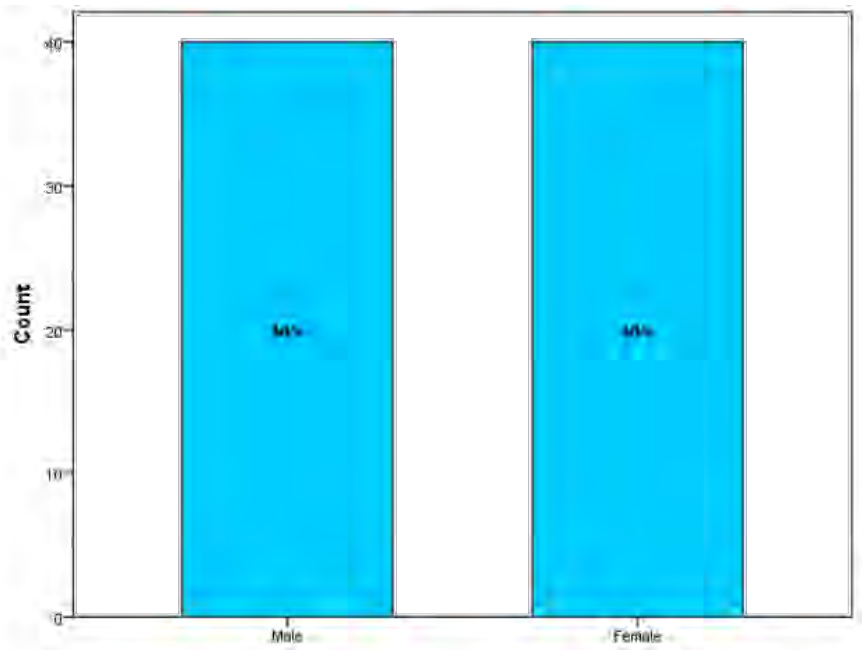


Figure 3.1.1: Bar Diagram for Gender of Respondents (WD)

The bar chart in Figure 3.1.1 displays the distribution of the male and female respondents.

Parental cousin marriage is the reason of prevailing recessive disorders in Pakistan like Wilson's disease. The bar chart in Figure 3.1.2 provides the percentages of the respondents whose parents have cousins' marriage. The above result shows that most of the respondent's (80%) parents have cousin marriage and the rest 20% haven't parental cousin marriage.

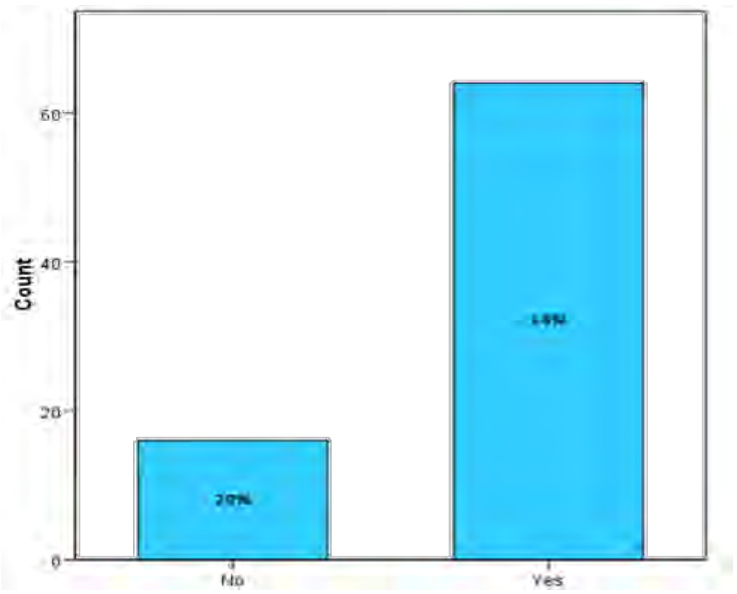


Figure 3.1.2: Bar Chart Showing the Status of Parental Cousin Marriage (WD)

Figure 3.1.3 gives the information about the respondents who have the family history of WD. The bars of the bar chart display that 52.50% of the respondents haven't any family history of WD and 47.50% of the respondents have the family history of the disease.

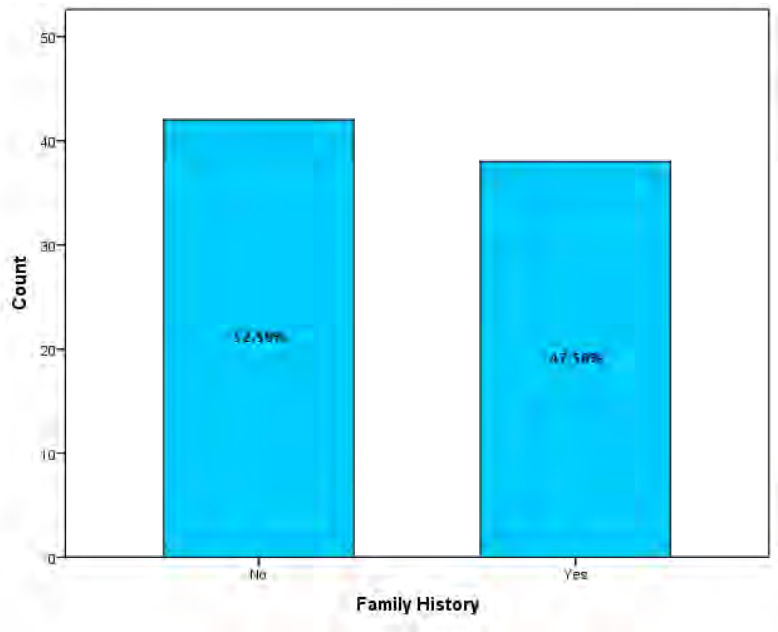


Figure 3.1.3: Bar Chart to show the Family History of WD

Figure 3.1.4 provides the information about the respondents who develops the kayser fleischer rings. The result shows that the 41.25% haven't developed kayser fleischer rings and the remaining 58.75% have kayser fleischer rings.

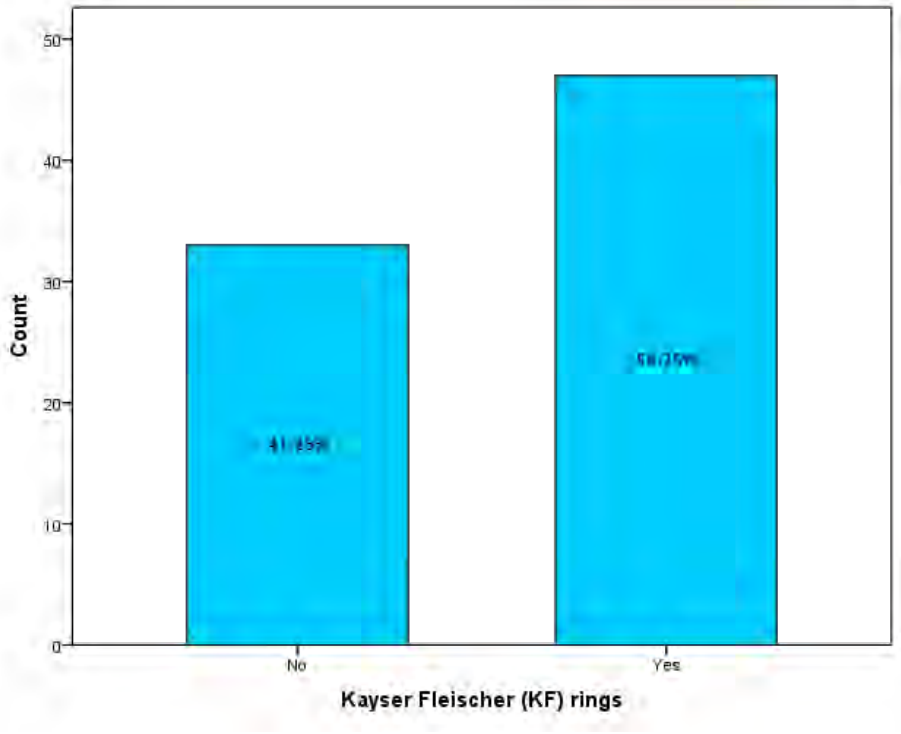


Figure 3.1.4: Bar Chart for showing the Kayser Fleischer rings (WD)

Diagnosis of WD occurred between the ages of 5 and 25 in the probands of families that were included. An average of onset in cases enrolled in this study was 11.3 years. The pie chart in Figure 3.1.5 provides the information about the respondents age at the time of diagnosis in different age groups. The result show that most of the respondent (52.50%) age at the time of diagnosis was between 6 to 10 years. It also observed that the maximum respondents ages at the time of diagnosis are the below 15 years.

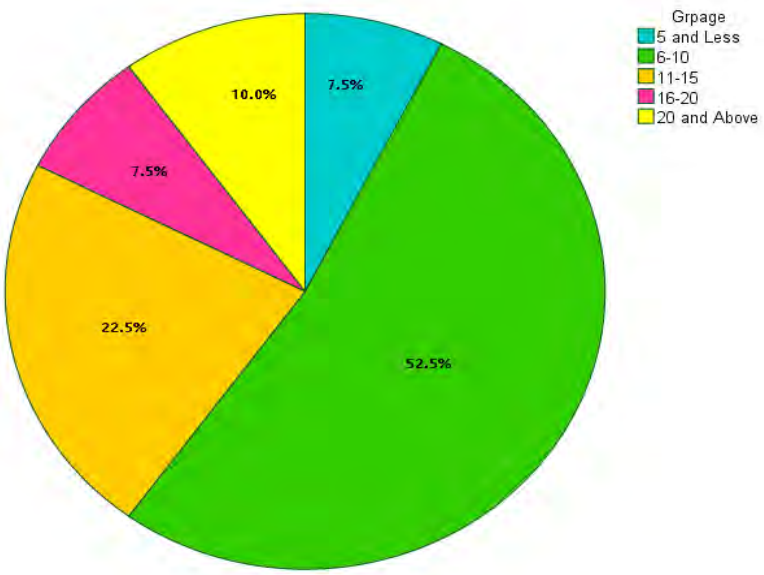


Figure 3.1.5. Pie Chart for showing the respondent age at diagnosis of WD

Figure 3.1.6 gives the information of the respondent family education. Pie charts show that 55.00%, 26.25% and 18.75% of the respondent's families have higher secondary education, upper/post-secondary and vocational education, respectively. Furthermore, most of the respondent's families have higher secondary education.

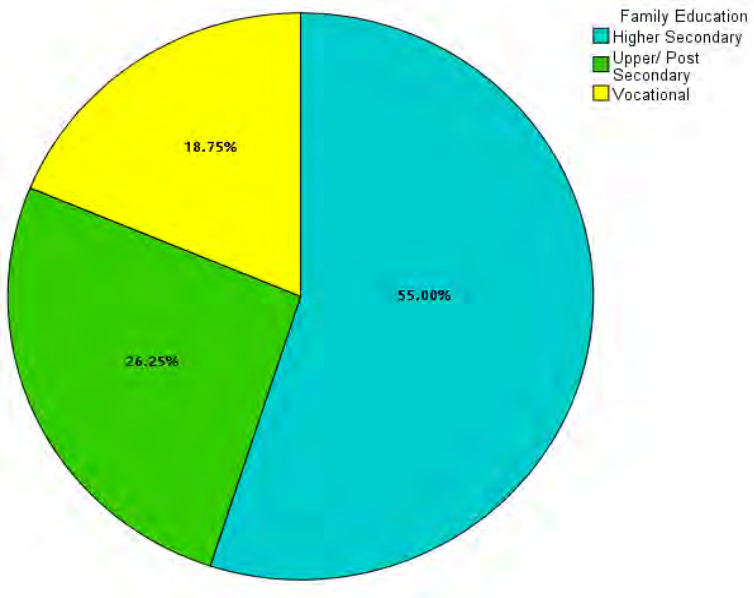


Figure 3.1.6: Pie Chart for showing the respondent family education at diagnosis of WD

Pie chart in Figure 3.1.7 provides the information of the respondent whose families are supportive towards treatment. The result shows that 55.00%, 23.75% and 21.25% are respectively, negative, neutral and supportive. It also displayed that most respondent's family position towards treatment is negative.

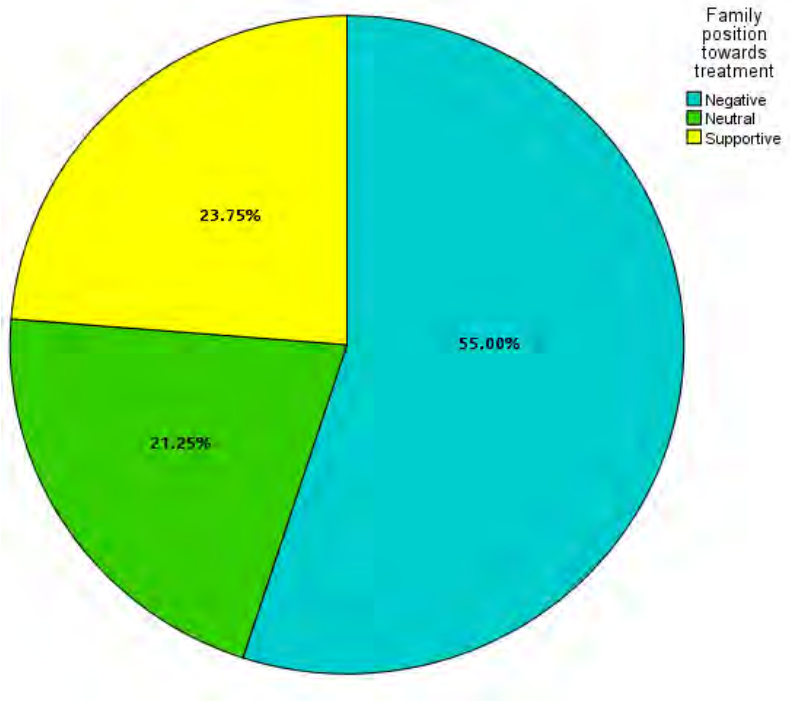


Figure 3.1.7: Pic Chart for showing family position towards treatment of WD

Figure 3.1.8 displays the information of the respondent whose families have the knowledge about WD. The chart above shows that 58.75%, 21.25% and 20.00% are the families who have good, little and moderate knowledge about WD. It further indicated that a very less percentage of the respondent's families have knowledge of WD.

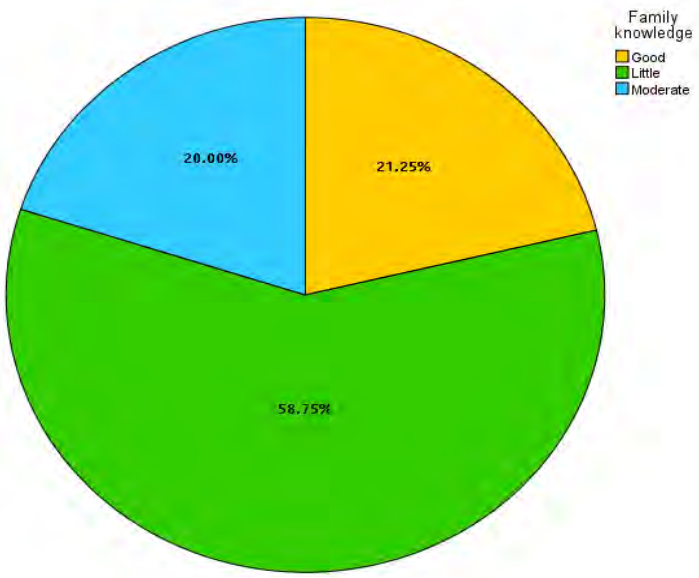


Figure 3.1.8. Pie Chart for showing the respondent's family knowledge about WD

Figure 3.1.9 gives the information of the diagnostic test of the WD. It shows that 92.41% of the diagnosis of the of WD through CP/UC level and remaining 7.59% through Biopsy.

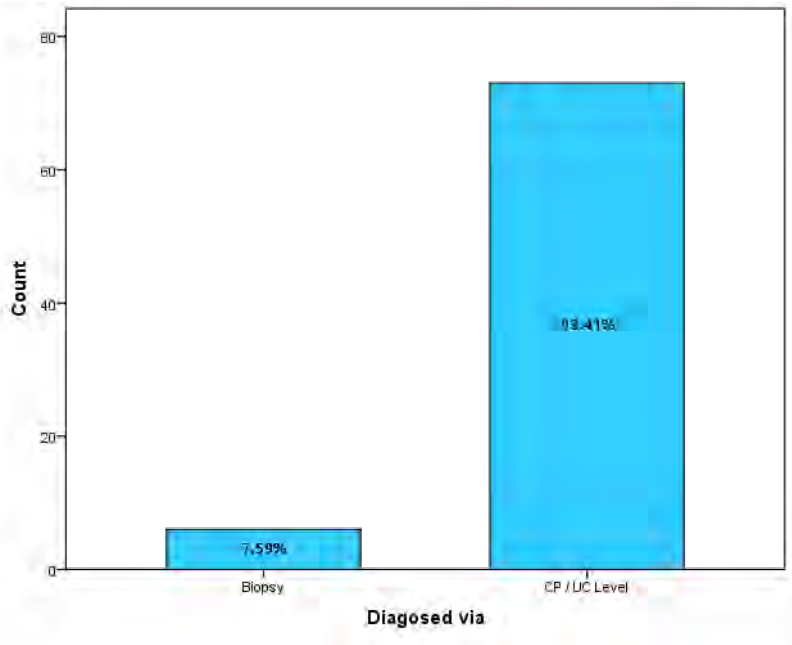


Figure 3.1.9. Bar Chart to display the diagnostic test of WD

Wilson's disease is categorised in 4 stages based on symptoms. Figure 3.1.10 displays the information of the respondent stage of illness. The overall result show that 15.0%, 57.5%, 15.0% and 12.5% are at stage-I, stage-II, stage-III and stage-IV, respectively. It further indicated that most of are at the stage-II.

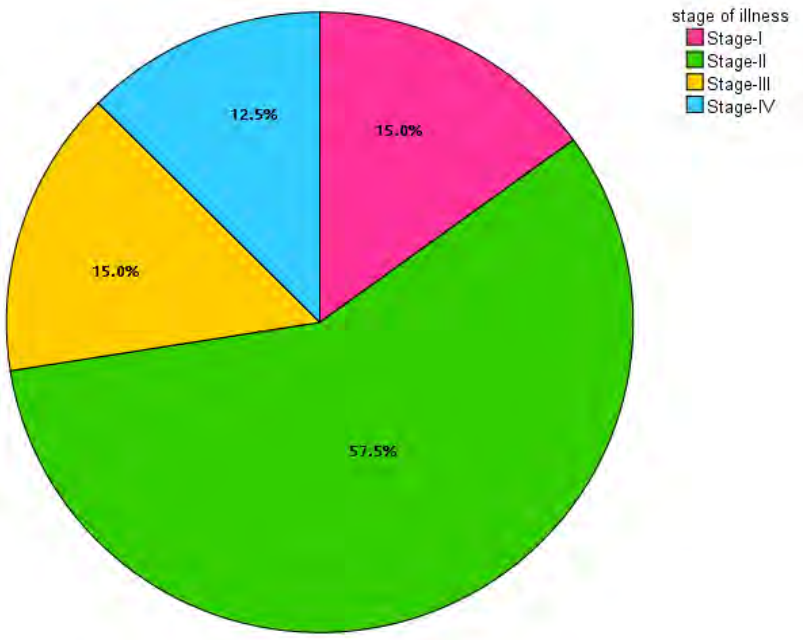


Figure 3.1.10. Pie Chart for showing the stages of WD

Figure 3.1.11 gives the information of the respondent who received therapy. The bars show that 65.0%, 21.3%, 12.5% and 1.3% received Trientine therapy, Zink Supplements therapy, 12.5% Penicillamine therapy and liver transplant, respectively.

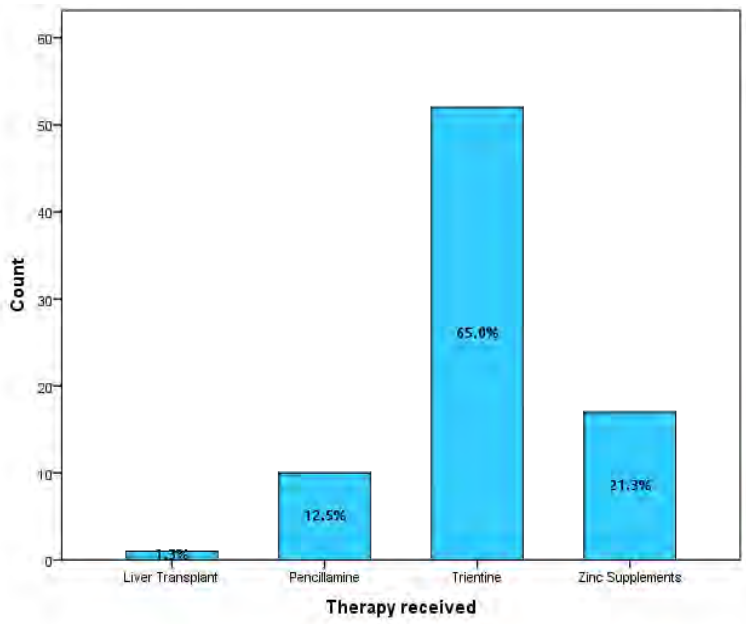


Figure 3.1.11. Bar Chart for displaying the therapies respondents received (WD)

The bar chart in Figure 3.1.12 gives the information about the reversing symptoms. Results shows that 76.25% have not reversing symptoms while 23.75% have reversing symptoms.

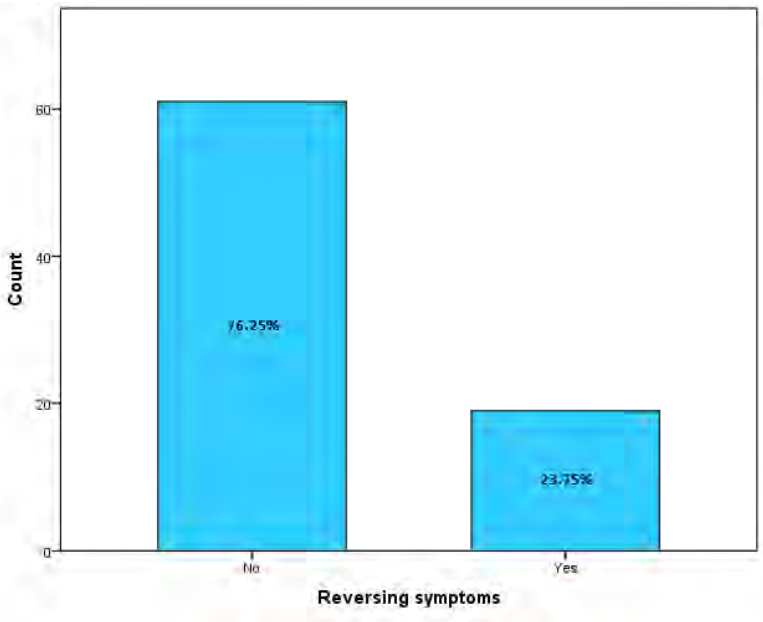


Figure 3.1.12. Bar Chart for displaying the respondents reversing symptoms (WD)

3.1.3. Data of Clinical Variables of enrolled WD cases

Patients having neurological illness were above 12 years of age, Children below 12 years mostly had hepatic disorders; with the mean age of onset of 11.3years. Figure 3.1.13 represents younger age of onset of patients with initial liver disease, compared to patients with neurological manifestations in a Pakistani cohort.

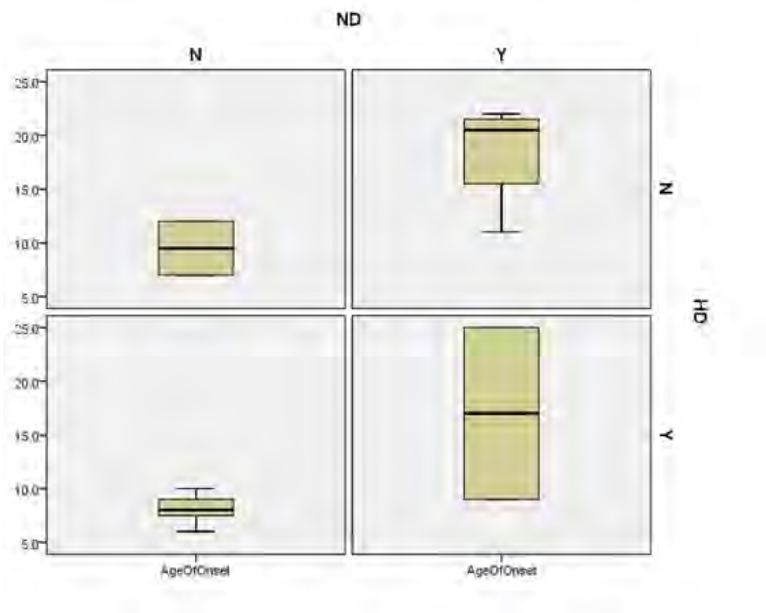


Figure 3.1.13. Box plot demonstrating younger age of onset of patients with initial liver disease, compared to patients with neurological manifestations in our Pakistani cohort

For WD significance of risk factors were checked among persistent and non-persistent groups. To check the significance of qualitative variables chi square test was used. For variables whose frequency was <5, Irwin Fisher's Exact test was used. For quantitative variables Shapiro-Wilk test was used to test the normality of data. Table 3.1.1 provide the results of the treatment Outcome by treatment Persistence. A sample of $n = 80$ is taken of which half are male and half are female respondents. The results showed that 20% are persistent for treatment procedures and rest of 80% are non-persistent. In 20% of persistent group 43.75% are male and 43.75% are female and similarly, in non-persistent 51.56% and 48.44% are male and female, respectively. The p-value (0.576) against the Chi-square test showed that there is no association among the gender with the persistent and nonpersistent groups (p>0.05). The average age group of all respondents lies within

the interval of 11.30 ± 5.44. The average age groups of persistent and nonpersistent respondents are, respectively 12.09 ± 4.58 and 10.56 ± 5.08. The p-value (0.014) of the t-test showed the significance of the ages diagnosis groups (p<0.05). 23.8% respondents are those whose families are supportive; 55% families are not supported for taking treatment. 93.8% are persistent belongs to supportive family and 68.8% nonpersistent are from nonsuppurative family which shows that there is a strong relationship ($p = 0.0000 < 0.01$) between family support towards treatment and drug persistence. Maximum respondents (55.0%) have higher education of 43.8% and 57.8% are, respectively from persistent and nonpersistent. 26.2% and 18.8% respondents have post-secondary (31.2% are persistent and 25.0% are nonpersistent) and vocational (17.2% are persistent and 25.0% are nonpersistent), respectively. P-value of the test is 0.586>0.05 showed that the family education isn't associated with drug persistent. The 21.2% (persistent=87.4%, nonpersistent=4.7%), 20.0% (persistent=12.5%, nonpersistent=21.9%), and 58.8% (persistent=0.0%, nonpersistent=73.4%) are respondents' families have good, moderate, and little knowledge about WD, respectively. The p-value (0.0000) of the test showed that the highly significant relationship between family knowledge about WD and drug usage in our study cohort. The cross tabulation of stage of illness and persistent displays that maximum respondents are at stage-II 57.5% from which 43.8% persistent and 60.9% are nonpersistent. Also, the p=0.120 showed the insignificance of the drug persistence and stage illness at 5% level.

Table 3.1.1. Association of drug persistence with awareness regarding disease and improvement of symptoms

	Total	**Persistent**	**Non-Persistent**	***p-value***
Population n (%)	80 (100%)	16 (20%)	64 (80%)	
Age at Diagnosis $Mean \pm SD(years)$	11.30 ± 5.44	12.09 ± 4.58	10.56 ± 5.08	0.014
Family Position Towards Treatment				0.000
Negative n(%)	44 (55%)	0 (0.0%)	44 (68.8%)	
Neutral n(%)	17 (21.2%)	1 (6.2%)	16 (25.0%)	
Supportive n(%)	19 (23.8%)	15 (93.8%)	4 (6.2%)	
Family Education				0.586
Higher n(%)	44 (55.0%)	7 (43.8%)	37 (57.8%)	
Upper/ Post-Secondary n(%)	21 (26.2%)	5 (31.2%)	16 (25.0%)	
Vocational n(%)	15 (18.8%)	4 (25.0%)	11(17.2%)	
Family Knowledge about WD				0.000
Good n(%)	17 (21.2%)	14 (87.4%)	3 (4.7%)	
Moderate n(%)	16 (20.0%)	2 (12.5%)	14 (21.9%)	

Little $n(\%)$	47 (58.8%)	0 (0.0%)	47 (73.4%)	
Stage of Illness				0.120
Stage-I $n(\%)$	12 (15.0%)	1 (6.2%)	11 (17.2%)	
Stage-II $n(\%)$	46 (57.5%)	7 (43.8%)	39 (60.9%)	
Stage-III $n(\%)$	12 (15.0%)	5 (31.2%)	7 (10.9%)	
Stage-IV $n(\%)$	10 (12.5%)	3 (18.8%)	7 (10.9%)	
Steatosis				0.554
Yes $n(\%)$	68 (85.0%)	14 (87.5%)	54 (84.4%)	
No $n(\%)$	12 (15.0%)	2 (12.4%)	10 (15.6%)	
Cirrhosis				0.108
Yes $n(\%)$	31 (38.8%)	9 (56.2%)	22 (34.4%)	
No $n(\%)$	49 (61.3%)	7 (43.8%)	42 (65.6%)	
Jaundice				0.153
Yes $n(\%)$	70 (87.5%)	14 (87.5%)	56 (87.5%)	
No $n(\%)$	4 (5.0%)	2 (12.5%)	2 (3.1%)	
NA $n(\%)$	6 (7.5%)	0 (0.0%)	6 (9.4%)	
Total Serum Bilirubin $Mean \pm SD$	7.68 ± 7.11	6.63 ± 6.36	7.94 ± 7.305	0.210
Alanine Transaminase $Mean \pm SD\ (u/l)$	195.71 ± 269.31	159.31 ± 280.38	204.81 ± 267.99	0.691
Aspartate Transaminase $Mean \pm SD$	266.33 ± 407.31	191.44 ± 152.79	285.05 ± 447.99	0.732
Urinary Copper $Mean \pm SD(\mu g/24hr)$	1191.21 ± 279.96	1261.19 ± 243.16	1173.72 ± 2287.86	0.266
Serum Ceruplasmin Level $Mean \pm SD$	2.26 ± 5.61	2.13 ± 5.81	2.30 ± 5.60	0.689
Seizures				0.002
Yes $n(\%)$	17 (21.2%)	8 (50.0%)	9 (14.1%)	
NA $n(\%)$	63 (68.8%)	8 (50.0%	55 (85.9%)	
Paralysis				0.638
Yes $n(\%)$	2 (2.5%)	0 (0.0%)	2 (3.1%)	
NA $n(\%)$	78 (97.5%)	16 (100%)	62 (96.9%)	
Decreased Alert				0.010
Yes $n(\%)$	28 (35.0%)	10 (62.5%)	18 (28.1%)	
NA $n(\%)$	52 (65.0%)	6 (37.5%)	46 (71.9%)	
Poor Cognitive Ability				0.015
Yes $n(\%)$	29 (36.2%)	10 (62.5%)	19 (29.7%)	
NA $n(\%)$	51 (63.7%)	6 (37.5%)	45 (70.3%)	
Dysarthria				0.448
Yes $n(\%)$	13 (16.2%)	1 (6.2%)	12 (18.8%)	
No $n(\%)$	67 (83.8%)	15 (93.8%)	52 (81.2%)	
Ataxic Gait				0.594
Yes $n(\%)$	6 (7.5%)	2 (12.5%)	4 (6.2%)	
No $n(\%)$	74 (92.5%)	14 (87.5%)	60 (93.8%)	
Abnormal Saliva Flow				0.594
Yes $n(\%)$	6 (7.5%)	2 (12.5%)	4 (6.2%)	
No $n(\%)$	74 (92.5%)	14 (87.5%)	60 (93.8%)	
Blood Vomiting				0.136
Yes $n(\%)$	16 (20.0%)	6 (37.5%)	10 (15.6%)	
No $n(\%)$	1 (1.2%)	0 (0.0%)	1 (1.6%)	
NA $n(\%)$	63 (78.8%)	10 (62.5%)	53 (82.8%)	

Depression				0.815
Yes $n(\%)$	28 (35.0%)	5 (31.2%)	23 (35.9%)	
No $n(\%)$	51 (63.7%)	11 (68.8%)	40 (62.5%)	
NA $n(\%)$	1 (1.2%)	0 (0.0%)	1 (1.2%)	
Hepatic Necrosis				1.000
Yes $n(\%)$	71 (88.8%)	14 (87.5%)	57 (89.1%)	
No $n(\%)$	9 (11.2%)	2 (12.5%)	7 (10.9%)	
Psychosis				0.005
Yes $n(\%)$	12 (15.0%)	6 (37.5%)	6 (9.4%)	
No $n(\%)$	68 (85.0%)	10 (62.5%)	58 (90.6%)	
Irritability				0.642
Yes $n(\%)$	29 (36.2%)	5 (31.2%)	24 (37.5%)	
No $n(\%)$	51 (63.7%)	11 (68.8%)	40 (62.5%)	
Reduction in Amount of CU Through Diet				0.685
Yes $n(\%)$	69 (86.2%)	13 (81.2%)	56 (87.5%)	
No $n(\%)$	11 (13.8%)	3 (18.8%)	8 (12.5%)	
Penicillamine-Induced Systematic Lupus Erythematosus				1.000
Yes $n(\%)$	4 (5.0%)	1 (6.2%)	3 (4.7%)	
No $n(\%)$	76 (85.0%)	15 (93.8%)	65 (95.3%)	
Penicillamine Intolerance				1.000
Yes $n(\%)$	10 (12.5%)	2 (12.5%)	8 (12.5%)	
No $n(\%)$	70 (87.5%)	14 (87.5%)	56 (87.5%)	
Reversing Symptoms				0.514
Yes $n(\%)$	19 (23.8%)	5 (31.2%)	14 (21.9%)	
No $n(\%)$	61 (76.2%)	11 (68.8%)	50 (78.1%)	
Parkinsonism				0.053
Yes $n(\%)$	20 (25.0%)	7 (43.8%)	13 (20.3%)	
No $n(\%)$	60 (75%)	9 (56.2%)	51 (79.7%)	
Therapy Received				0.253
Liver Transplant $n(\%)$	1 (1.2%)	1 (6.2%)	0 (0.0%)	
Penicillamine $n(\%)$	10 (12.5%)	2 (12.5%)	8 (12.5%)	
Trientine $n(\%)$	52 (65.0%)	10 (62.5%)	42 (65.6%)	
Zinc Supplements $n(\%)$	17 (21.2%)	3 (18.8%)	14 (21.9%)	
Zinc Treatment				0.736
Yes $n(\%)$	17 (21.2%)	4 (25.0%)	13 (20.3%)	
NA $n(\%)$	63 (78.8%)	12 (75.0%)	51 (79.7%)	
Trientine				1.000
Yes $n(\%)$	52 (65.0%)	10 (62.5%)	42 (65.6%)	
NA $n(\%)$	28 (35.0%)	6 (37.5%)	22 (34.4%)	
Penicillamine				1.000
Yes $n(\%)$	10 (12.5%)	2 (12.5%)	8 (12.5%)	
NA $n(\%)$	70 (87.5%)	14 (87.5%)	56 (87.5%)	
Factors that Influence the Treatment Adherence				0.000
Adverse Effect $n(\%)$	19 (35.2%)	1 (6.2%)	18 (47.4%)	
Education Level $n(\%)$	1 (1.9%)	0 (0.0%)	1 (2.6%)	
Finance $n(\%)$	19 (35.2%)	0 (0.0%)	19 (50.0%)	
Positive Family Support $n(\%)$	15 (27.8%)	15 (93.8%)	0 (0.0%)	
Treatment Duration $Mean \pm SD(months)$	12.25 ± 4.73	12.09 ± 4.58	12.09 ± 4.58	0.557
Status				0.514
Alive $n(\%)$	19 (23.8%)	5 (31.2%)	14 (21.9%)	
Deceased $n(\%)$	61 (76.2%)	11 (68.8%)	50 (78.1%)	

*SD=standard deviation *NA=not adopted

Table 3.1.2: Risk factor analysis with stage of illness of disease (WD)

		Stage of illness				
Risk factors		**Stage I**	**Stage II**	**Stage III**	**Stage IV**	**p value**
Age at diagnosis	5 or less	1(1.3%)	5 (6.3%)	0	6(7.5%)	0.00012
	6-10	9 (11.3%)	30(37.5%)	0(0%)	42(52.5%)	
	11-15	2(2.5%)	8(10.0%)	4(5%)	18(22.5%)	
	16-20	0(0%)	0	4(5%)	6(7.5%)	
	20 above	0	3	4(5%)	8(10%)	
Therapy received	Liver transplant	0 (0%)	0 (0%)	0(0%)	1 (1.3%)	0.016
	Penicillamine	2(2.5%)	8(10%)	0(0%)	0(0%)	
	Treatine	5(6.3%)	27(33.8%)	12(15%)	8(10%)	
	Zinc supplements	5(6.3%)	11(13.8%)	0(0%)	1(1.3%)	

Table 3.1.2 provides the risk factor analysis of the WD at different stages of illness, like stage-I, stage-II, stage-III and stage-IV. Stages of illness have a strong relationship with the group ages at 1% level. With the group ages 5 or less, 6-10, 11-15, 16-20 and 20 above 1.3%, 11.3%, 2.5%, 0% and 0% are respectively, at stage-I. Similarly, all results for stage-II to stage-III of different group ages are given in Table 3.2. Only 1.3% has received liver transplant therapy at stage-IV. 2.5% and 10% of the respondents have received penicillamine therapy at stage-I and stage-II, respectively. The respondents who received treatine therapy at stage-I, stage-II, stage-III and stage-IV are respectively, 6.3%, 33.8%, 15% and 10%. The percentages 6.3%, 13.8%, 1.3% of the respondents have received Zinc supplements therapy at stage-I, stage-II, and stage-IV, respectively. The p-value 0,016 of the chi-square test show the significance of the stages of illness with the therapy received at 5% level of significance.

Table 3.1.3 represents the risk factor analysis with the status alive or deceased. For group ages 5 or less, 6-10, 11-15, 16-20 and 20 above, 2.5% alive and 5% deceased, 13.8% alive and 38.8% deceased, 5% alive and 17.5% deceased, 0% alive and 7.5% deceased, 2.5% alive and 7.5% deceased, similarly. The p-value of the chi-square test shows the insignificance of the status alive/deceased with different age groups. Majority (76.2%) of the patients who received therapy are deceased. 23.8% of the patients who received therapy are alive. The p-value 0.163 of the test statistic shows the independence of the status from received therapy. The percentage of the alive patients at stage-I and stage-II are 11.3%, 1.3% are alive at stage-IV and no one is alive at the stage-III. Similarly, the percentages of deceased at stage-I, stage-II, stage-III and stage-IV are respectively, 3.8%, 46.3%, 15.0% and 11.3%. the 46.3% show that most of the patients diseased at stage-II. The p-value of the test is 0.0000 which shows the significance of the status alive/diseased with the stages of illness.

Table 3.1.3. Risk factor analysis with survival of patients with (WD)

Risk factors		**Status**		
		Alive	**Decreased**	**p value**
Age groups	5 or less	2(2.5%)	4(5%)	0.673
	6-10	11(13.8%)	31(38.8%)	
	11-15	4(5%)	14(17.5%)	
	16-20	0(0%)	6(7.5%)	
	20and above	2(2.5%)	6(7.5%)	
Therapy	Transplant	1(1.3%)	0(0.0%)	0.163
	Penicillamine	2(2.5%)	8(10.0%)	
	Treatine	10(12.5%)	42(52.5%)	
	Zinc supplements	6(7.5%)	11(13.8%)	
Stage of illness	Stage I	9(11.3%)	3(3.8%)	0.000
	Stage II	9(11.3%)	37(46.3%)	
	Stage III	0(0.0%)	12(15.0%)	
	Stage IV	1(1.3%)	9(11.3%)	

3.1.4. Genetic screening of selected exons of *ATP7B* gene (WD)

The study was carried out at Molecular Biology Lab, Quaid-I-Azam University, Islamabad, Pakistan. Twenty out of 80 enrolled WD patients along with available family members were included in molecular analysis, based on the availability of funds and willingness to participate molecular analysis. Blood of affected patients and their relatives was collected along with the genetic history and clinical data. Sampling was done after taking the informed written consent from patients and their family members (Annexure II). Unbiased patients pool i.e., Wilson patients with/without cousin marriage, Male/females were included. All the patients included had Global Assessment scale for Wilson Disease (GAS) score of 4 or more.

Phenol-chloroform isolation technique (organic method) was used for DNA extraction (Sambrook *et al*., 1989). Direct sequencing of selected hotspot exons of *ATP7B* gene of probands was done after amplification using primers mentioned in Table 2.2, to check reported and novel mutations. PCR amplification was carried out in the PCR tubes, 200 µL (Axygen, USA).

3.1.5. PCR based Sanger sequencing of *ATP7B* gene

Sequencing of selected exons of the *ATP7B* gene in 20 enrolled patients affected with Wilson's disease, revealed 22 variants in intronic, exonic and untranslated regions (UTR's) (Table 4). Out of 22 identified variants 7 were the potent disease-causing variants, and 15 polymorphisms as predicted by mutation taster. Out of 7 identified disease-causing variants six were in homozygous condition and one in heterozygous condition. 3 out of 7 identified disease-causing variant were first time reported in this study whereas, 4 are previously reported. The study report 5 known polymorphisms and 10 novel polymorphisms from local population chromatograms shown in Figure 3.1.17 & 3.1.18.

Novel disease causing variants include p. V761E (valine 761 leucine), c.2282T>A (WD-18) in exon 8 with Polyphen 2 score of 0.378 with predictive Damaging effect; p. I774L (Isoleucine 774 Leucine), c.2320A>C (WD-18,5,19) in exon 8 with a Polyphen 2 score of 0.994 with predictive Damaging effect; p.I976N (Isoleucine 976 Asparagine), c.2927T>A (WD-11) in exon 13, predicted to be Probably Damaging as per mutation taster and

Polyphen 2 prediction with score 1.000. (Figure 3.1.15). Insilico predictions of Novel disease-causing variant on protein structure through HOPE, is shown in (Figure 3.1.I5-B). The entire identified disease-causing variant have been found to affect Cu++ channel TMD (Ch/Tm4) of the protein. Segregation analysis showed that the disease-causing variants are inherited in recessive pattern.

Reported disease causing variants identified in the study include p. L227Yfs*35 exon 2 (WD-1), previously reported from Indian population by Aggarwal in 2013 (Aggarwal *et al*., 2013); reported disease causing variant p. A1003A (rs1801297) (WD-8 and 13) in exon 13 of *ATP7B* gene previously reported by (El-Mougy *et al*., 2014); reported disease causing variant p.D765N (Aspartic acid 765 Asparagine), (rs28942075) (WD-8) was identified in exon 8 that was previously reported from Iran in 2013 (Dastsooz, Dehghani, *et al*., 2013); reported disease causing variant p. S986T (WD-10) that was previously reported from Russia was found in exon 13 (Figure 3.1.16) (Dastsooz, Imanieh, *et al*., 2013).

Pathogenic-variation c.6T>C (p. L227Yfs*35), causes leucine converted to tyrosine and then frameshift and then stop codon after 35 nucleotides; detected in exon 2 of one *ATP7B* gene (WD-1), affecting Cu binding domain of the protein. According to Insilico analysis this variation is listed as Probably Damaging with Polyphen 2 score of 0.995. Human Splicing Finder (HSF) considered the variant as possible source of splicing alteration, through altering an exonic splicing silencer (ESS) site and breaking an exonic splicing enhancer (ESE) (Figure 3.1.14).

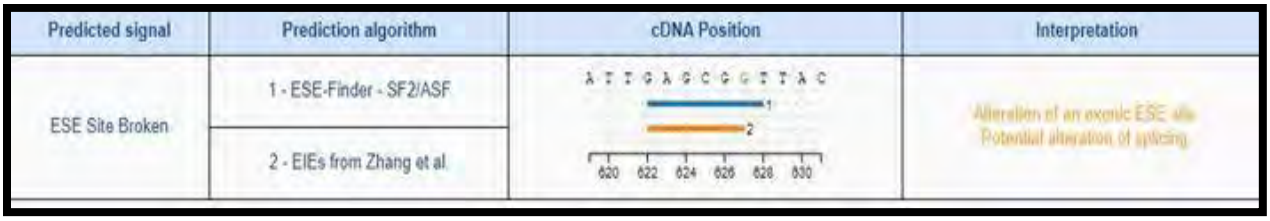


Figure. 3.1.14 Pathogenic effect prediction output of HSF program for mutation L227Yfs*35.

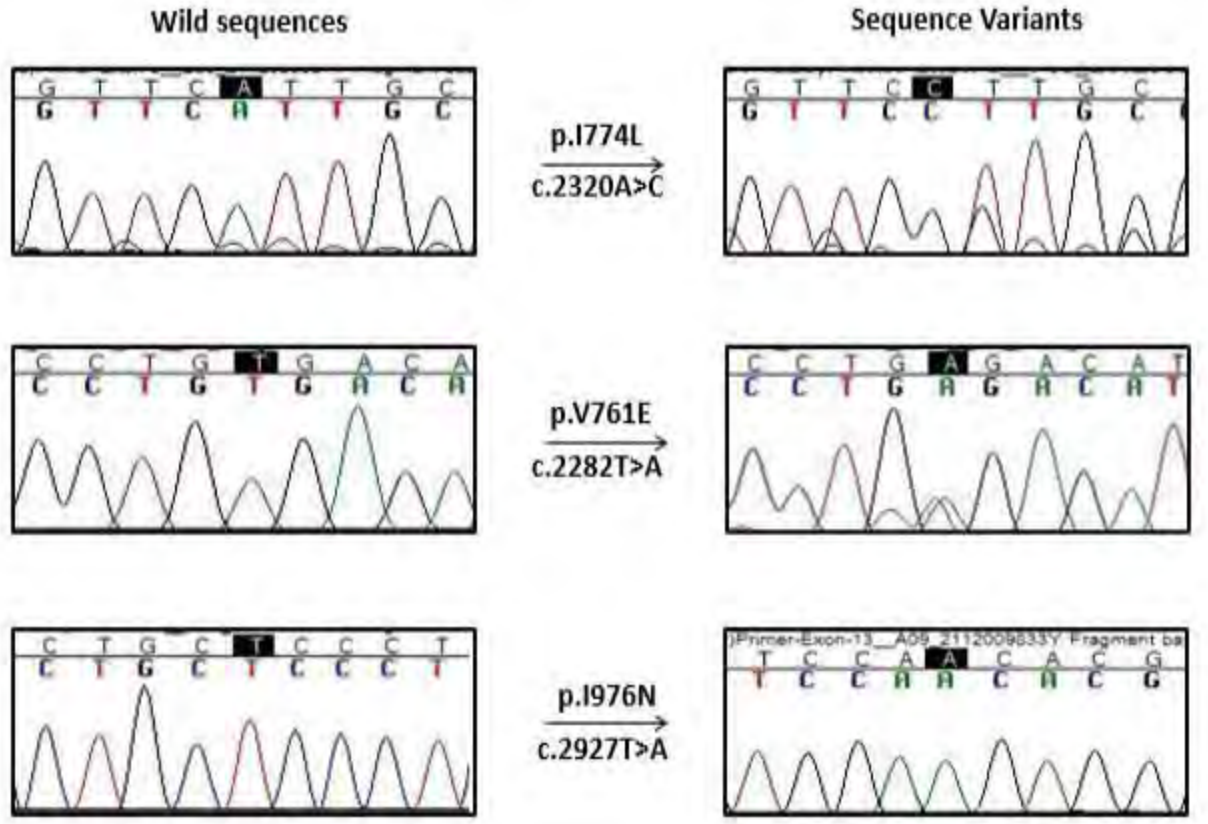
The novel variants identified in the suspected gene include (c.251A>C, c.15T>A, c.6T>C, c.238C>T, g.46426_46426delC, g.52891T>A, g.52913C>G, g.65292_65293insG, g.53127G>C and g.53202T>A (Figure 3.1.17). The known variants identified in the

suspected gene include c.83C>A, c.39_40insCGCCG, c.39_40insCGGCG, c.1366G>C and c.1544-53A>C (Figure 3.1.18).

Table: 3.1.4 Summary of all the disease-causing variants and polymorphisms identified in *ATP7B* gene in this study

Sr No.	Variant	location	AA change	Insilico	Polyphen 2	Domain	PF %	Reference ID	Novelty
1	c.83C>A	5'UTR*	-	P	-	5'UTR	35	rs2277448	-
2	c.39_40ins CGCCG	5'UTR	-	-do-	-	5'UTR	10	rs3832920	-
3	c.251A>C	Intron 1	-	-do-	-	Cu** Binding	5	-	N
4	c.15T>A	5'UTR	-	-do-	-	5'UTR	5	-	N
5	c.6T>C	Exon 1	-	-do-	-	Cu Binding	10	-	N
6	c.39_40ins CGGCG	5'UTR	-	-do-	-	5'UTR	5	rs3832920	-
7	c.238C>T	Intron 1	-	-do-	-	Cu Binding	5	-	N
8	c.678_678d elG	Exon-2	p.L227 Yfs*35	DC	PD(0.995)	Cu3	5	-	-
9	c.1366G>C	Exon 3	p.V45 6L	P	B(0.001)	Cu4/Cu 5 Binding	40	rs1801244	-
10	c.1544-53A>C	Intron 3	-	-do-	-	Cu Binding	100	rs2147363	-
11	g.52891T> A	Intron 7	-	P	-	Cu++ channel	5	-	N
12	g.52913C> G	Intron 7	-	P	-	Cu++ channel	5	-	N
13	c.2282T>A	Exon 8	p.V76 1E	DC	B(0.378)	Cu++ channel	5	-	N
14	g.53122G> A	Exon 8	p.D76 5N	DC	PD(1)	Cu++ channel	5	rs28942075	-
15	g.65292_65 293insG	Intron 12	-	P	-	Cu++ channel	5	-	N
16	g.65107T> A	Exon 13	p.S986 T	DC	PD(0.995)	Cu++ channel	5	-	-
17	g.53127G> C	Exon 8	-	DC	-	Cu++ channel	40	-	N
18	g.53202T> A	Intron 7	-	P	-	Cu++ channel	10	-	N
19	g.65160G> A	Exon 13	p .A100 3A	DC	-	Cu++ channel	40	rs1801297	-
20	g.65078T> A	Exon 13	p.I976 N	DC	PD(1)	Cu++ channel	5	-	N
21	g.53149A> C	Exon 8	p.I774 L	DC	PD(0.994)	Cu++ channel	15	-	N

PF: patient frequency, P: Polymorphism, N: Novel, DC; Disease Causing, PD: Probably Damaging, B: Benign, V: valine, L: Leucine, Y: Tyrosine, UTR: Untranslated region*un-translated region **copper, D: Aspartic acid, S: Serine, T: Threonine, E: Glutamic acid, N: Asparagine, DC: Disease Causing, P: Polymorphism, PD: Probably Damaging, B: Benign



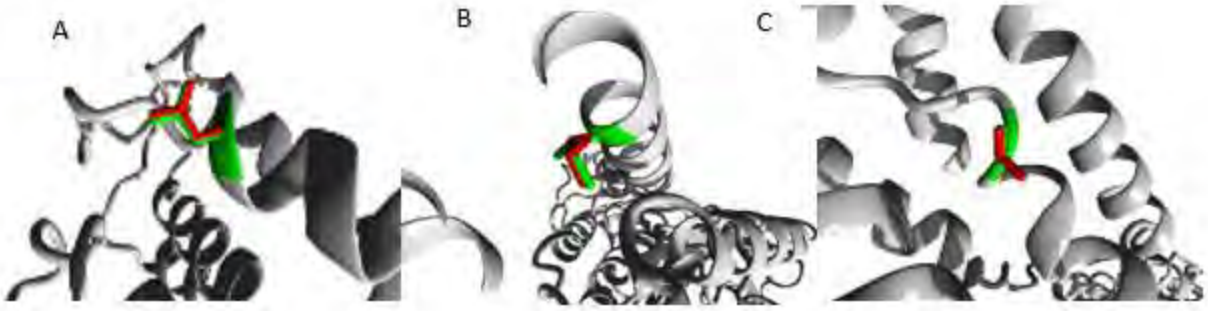


Figure. 3.1.15. a- Chromatograms showing Wild type sequences and the sequence of Novel disease causing variants *ATP7B* gene identified in Exon 8 and Exon 13. p.I774L (Isoleucine774Leucine, p.V761E (Valine761Glutamic acid) p.I976N

(Isoleucine976Asparagine); b-In-silico prediction of (A) p.I774L (Isoleucine774Leucine) (B) p.V761E (Valine761Glutamic acid (C) p.I976N (Isoleucine976Asparagine) as generated by HOPE. Wild type amino acid residue is colored as green while mutant is colored as red.

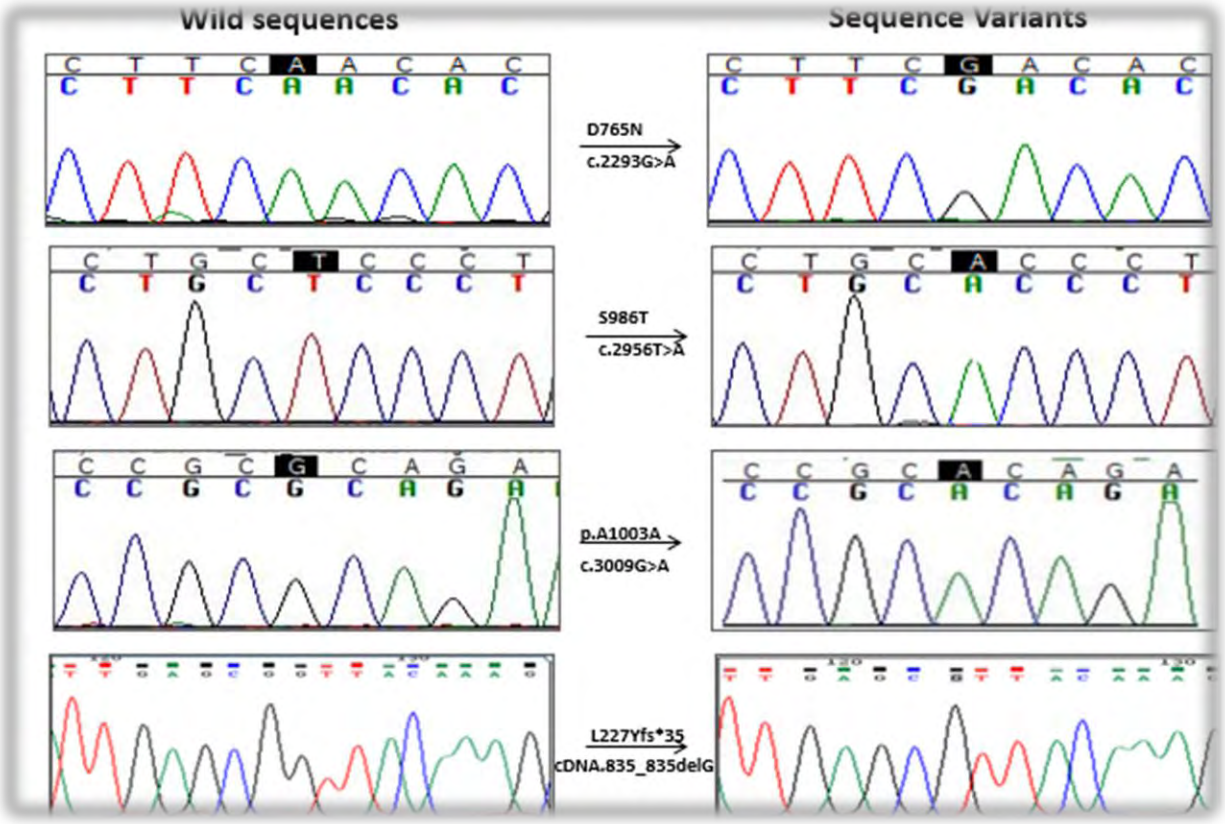


Figure 3.1.16. Chromatograms showing reported disease-causing variants identified in *ATP7B* gene in this study. p.D765N (Aspartic acid765Asparagine), p.S986T (Serine986Threonine), p.A1003A(Alanine1003Alanine), p.L227Yfs*35 (Leucine227Tyrosine fs*35).

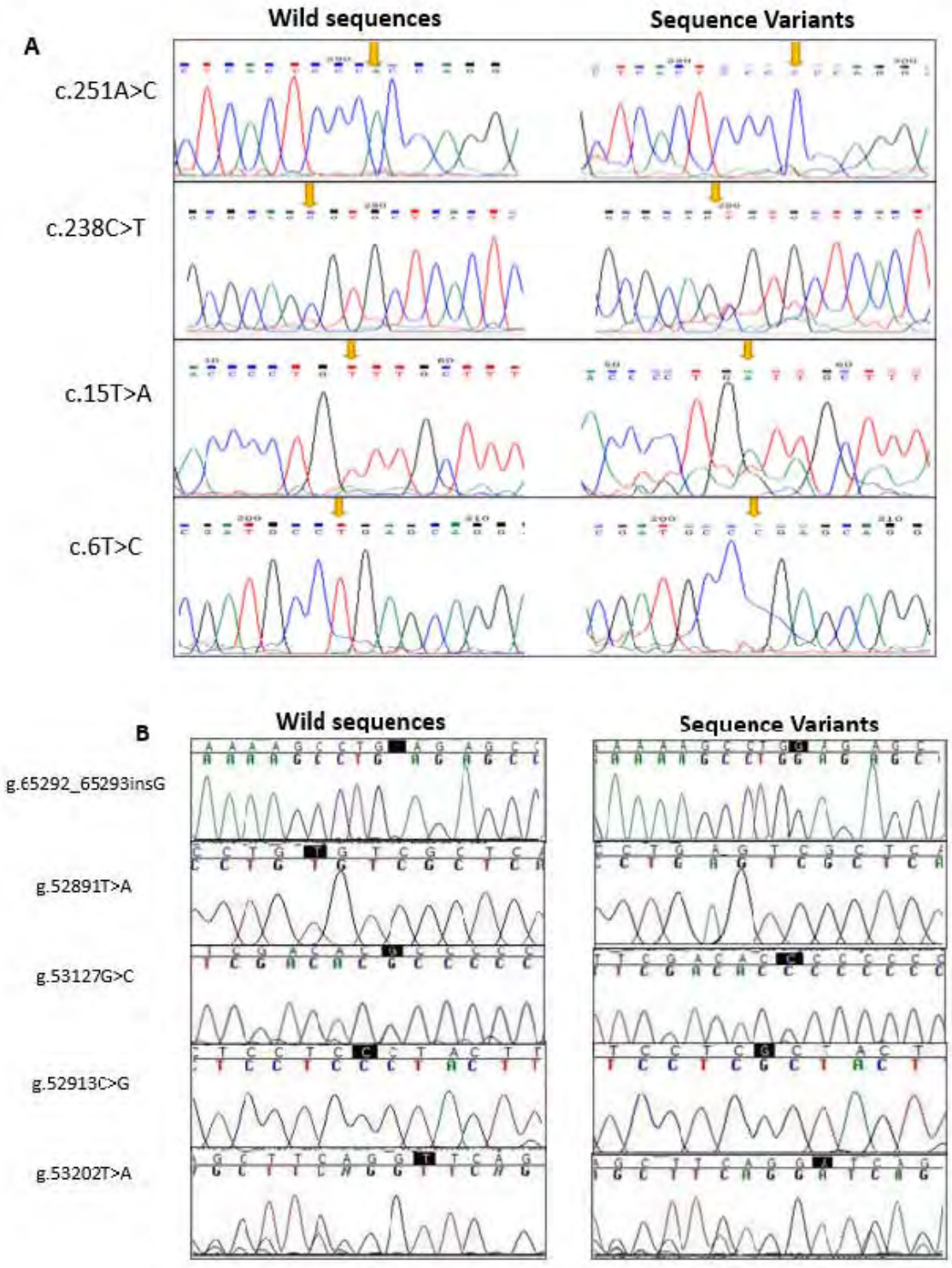


Figure 3.1.17. A Representing Chromatograms for identified novel polymorphisms in coding region of *ATP7B* gene. B Representing Chromatograms for identified novel polymorphisms in non-coding region of *ATP7B* gene.

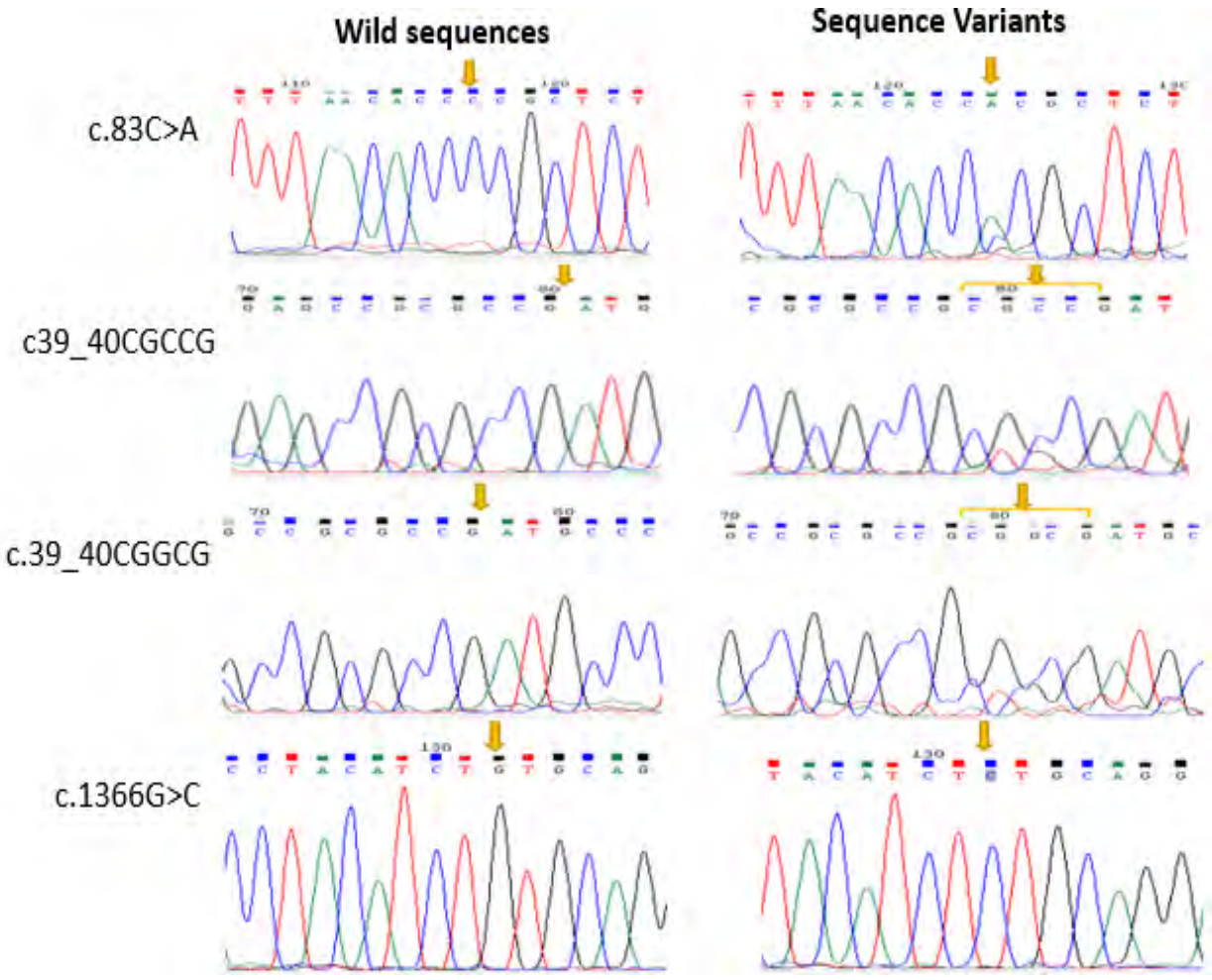


Figure 3.1.18. A & B Chromatograms for identified reported polymorphisms identified in *ATP7B* gene

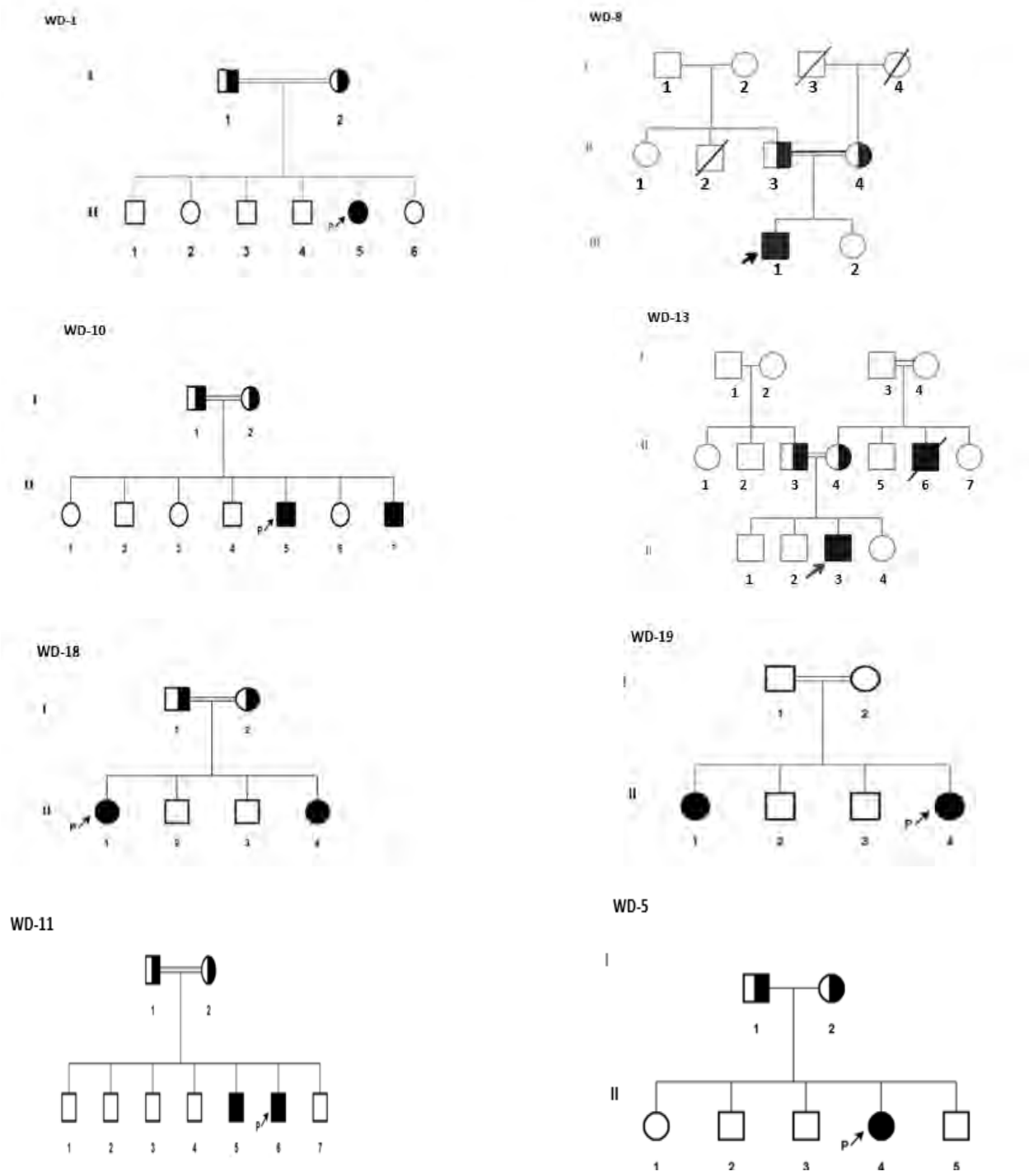


Figure 3.1.19. Pedigrees of the families in which disease-causing variants in *ATP7B* gene were identified

3.2. MUTATIONAL ANALYSIS AND CLINICAL INVESTIGATIONS OF PATIENTS AFFECTED WITH GSD 1a

3.2.1. Baseline characteristics of study participants

40 patients diagnosed by expert physicians as Glycogen storage disease Type 1a (GSD Ia; MIM# 232200) (von Gierke) were enrolled in the study. Clinical data and demographic details of patients were recorded. Direct sequencing of all the coding exons and exon/ intron boundaries in 20 selected patients with Glycogen storage disease Type 1a was performed to screen for disease causing variants.

3.2.2. Clinical details of the enrolled GSD patients

Glycogen storage diseases (GSDs) are characterized by abnormal inherited glycogen metabolism in the liver, muscle, and brain (Shin, 2006). Glycogen storage disease Type 1a has a glucose 6-phosphatase deficiency that occurs due to alteration in nucleotide sequence of *G6PC* gene (*G6PC*; GDB 231927)., a nine-helical endoplasmic reticulum transmembrane protein required for maintenance of glucose homeostasis. G6Pase plays a central role in both glycogenolysis and gluconeogenesis, hydrolysing glucose-6-phosphate (G6P) to glucose. In 1993 the gene encoding this unit was identified in band q21 of chromosome 17 and a steadily growing list of mutations has been reported (Lei *et al*., 1993). *G6PC* contains five exons, spans 12.5 kb and encodes an endoplasmic reticulum membrane associated protein containing 357 amino acids (Lei *et al*., 1993). To date 146 mutations have been reported according to Human Genetic Mutation database http://www.hgmd.cf.ac.uk/ac/gene.php?gene=*G6PC*. Deficiency of G6Pase activity in liver, kidney, and intestine results in accumulation of glycogen in these organs. As a result of inadequate glucose production, patients have severe fasting hypoglycaemia with secondary biochemical abnormalities: hyperlactacidaemia, hyperuricaemia and hyperlipidaemia. Untreated patients have a protruding abdomen because of marked hepatomegaly (storage of glycogen and fat), short stature, truncal obesity, a rounded doll-like face, wasted muscles, and bleeding tendency due to impaired platelet function (Lei *et al*., 1993; Scriver, 2001). Initial symptoms in glycogen storage disease are frequently non-specific. Hypoglycemia, the diagnosis of glycogen storage disease type Ia was

subsequently confirmed on the basis of undetectable glucose-6-phosphatase activity in a liver biopsy (Talente *et al*., 1994). Other clinical symptoms include Hepatomegaly, lactic acidosis, Hyperuricemia, seizures, epistaxis, and hypertriglyceridemia.

Forty patients with glycogen storage disease type Ia were enrolled from Paediatric Department and Liver Unit of Pakistan institute of medical sciences PIMS, and Children Hospital Lahore CHL, over the period January 2018 to August 2020 as diagnosed by physicians. Based on the generation skip and affected individuals having unaffected parents' autosomal recessive inheritance of the glycogen storage disease type Ia was demonstrated in enrolled cases.

Glycogen storage disease Type 1a was detected by expert physicians based on characteristic clinical features including Short Stature, Hypoglycemia, Hepatomegaly, anemia, proteinuria, micro albuminuria, hepatic adenomas, Lactic Acidosis, Hyperuricemia, Seizures, Epistaxis, Hypertriglyceridemia, delayed motor development, cushingoid appearance, impaired platelet function, wasted muscles, hyperlipidemia, liver adenomas, osteopenia, carcinomas, progressive renal disease, delayed pubertal development, inflammatory bowel disease.

The age of diagnosis of glycogen storage disease type Ia in probands of enrolled families was between 0.5-25 years. The average age of all respondents lies within the interval of $7.90 \pm 3(4.98)$ among which maximum respondents i.e., 35.0%, have ages between 5.1-10.0 years, 25.0% have ages 10.1-15.0 years, 20.0% have ages between 2.1-5.0 years, 15.0% have ages between 0.0-2.0 years, and 5.0% respondents have ages between 15.1 years and above. More males i.e., 26/40 were affected (65%) than females. Parental cousin marriage is the reason of prevailing recessive disorders in Pakistan like glycogen storage disease type Ia. Parental cousin marriage was observed among parents of 35 (87.5.%) patients. 23 cases had the history of the disease in their families i.e. (57.5%) cases were familial. Twenty-three (57.5%) of the index cases died of the disease severity. Hypoglycemia (Less than or equal to 60mg/dl**)** was observed in all the 40 cases that is 100% frequency. Proteinuria i.e., increased level of protein (a normal value in healthy adults is less than 150 mg/ 24 hour) in the urine is observed in 70% cases i.e., 28 out of 40 cases. High Urine micro albuminuria excretion of more than 300 mg of albumin per 24 hours was observed

in 10 cases with frequency of 25%. Hypertriglyceridemia (a condition in which triglyceride levels are elevated) more 180 mg/dl, was noticed in all GSD 1a cases. (Table 3.2.1).

Table 3.2.1. Demographic profile and clinical data of 40 included GSD1a Patients

Variables	Category	***n*** (%)
Gender	Male	26 (65.0)
	Female	14 (35.0)
Age	Mean± 3SD (in Years)	7.90±3(4.98)
	0.0-2.0 Years	6 (15.0)
	2.1-5.0 Years	8 (20.0)
	5.1-10.0 Years	14 (35.0)
	10.1-15.0 Years	10 (25.0)
	15.1-20.0 Years	1 (2.5)
	>20.0 Years	1 (2.5)
Family History of GSD	Yes	23 (57.5)
	No	17 (42.5)
Parental Cousin Marriage	Yes	35 (87.5)
	No	5 (12.5)
Hypoglycemia	Mean± 3SD (mg/dL)	58.0±3(6.59)
Proteinuria	Mean± 3SD	192.23±3(53.19)
High Micro Albuminuria	Mean± 3SD	166.73±3(105.12)
Hypertriglyceridemia	Mean± 3SD	279.08±3(59.99)
Status	Alive	17 (42.5)
	Dead	23 (57.5)

Short stature was observed in 12 out 40 cases (30 %). Figure 3.2.1 displays the prevalence of short stature according to different age groups. It is observed that, the maximum prevalence of short stature i.e., 49.0% lies among the group having age 5.1-10.0 years. Similarly, 25.0% prevalence of short stature is observed in age groups 2.1-5.0 years and 10.1-15.0 years, respectively. Moreover, there is no prevalence of short stature among the age groups of 0.0-2.0 years, 15.1 years and above.

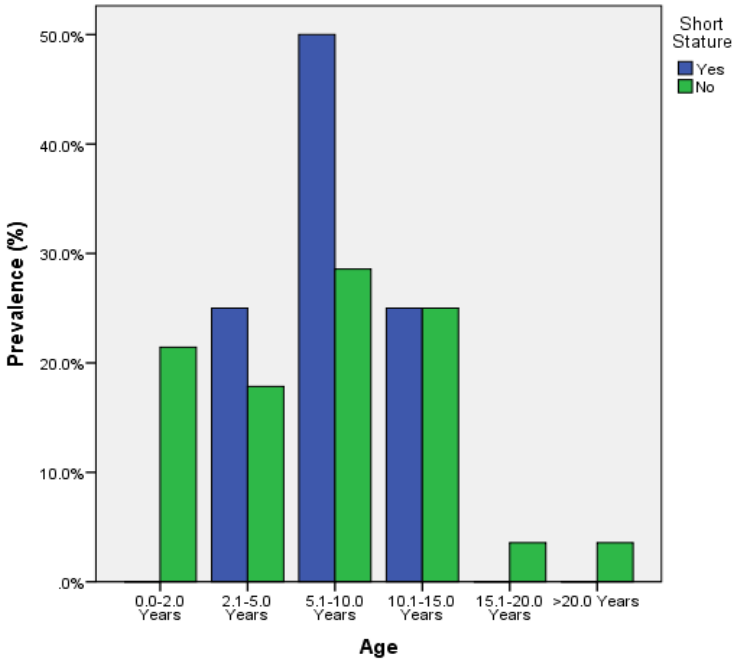


Figure 3.2.1. Presence of short stature among different age groups

Hepatomegaly i.e., an enlarged liver is one that's bigger than normal is observed in 100 % patients. Figure 3.2.2 displays the prevalence of hepatomegaly among different age groups. It is observed that, the maximum prevalence of hepatomegaly i.e., 35.0% lies among the group having age 5.1-10.0 years. Similarly, 25.0% prevalence of hepatomegaly is observed in age groups 10.1-15.0 years. Moreover, 15%, 2.5%, and 2.5% prevalence of hepatomegaly is observed in age groups 0.0-2.0 years, 15.1-20.0 years and above 20years, respectively.

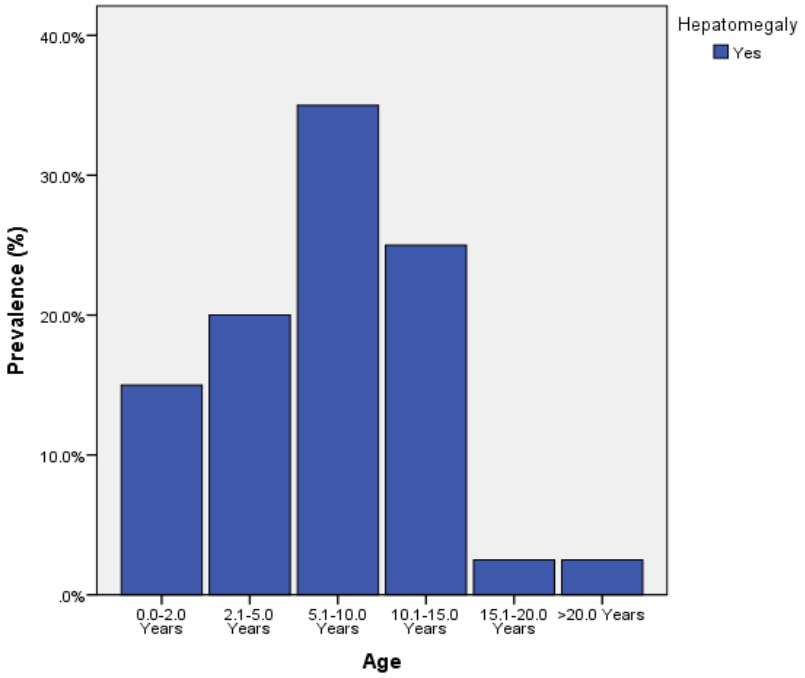


Figure 3.2.2. Presence of hepatomegaly among different age groups

Anaemia was observed in 80% cases i.e., 32/40. Figure 3.2.3 displays the prevalence of anaemia among different age groups. It is observed that, the maximum prevalence of anaemia i.e., 34.4% lies among the group having ages 5.1-10.0 years. Similarly, 28.1%, 18.8% and 12.5% prevalence of anaemia is observed in the age groups of 10.1-15.0 years, 2.1-5.0 years, and 0.0-2.0years, respectively. Moreover, in age groups 15.1-20.0 years and above 20 years all the respondents suffer from anaemia.

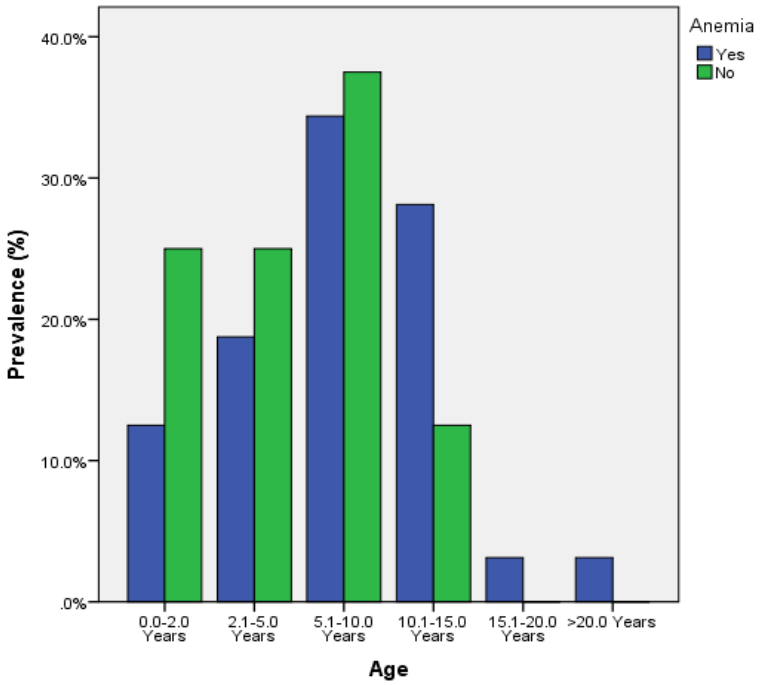


Figure 3.2.3. Presence of Anemia among different age groups of GSD1a Patients

Hepatic adenomas were seen in 5 percent patients. Figure 3.2.4 displays the prevalence of liver adenomas among different age groups of GSD1a patients. It is observed that, the most prevalent age groups of liver adenomas are 5.1-10.0 years and 10.1-15.0 years having 50.0% prevalence of liver adenomas in each. Also, the rest of the age groups do not have the prevalence of liver adenomas.

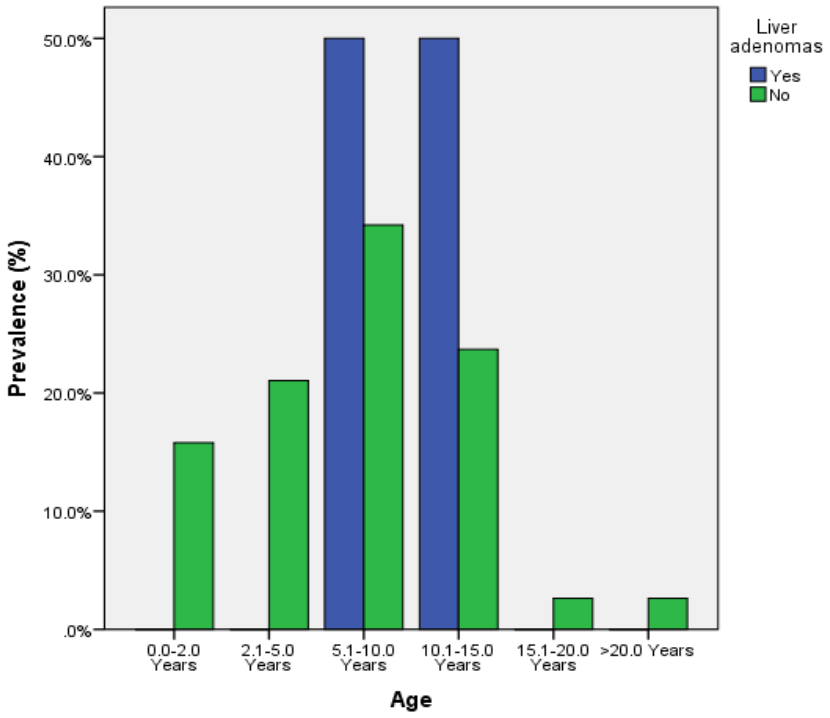


Figure 3.2.4. Presence of Liver Adenomas among different age groups of GSD1a Patients

Lactic Acidosis (PH less than 7.2) is observed in all enrolled cases. Figure 3.2.5 displays the prevalence of lactic acidosis among different age groups of GSD1a patients. It is observed that in age group of 0.0-2.0 years: 66.7% respondents have 7 pH, 9.1% respondents have 6 pH, in the age group of 2.1-5.0 years: maximum i.e. 33.3% respondents have 4 pH, 18.2% respondents have 6 pH, whereas 5 and 7 PH of lactic acidosis is observed in 16.7% of the respondents. Similarly, in the age group 5.1-10.0years: 50.0% respondents have 5 pH, 45.5% have 6 pH and 16.7% respondents have the prevalence of lactic acidosis with 4 pH level. Moreover, in age group of 10.1-15.0 years: 33.3% prevalence of lactic acidosis is observed for 4 & 5 both pH levels, 22.7% and 16.7% prevalence is observed with 6 & 7 pH levels, respectively. Also, 16.7% prevalence with 4 pH and 4.5% prevalence with 6 pH is observed in 15.1-20.0 years and above 20 years age groups, respectively.

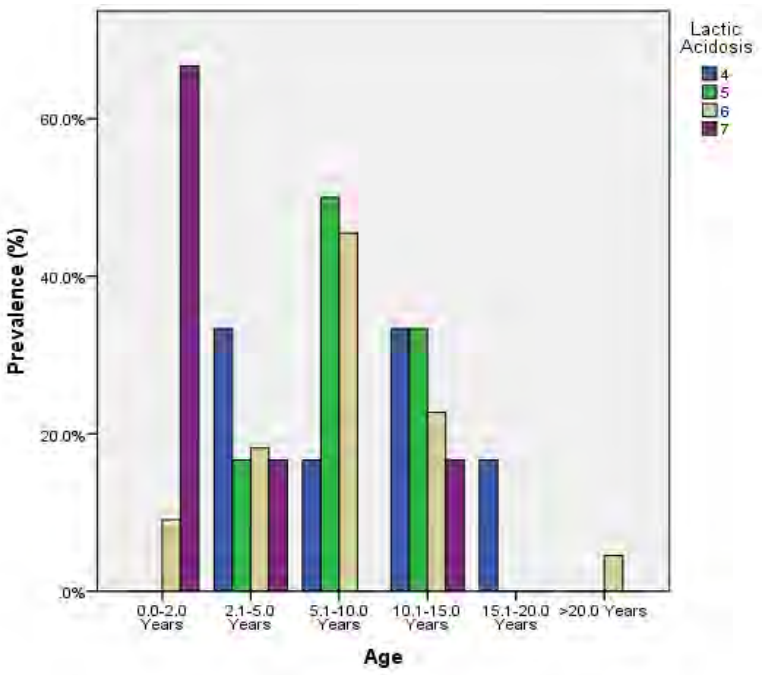


Figure 3.2.5. Presence of Lactic Acidosis among different age groups of GSD1a Patients

Hyperuricemia is an elevated uric acid level in the blood, the normal upper limit is 6.8mg/l was observed in 9 out of 40 cases with frequency of 22.5% (Table 3.2.1). Figure 3.2.6 displays the prevalence of hyperuricemia among different age groups of GSD1a patients. It is observed that in age group of 0.0-2.0 years: 40.0% respondents have 5 and 8 pH, 12.5%, and 5.0% respondents have 7 and 6 pH, respectively. In the age group of 2.1-5.0 years: 25.0% respondents have 6 pH, 20.0% respondents have 5 and 7 pH, whereas 12.5% of the respondents have prevalence of hyperuricemia with 7 pH level. Similarly, in the age group 5.1-10.0years: 62.5% respondents have 7 PH, 40.0% have 5 pH, 30% have 6 pH and 20.0% respondents have the prevalence of hyperuricemia with 8 pH level. Moreover, in age group of 10.1-15.0 years: 100% prevalence of hyperuricemia for 4 pH level is observed, while, 30.0%, 12.5% and 20.0% prevalence is observed with 6, 7 and 8 pH levels, respectively. Also, 5.0% prevalence with 6 pH is observed in 15.1-20.0 years and above 20 years age groups, respectively.

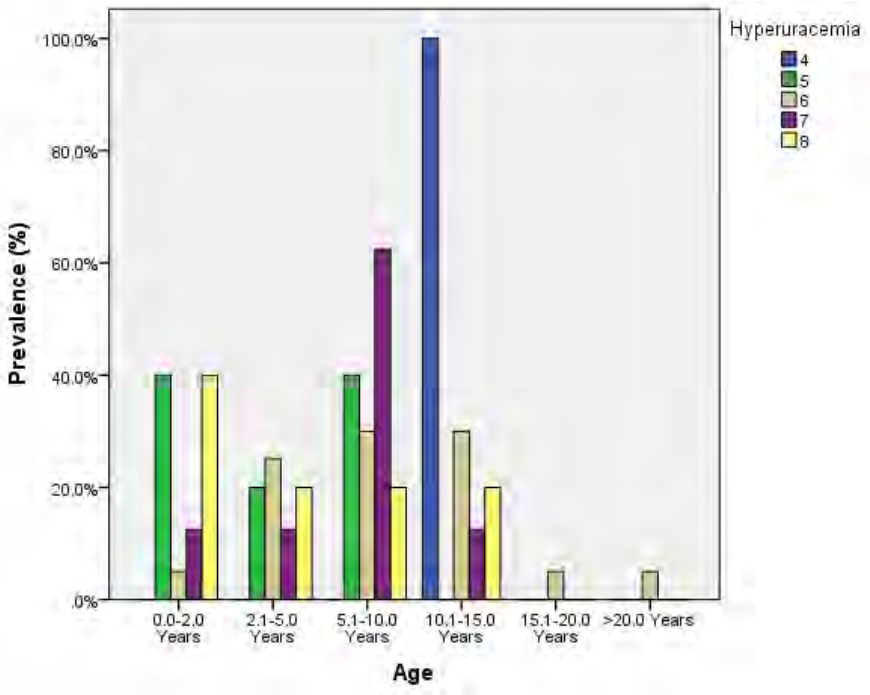


Figure 3.2.6. Presence of Hyperuricemia among different age groups of GSD1a Patients

Seizures i.e., a sudden, uncontrolled electrical disturbance in the brain were observed in 10 out of 40 patients with frequency of 25%. Figure 3.2.7 displays the prevalence of seizures among different age groups of GSD1a patients. It is observed that, the most prevalent age groups of seizures is 10.1-15.0 years having 40.0% prevalence of seizures. Also, 20.0%, 10.0%, and 30.0% prevalence of seizures is observed in age groups 0.0-2.0years, 2.1-5.0years, and 5.1-10.0years, respectively. Moreover, no seizures prevalence is observed among the patients having ages 15.1 years and above.

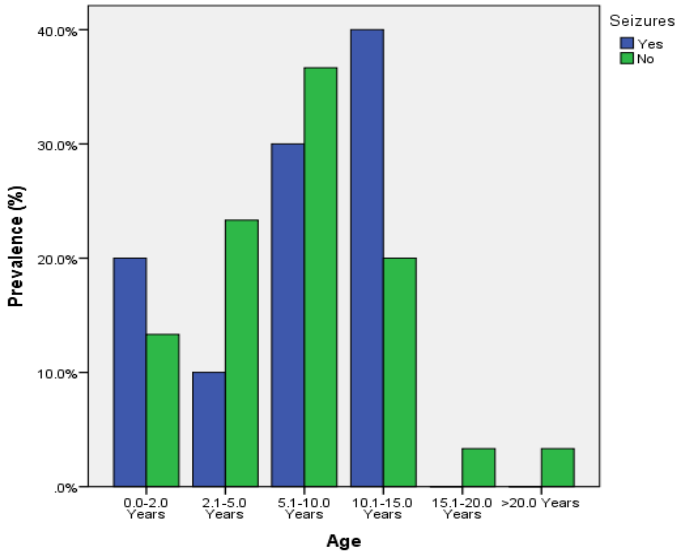


Figure 3.2.7. Presence of Seizures among different age groups of GSD1a Patients

Epistaxis a nosebleed is the loss of blood from the tissue that lines the inside of your nose is observed in 7 out of 40 patients with 17.5% frequency. Figure 3.2.8 displays the prevalence of epistaxis among different age groups of GSD1a patients. It is observed that, the most prevalent age groups of seizures are 2.1-5.0years and 10.1-15.0 years having 40.0% prevalence of epistaxis in each. Also, 15.0% prevalence of epistaxis is observed in age groups 5.1-10.0 years. Moreover, rest of the age groups do not have the prevalence of epistaxis.

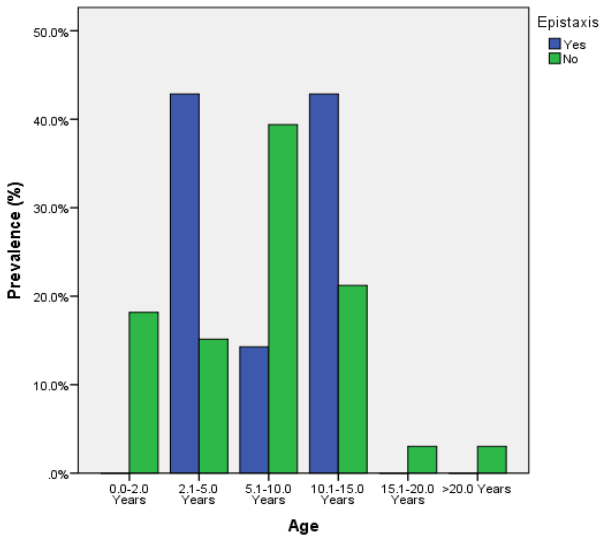


Figure 3.2.8. Presence of Epistaxis among different age groups of GSD1a Patients

Delayed motor development Problem with crawling or walking, or fine motor skills, such as using fingers to grasp a spoon, is observed in 5 out of 40 cases i.e. 12.5% cases. Figure 3.2.9 displays the prevalence of delayed motor development among different age groups of GSD1a patients. It is observed that, the most prevalent age group of delayed motor development is 5.1-10.0 years having 40.0% prevalence. Similarly, 20.0% prevalence is observed in each age groups 0.0-2.0 years, 10.1-15.0 years, and above 20 years. Moreover, there is no prevalence of delayed motor development in the age groups: 2.1-5.0 years and 15.1-20.0 years.

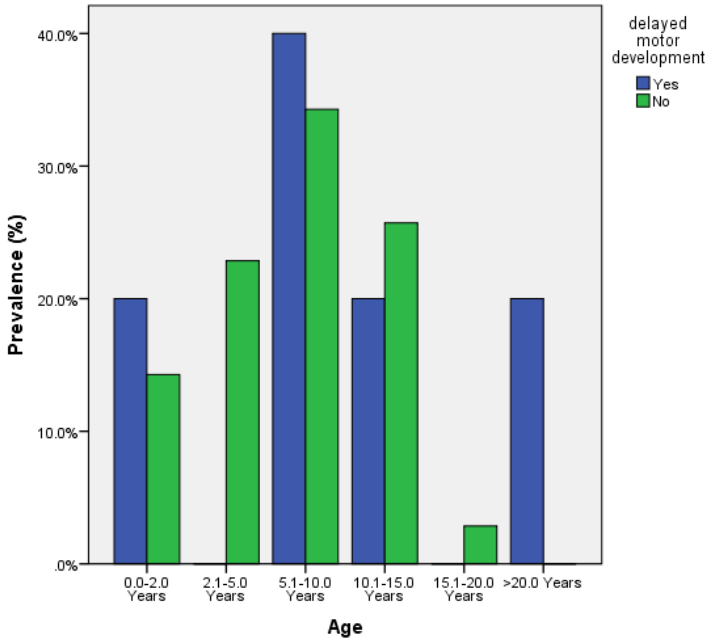


Figure 3.2.9. Presence of Delayed Motor Development among different age groups of GSD1a Patients

Osteopenia (loss of bone mineral density (BMD) that weakens bones) was diagnosed in 9 out of 40 cases, i.e. 22.5% cases. Figure 3.2.10 displays the prevalence of osteopenia among different age groups of GSD1a patients. It is observed that, the most prevalent age group of osteopenia is 5.1-10.0 years with 35.0% prevalence. Similarly, 20.0% prevalence is observed in each age groups 0.0-2.0 years, 2.1-5.0 years, and 10.1-15.0 years. Moreover, there is no prevalence of osteopenia in rest of the age groups.

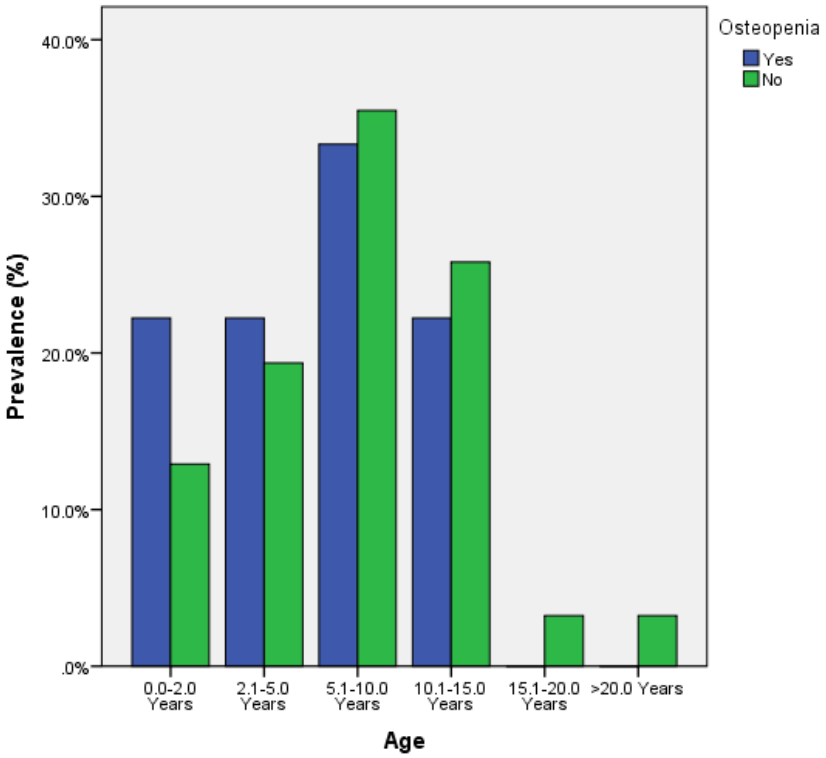


Figure 3.2.10. Presence of Osteopenia among different age groups of GSD1a Patients

Cushingoid appearance (Moon like face) was present in 3 cases with 7.5 percent frequency. Figure 3.2.11 displays the prevalence of cushingoid appearance among different age groups of GSD1a patients. 66. 7% and 33.3% prevalence of cushingoid appearance is observed in the age groups 10.1-15.0 years and 5.1-10.0 years, respectively. While, rest of the age groups have no prevalence of cushingoid appearance.

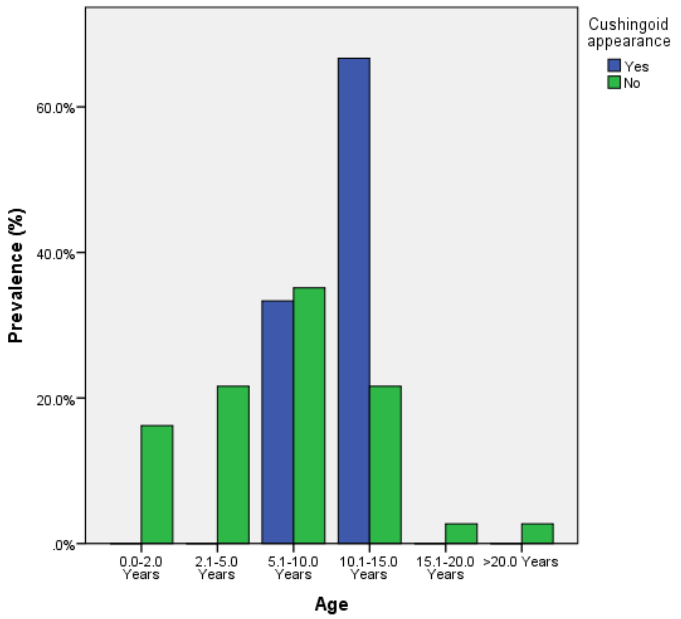


Figure 3.2.11. Presence of Cushingoid appearance among different age groups of GSD1a Patients

Inflammatory bowel disease (IBD), pain and swelling in the intestines was diagnosed in 15/40 i.e. 37.5% cases. Figure 3.2.12 displays the prevalence of inflammatory bowel disease among different age groups of GSD1a patients. 20.0%, 13.3%, 33.3%, 26.7% and 6.7% prevalence of inflammatory bowel disease is observed in the age groups 0.0-2.0 years, 2.1-5.0 years, 5.1-10.0 years, 10.1-15.0 years and 15.1-20.0 years, respectively. Whereas, the respondents having the age of above 20.0 years do not have inflammatory bowel disease.

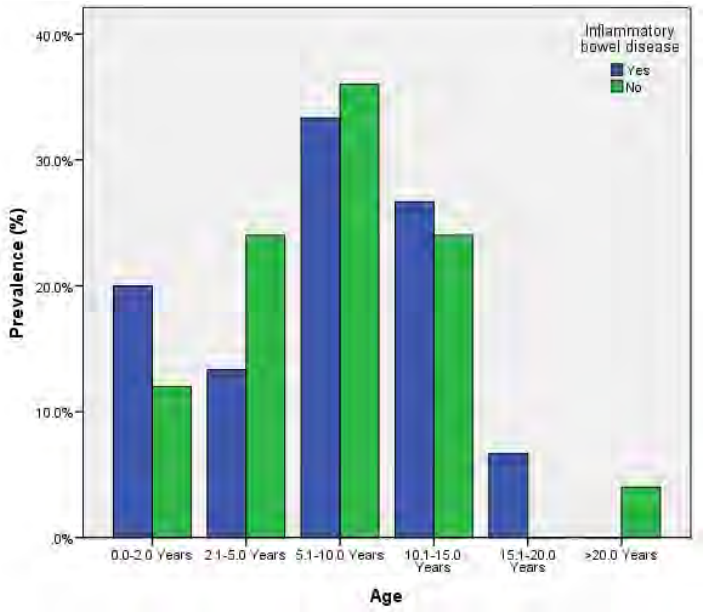


Figure 3.2.12. Presence of Inflammatory bowel disease among different age groups of GSD1a Patients

3.2.3. Genetic Screening of *G6PC* in 20 GSD 1a cases

For molecular analysis of GSD 1a all coding exons and intron-exon boundaries of *G6PC* gene were screened to identify pathogenic variant in 20 patients (family pedigrees shown in figure 3.2.18) based on availability of DNA samples. Mutational analysis was done using PCR-Sanger sequencing method and pathogenic effect predictions for identified variants were carried out using PROVEAN, MutationTaster, Polyphen 2, SIFT and HSF software. Upon sequencing of coding exons, their flanking intronic regions and 3' as well as 5' untranscribed regions (UTRs) of *G6PC* gene in 20 patients, 21 variants were detected including 8 novel disease causing variants i.e., p.Ala37Pro, p.Asp69Asn, p.Gln82Lys, p.Thr108Pro, g.50_51insT, p.Gln24Pro, p.Val45Leu and p.Cys284Tyr in the screened regions of *G6PC* gene. Out of 13 identified polymorphisms, 4 were identified in heterozygous condition while 9 were found in homozygous condition. This study revealed clinical presentation of GSD1a cases from Pakistan and identification of novel disease-causing sequence variants in coding region and intron-exon boundaries of *G6PC* gene.

Five novel homozygous disease causing variants include p.Ala37Pro (GSD11), p.Asp69Asn (GSD 5), g.50_51insT (GSD2) in exon 1 of *G6PC* gene; p.Gln82Lys (GSD

9) and p.Thr108Pro (GSD4) in exon 2 of *G6PC* gene (Chromatograms shown in figure3.2.14). Three novel identified heterozygous disease causing variants include p.Gln24Pro (GSD11), p.Val45Leu (GSD5) in exon 1 of *G6PC* gene and p.Cys284Tyr (GSD 19) in exon 5 of *G6PC* gene (Chromatograms shown in Figure 3.2.14)(Table 3.2.2).

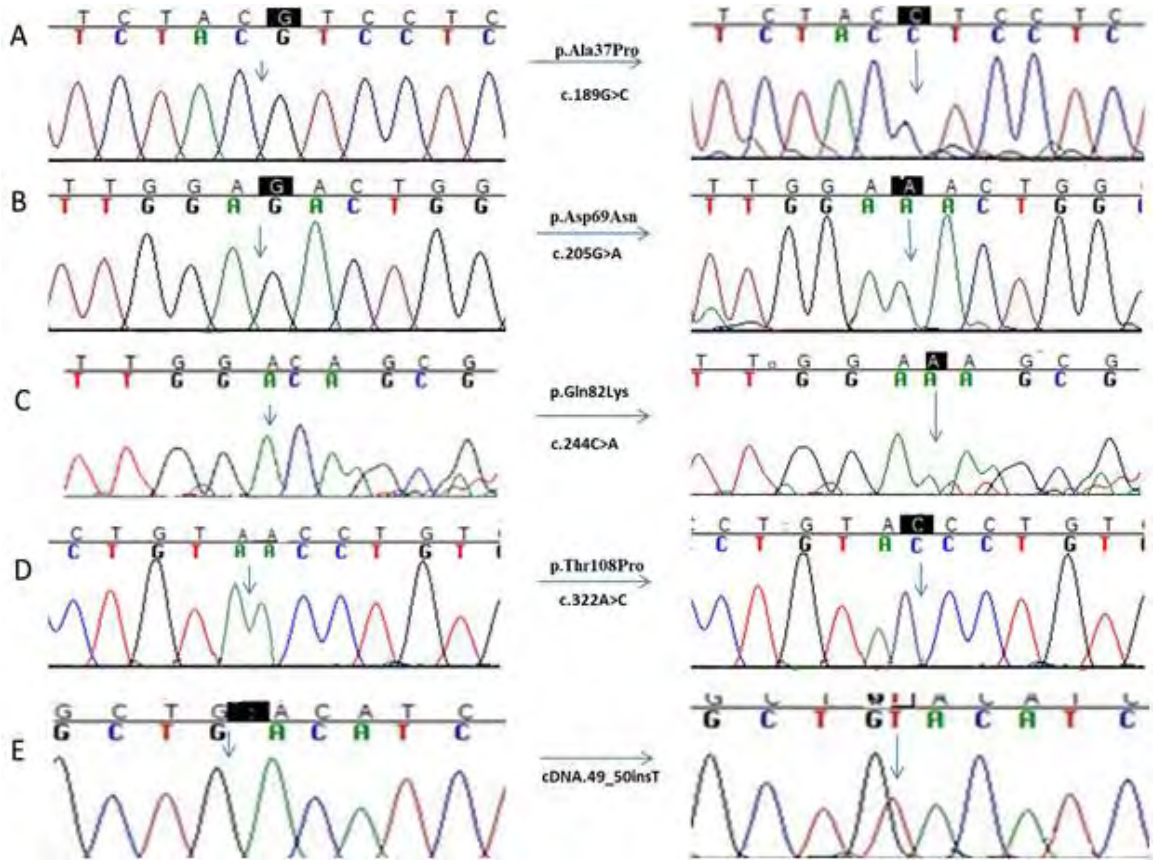


Figure 3.2.13. Chromatograms of homozygous disease-causing variants identified in *G6PC* gene (Wild type sequences on left side and sequence variants on right side). A. Chromatogram showing c.189G>C leading to p.Ala37Pro in patient GSD11 in exon 1 of *G6PC* gene. B. Chromatogram showing g.285G>A (c.205G>A) causing p.Asp69Asn in patient GSD 5 in exon 1 of *G6PC* gene. C. Chromatogram showing g.3148C>A (c.244C>A) causing p.Gln82Lys in patient GSD 9 in exon 2 of *G6PC* gene. D. Chromatogram showing g.3226A>C (c.322A>C) substitution resulting in p.Thr108Pro in patient GSD4 in exon 2 of *G6PC* gene. E. Chromatogram showing g.50_51insT (cDNA.49_50insT) identified in patient GSD2 in exon 1 of *G6PC* gene.

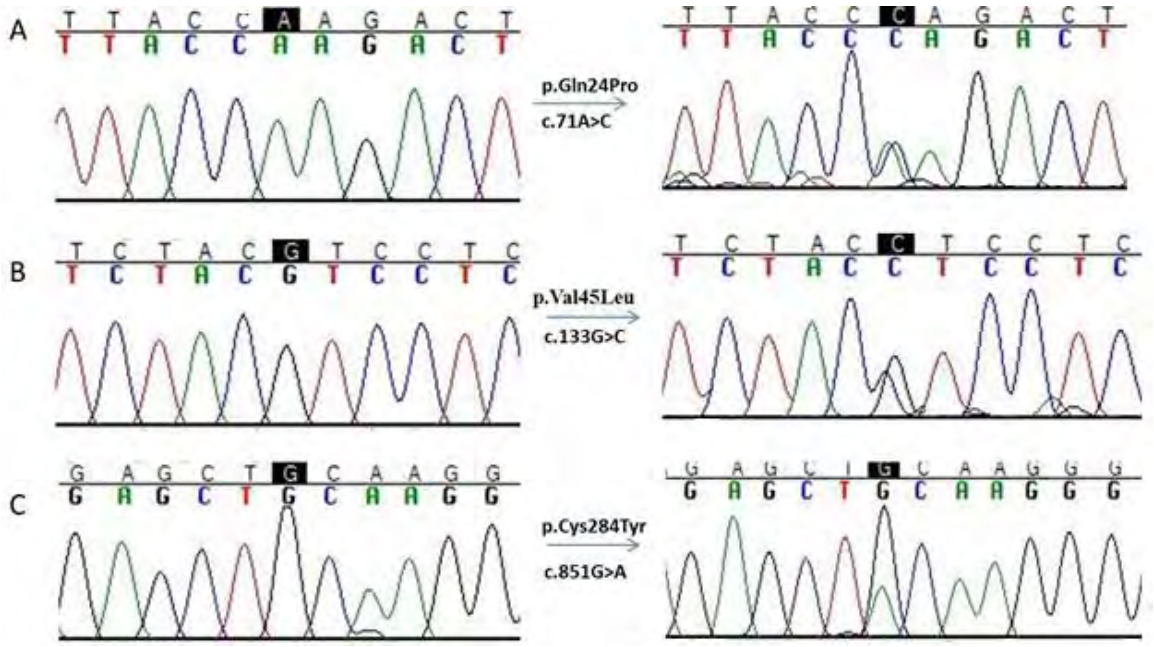


Figure 3.2.14. Chromatograms showing heterozygous disease-causing variants identified in *G6PC* gene. A (Wild type sequences on left side and sequence variants on right side).. Chromatogram showing g.151A>C (c.71A>C) causing p.Gln24Pro identified in exon 1 of *G6PC* gene. B. Chromatogram showing g.213G>C (c.133G>C) leading to p.Val45Leu identified in exon 1 of *G6PC* gene. C. Chromatogram showing g.10407G>A (c.851G>A) causing p.Cys284Tyr identified in exon 5b of *G6PC* gene.

The novel polymorphisms identified in GSD1a cases include g.3090_3090delA, g.6903_6904insA, g.8468_8469insA, g.8475_8476insC, g.6761T>A, g.10357G>A and g.10776T>G in homozygous states whereas two variants i.e., g.3266G>T and g.8481T>G were found in heterozygous states (Chromatograms shown in figure 3.2.16). Among three known polymorphisms, a homozygous variant g.3432A>G (rs2593595) was identified in 25% cases and two heterozygous variants i.e. g.8767T>C (rs161622) and g.10653T>C (rs2229611) were identified in 15% and 20% of cases respectively (Chromatograms shown in figure 3.2.15).

3.2.4. Insilico prediction

All variants were verified by mutation taster, SIFT, Polyphen2, HOPE and Mutalyzer (Table 3.2.2). These variants were not found in ExAC. Hope analysis showed that the

original wild-type residue and newly introduced mutant residue differ in properties for each novel missense variant playing role in disease pathogenicity (Figure 3.2.17).

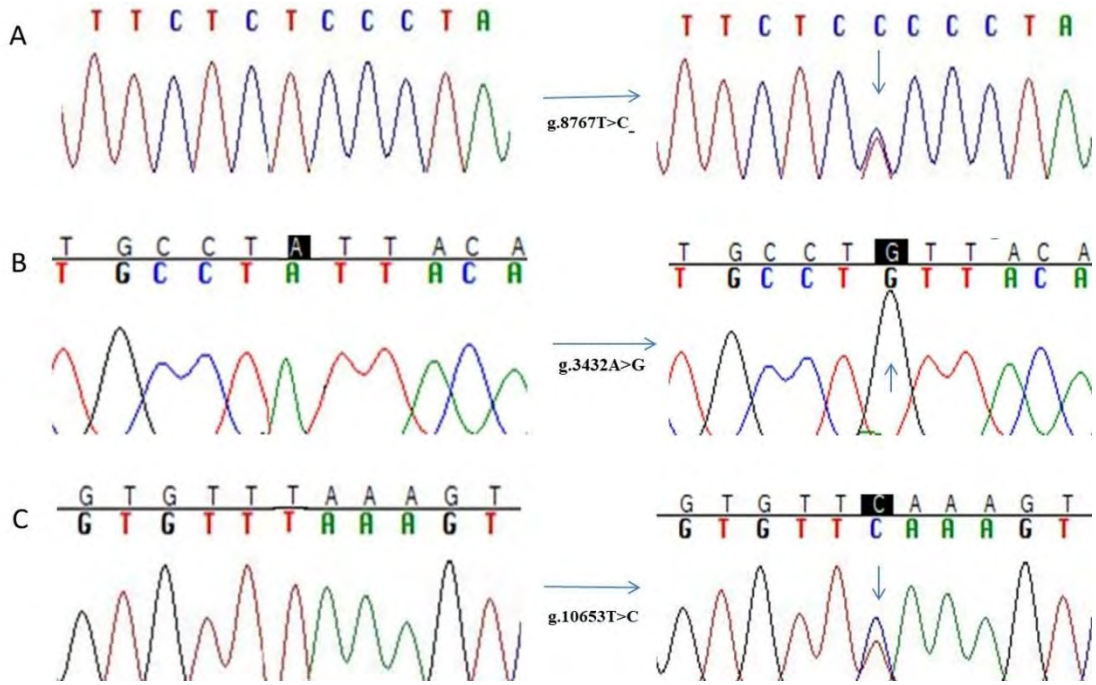


Figure 3.2.15. Chromatogram showing reported polymorphisms in *G6PC* gene (Wild type sequences on left side and sequence variants on right side). A- g.8767T>C identified in heterozygous state (rs161622), B. g.3432A>G identified in homozygous state (rs2593595) C. g.10653T>C identified in heterozygous state (rs2229611).

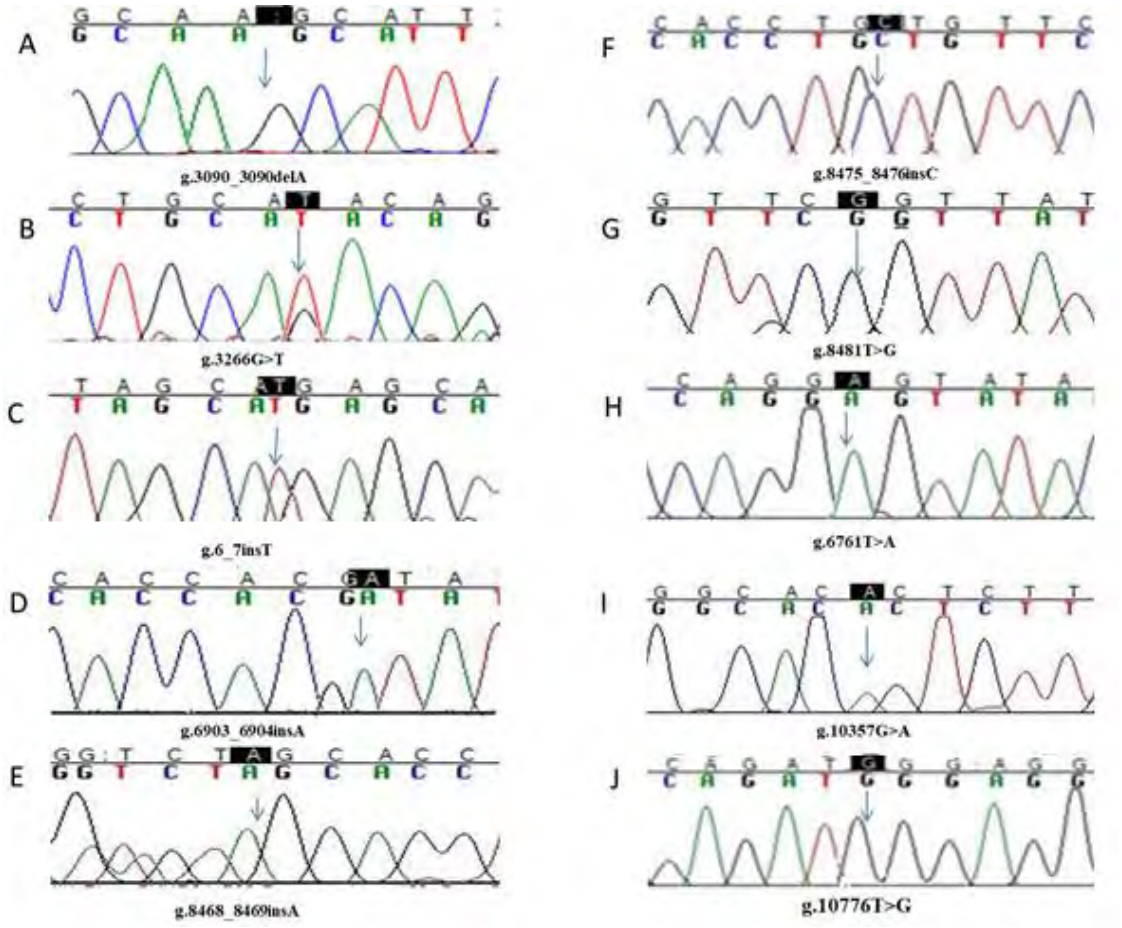


Figure 3.2.16. Chromatogram showing novel polymorphisms identified in *G6PC* gene in this study (Wild type sequences on left side and sequence variants on right side).. A. g. 3090_3090delA (homozygous). B. g.3266G>T (heterozygous), C. g.6_7insT (homozygous). D. g.6903_6904insA(homozygous). E. g.8468_8469insA (homozygous) F. g.8475_8476insC (homozygous), G. g.8481T>G (homozygous). H. g.6761T>A (homozygous). I. g.10357G>A (homozygous) J. g.10776T>G (homozygous).

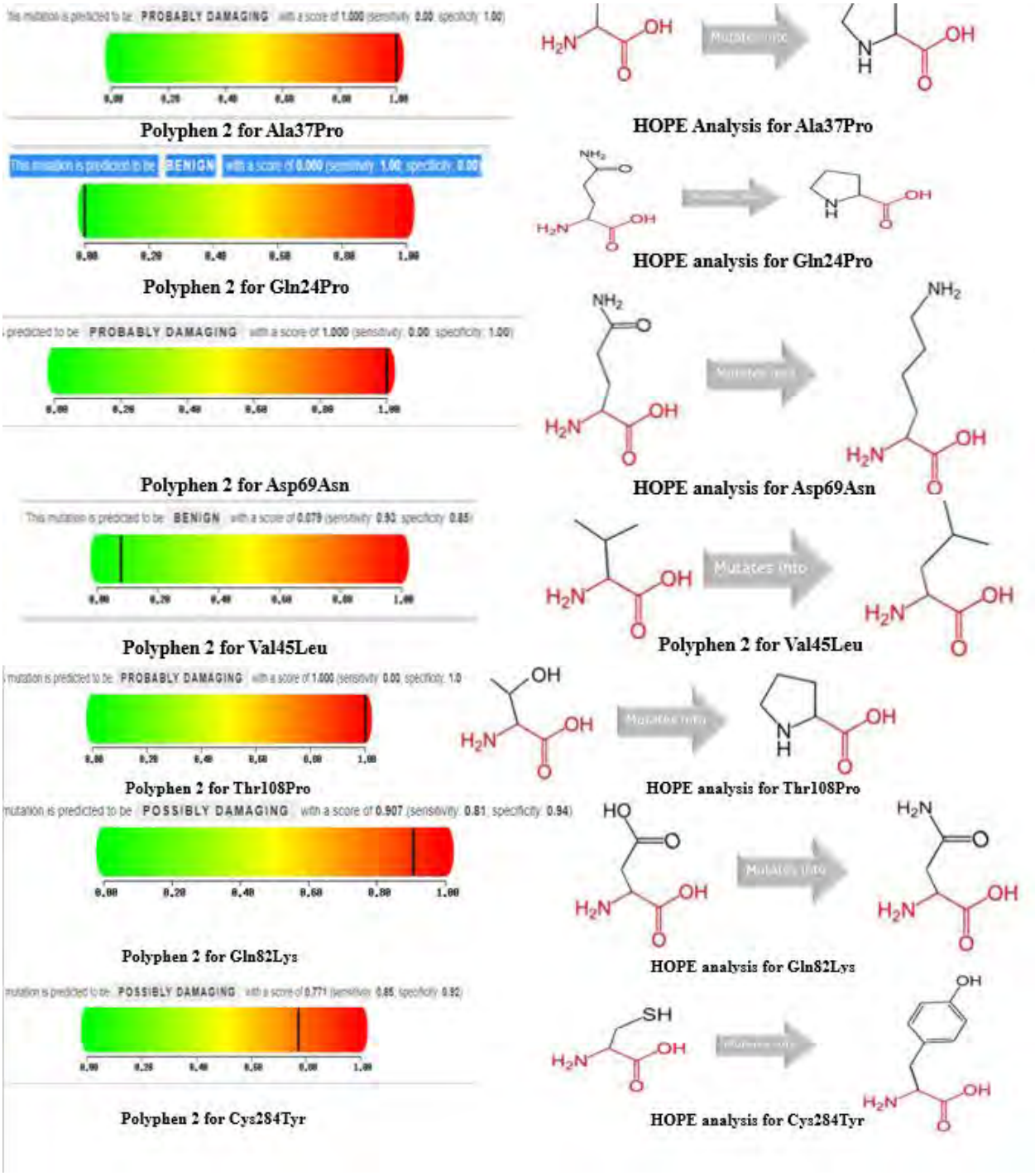


Figure 3.2.17. Results of Polyphen 2 (left) and HOPE (Protein structure prediction) tool (right) for identified disease causing variants in *G6PC* gene

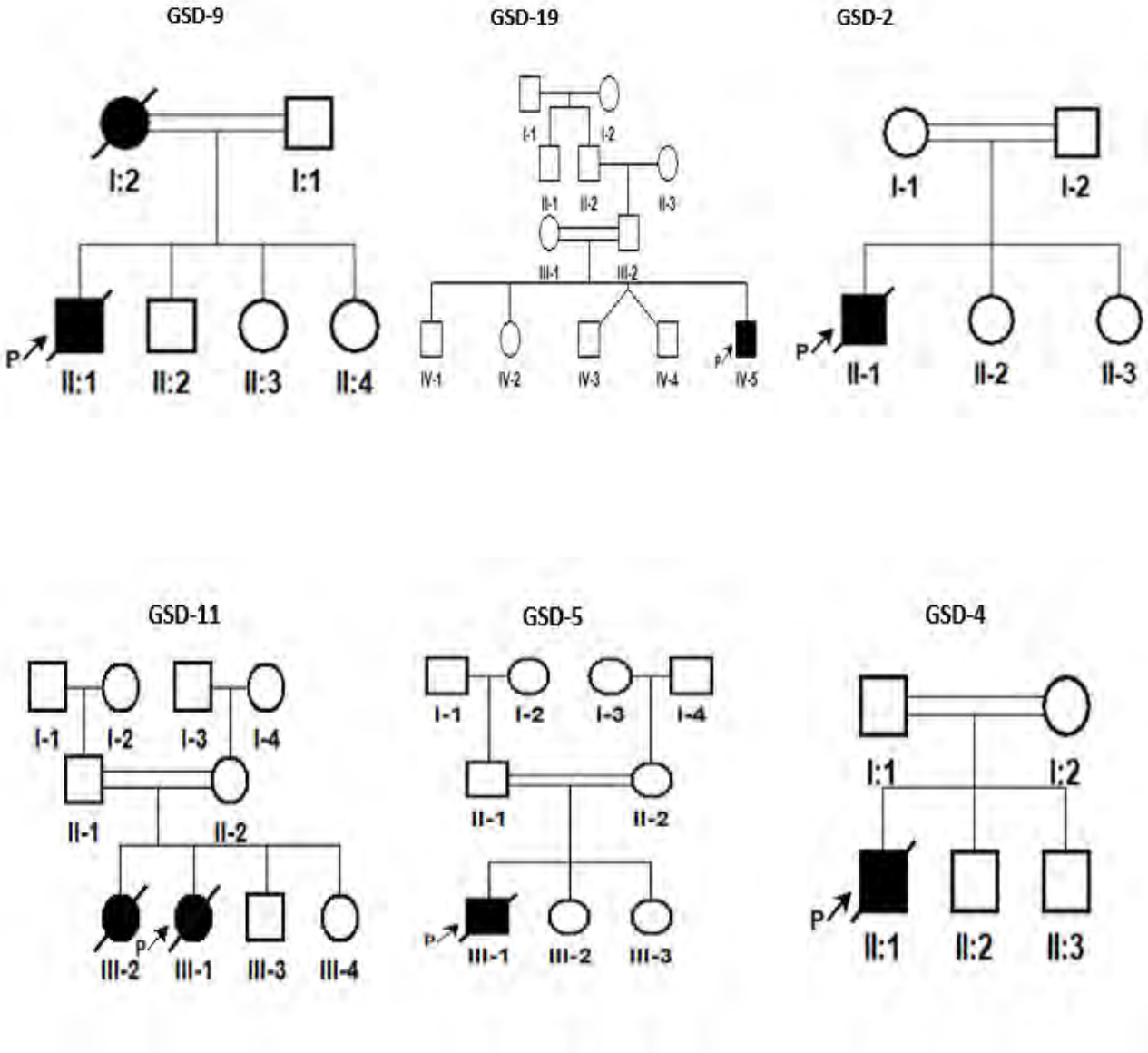


Figure 3.2.18. Pedigrees of the families in which disease-causing variants in *G6PC* gene were identified.

Table 3.2.2. A list of disease-causing variants identified in *G6PC* gene in this study

Serial No.	Nucleotide Change	Exon	AA	Zygosity	Polyphen 2		Functional Domain	SIFT P/S	Patient ID
					Prediction	Score			
1	g.3226A>C	E 2	p.Thr108Pro	Homo	PD	1	CDS	D 0.02.	GSD 04
2	g.3148C>A	E 2	p.Gln82Lys	Homo	PD	0.907	CDS	T 0.21	GSD 09
3	g.50_51insT	E1	N/A	Homo	N/A	N/A	5'UTR	N/A	GSD 02
4	g.151A>C	E1	p.Gln24Pro	Hetero	B	0	CDS	T 0.32	GSD 11
5	g.189G>C	E1	p.Ala37Pro	Homo	PD	0	CDS	APF 0.05	GSD 11
6	g.213G>C	E1	p.Val45Leu	Hetero	B	0.079	CDS	T 0.53	GSD 05
7	g.285G>A	E1	p.Asp69Asn	Homo	PD	1	CDS	APF 0.02	GSD 05
8	g.10407G>A	E 5	p.Cys284Tyr	Hetero	PD	0.771	CDS	T 0.08	GSD 19

E: Exon, PD: Probably Damaging, N/A: Not Applicable, B: Benign, T: Tolerant, D: Damaging APF: Affected Protein Function, CDS: coding sequence

Table 3.2.3. A list of polymorphisms identified in *G6PC* gene in this study

Serial No.	Nucleotide Change	Exon	Zygosity	Functional Domain	Patient frequency (%)	ID/Novelty
1	g.8767T>C	E 4	Hetero	I	15	rs161622
2	g.3090_3090delA	E 2	Homo	I	10	N
3	g.3266G>T	E 2	Hetero	I	10	N
4	g.3432A>G	E 2	Homo	I	25	rs2593595
5	g.6_7insT	E1	Homo	5'UTR	10	N
6	g.6903_6904insA	E 3	Homo	I	5	N
7	g.8468_8469insA	E4	Homo	I	10	N
8	g.8475_8476insC	E 4	Homo	I	5	N
9	g.8481T>G	E 4	Hetero	I	5	N
10	g.10653T>C	E 5	Hetero	3'UTR	20	rs2229611
11	g.6761T>A	E 3	Homo	CDS	5	N
12	g.10357G>A	E 5	Homo	CDS	5	N
13	g.10776T>G	E 5	Homo	3'UTR	5	N

E: Exon, N: Novel, I: Intronic, CDS: Coding Sequence, UTR: Untranslated region.

3.3. INTRAHEPATIC CASES OF PREGNANCY; CLINICAL FEATURES AND MOLECULAR ANALYSIS

Cholestasis is a pathological condition in which the liver's ability to produce, secrete, or excrete bile is compromised, resulting in an overabundance of bile entering the duodenum and blood. Patients affected by this condition often exhibit symptoms such as pruritus, weariness, darker urine, and jaundice. Patients in the early stages of cholestasis are often asymptomatic and only present with abnormally high levels of serum alkaline phosphatase (ALP) and gamma- glutamyl transferase (GGT).

3.3.1. Demographic and clinical characterization of ICP cases enrolled in this study

A total of 50 cases and 50 controls were enrolled in this study. The table 3.3.1 displays the demographic characteristics of Intrahepatic cholestasis of pregnancy cases. The average age of all respondents (cases and controls) lies within the interval of 23.58±3.1. Results also shows that 40% of the cases are those who have family history of ICP, and 60% cases do not have any family history of ICP. Similarly, maximum respondents i.e., 64% are those who have parental cousin marriage and the rest of all i.e., 36% haven't their parental cousin marriage. Among cases the average level of ALT lies within the interval of 344.52±598.3 mg/dL. The average level of ALP lies within the interval of 311.02±156.4 mg/24Hr. Similarly, the average level of BMI lies within the interval of 25.754±1.7, 70% of the women having overweight BMI. Blood group B+ was observed to be most common in patients with percentage 42%. Most of the cases were belonging to Middle class social status (60%), as the study was carried out at public sector hospitals. ICP history was observed in 26% of the Multigravida women out of 60%. The most common indicating clinical symptoms of ICP pruritus and jaundice was observed in 92% of the women, out of which 72% were taking medications. History of miscarriages was recorded to be 12% in cases while IUD was observed in 42% respectively.

Table 3.3.1. Descriptive analysis (Frequency and percentages of non-genetic risk factors) of variables recorded from ICP cases.

Variables	**Category**	***n*** (%)
Blood Group	A-	4(8)
	A+	5(10)
	AB-	4(8)
	AB+	6(12)
	B-	4(8)
	B+	21(42)
	O-	1(2)
	O+	5(10)
Social status	HC	8(16)
	MC	30(60)
	P	12(24)
Ethnicity	Sindh	4(8)
	KPK	12(24)
	MHJR	2(4)
	N/A	1(2)
	PNJ	31(62)
Education	Middle	6(12)
	Matric	11(22)
	Inter	14(28)
	Master	14(28)
PCM	N	18(36)
	Y	32(64)
Parity	MG	30(60)
	PG	20(40)
ICP history in MG	N	32(64)
	N/A	5(10)
	Y	13(26)
Family history of ICP	N	30(60)
	Y	20(40)
Pruritus	N	4(8)
	Y	46(92)
Medication	N	14(28)
	Y	36(72)
	0-1	14(28)
	2-3 and above	36(72)
Folic acid intake	N	6(12)
	Y	44(88)
Polyhydramniosis	N	40(80)
	Y	10(20)
Treatment taken for pregnancy	N	45(90)
	Y	5(10)
Still birth/IUD	N	29(58)
	Y	21(42)
History of miscarriages	N	44(88)
	Y	6(12)
Vit D intake	N	27(54)
	Y	23(46)
Jaundice	N	4(8)
	Y	46(92)

BMI	Normal	15(30)
	Overweight	35(70)

Table 3.3.2. Mean and SD of continuous variables for ICP cases

Continuous variables	**Mean±SD**
Age at time of marriage	23.58±3.1
BMI	25.754±1.7
ALT	344.52±598.3
ALP	311.02±156.4

3.3.2. Analysis of Genetic Variants of ICP

50 patients affected with ICP were involved in this study. Detailed information about disease and demographic profile on a specified Performa was collected from ICP cases and controls. Clinical tests (LFTs) were performed by physician to check appropriate diagnosis criterion. DNA was extracted to perform molecular analysis. After quantification of DNA ARMS PCR was performed in Biometra thermal cycler machine. Two previously reported variants (c.504 C>T and c.1686 A>G) in the *ABCB4* gene, which have been previously associated with the ICP were identified in this study. Results were analysed on 2% agarose gel revealed homozygous and heterozygous variants. Out of 50 enrolled cases of ICP c.504 C>T was observed in 17 (34%) in both hetero and homozygous forms (Figure 3.3.1, Table 3.3.3) while c.1686 A>G was revealed in 8 (16%) patients in homo and heterozygous states (Figure 3.3.2, Table 3.2.4). Statistical analyses showed significant association of c.504 C>T with the disease onset while non-significant association of variant c.1686 A>G were recorded.

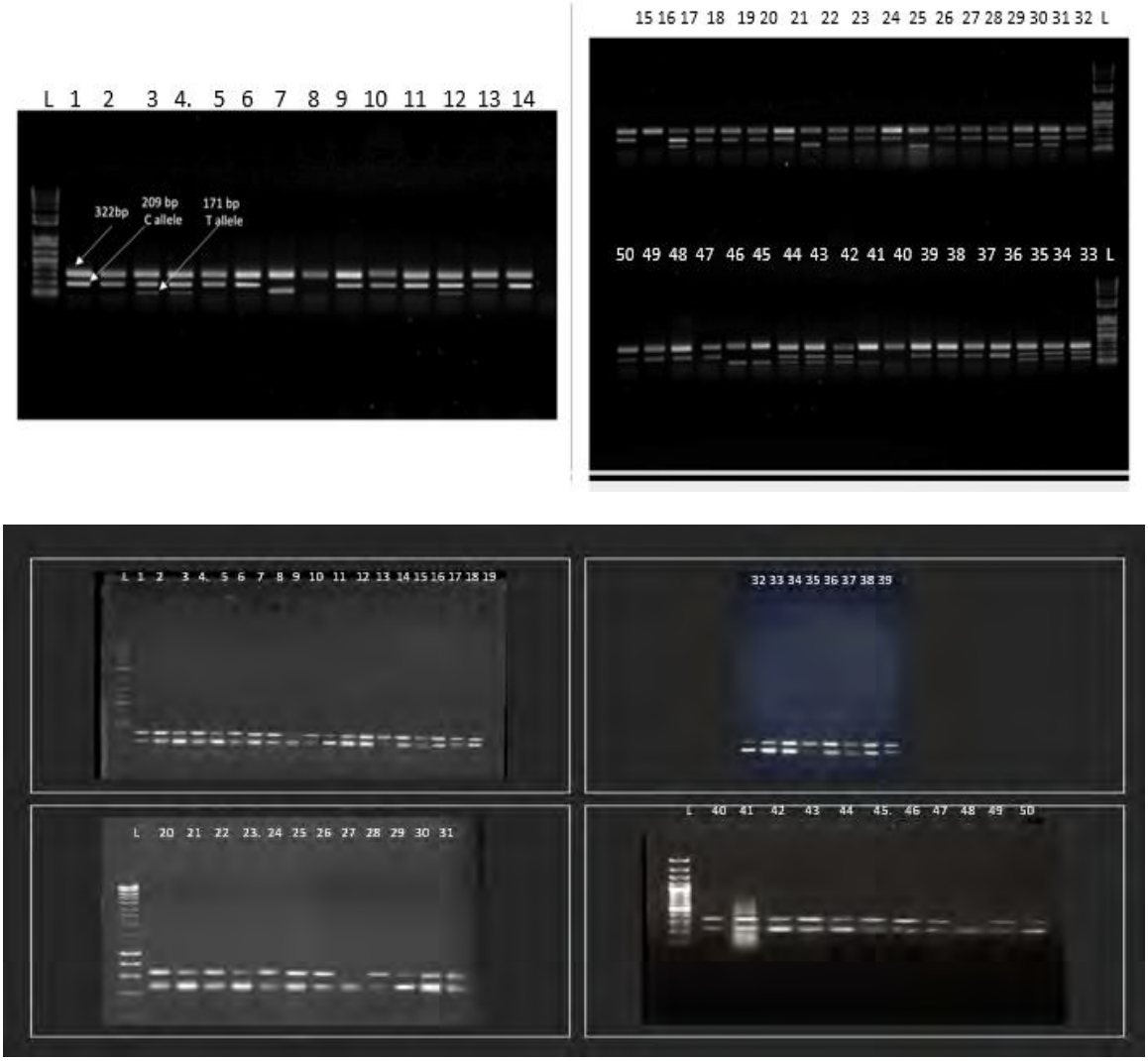
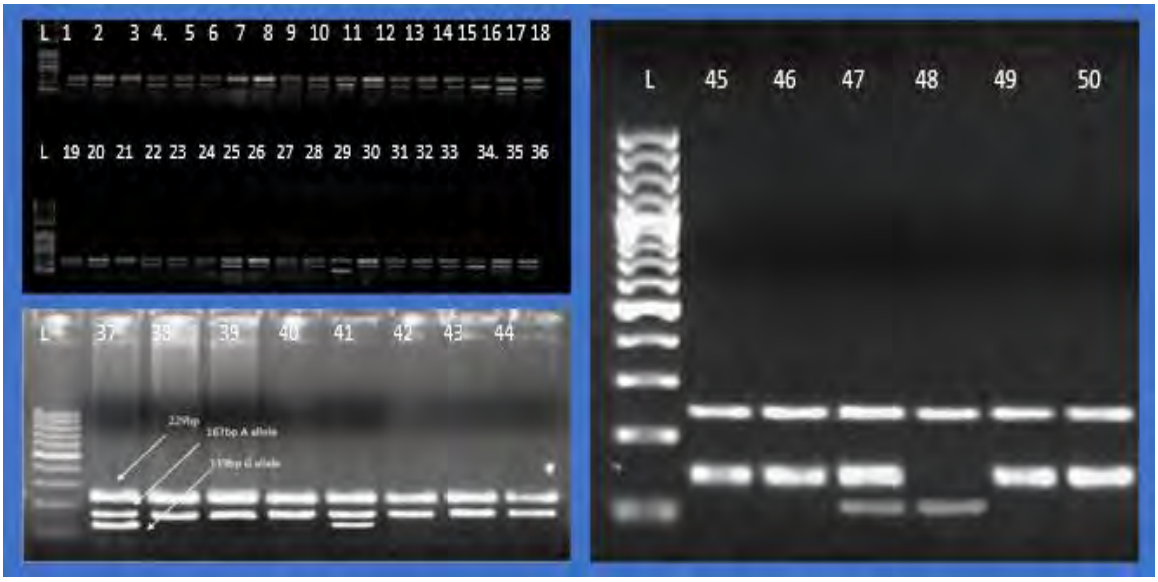


Figure 3.3.1. Agarose gel image of tetra ARMS-PCR products obtained using DNA of ICP (a) cases and (b) controls. A 2% agarose gel image showing PCR products for the amplified region containing *ABCB4* polymorphism (c.504 C>T). The PCR amplified products of CC homozygous ICP cases appear on the gel with band sizes 322 bp and 209 bp, CT heterozygous appears on gel with band size 322, 209 and 171bp, homozygous affected TT appears on gel with 322 and 171bp.



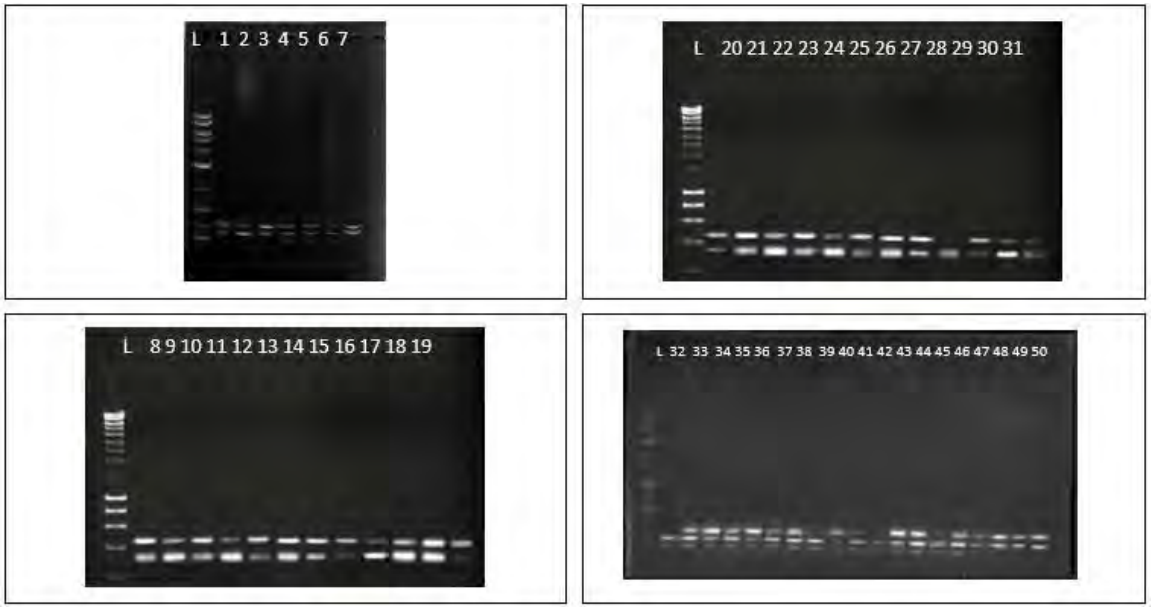


Figure 3.3.2. Agarose gel image of tetra ARMS-PCR products obtained using DNA of ICP(a) cases and (b) controls. A 2% agarose gel image showing PCR products for the amplified region containing *ABCB4* polymorphism (c.1686 A>G). The PCR amplified products of AA homozygous ICP cases appear on the gel with band sizes 229 bp and 167 bp, heterozygous AG appears on gel with 229, 167 and 119bp, homozygous affected GG appears on gel with 229 and 119bp.

Table 3.3.3. Association of *ABCB4* gene polymorphism (c.504 C>T) with ICP in Pakistani Population

Genotype	Patients Frequency (%)	Controls Frequency (%)	p value
CC (Normal)	31 (62)	50 (100)	
TT (Homozygous)	06 (12)	0 (0)	
TC (Heterozygous)	11 (22)	0 (0)	0.0063**

Table 3.3.4. Association of *ABCB4* gene polymorphism (c.1686 A>G) with ICP in Pakistani Population

Genotype	Patients Frequency (%)	Controls Frequency (%)	p value
AA	42 (84)	50 (100)	
GG	02 (4)	0 (0)	
AG	06 (12)	16 (32)	0.4200

CHAPTER 4

4. DISCUSSION

Though a lot of progress has been observed on molecular characterization of different viral infections leading to liver dysfunction (Attaullah *et al*., 2011; Sharawey *et al*., 2011) in Pakistan but wide spread studies on genetic basis and molecular characterization of inherited liver disorders including intrahepatic cholestasis, Wilson disease, glycogen storage disease, hereditary fructose intolerance, hemochromatosis, congenital hepatic fibrosis etc. has not taken place and there are only few epidemiological studies regarding patients with congenital liver disorders (Hafeez *et al*., 2016; Parkash & Akram, 2015). Therefore, overall goal of proposed study is to molecularly and genetically characterize inherited liver disorders present in Pakistani population. The study was approved from Bioethical committee of Quaid-i-Azam University Islamabad. Patients suffering from Wilson's disease, Glycogen storage disease type 1a and Intrahepatic cholestasis of pregnancy were enrolled in the study with informed consent from Pakistan institute of Medical Sciences Islamabad, Children Hospital Lahore and Wah Genetal Hospital, Wah cantt Pakistan.

4.1. Study of clinical presentation and genetics of Wilson's disease cases from local population.

Wilson disease is caused by impaired biliary copper excretion and is inherited as an autosomal recessive disorder. Mutations in the *ATP7B* which encodes a metal-transporting P-type adenosine triphosphatase, a transmembrane transporter of copper within hepatocytes are responsible for Wilson disease (Roberts & Schilsky, 2008). Symptoms of Wilson's disease, being highly unspecific, cannot be identified at initial level and patient remains phenotypically asymptomatic until late stages (Caca *et al*., 2001).

The aim of this study was the genetic screening and clinical analysis of Wilson's disease among local population. 80 patients with the Wilson's disease were enrolled in the study from the Pediatric Department and Liver Unit of Pakistan institute of medical sciences (PIMS) Islamabad, and Children Hospital Lahore (CHL), over the period January 2018 to January 2020. Clinical investigation was done in cases and statistical analysis was performed using SPSS 20.0. We selected unbiased patient pool i.e., both hepatic and neurological cases, without difference disease manifestations due to gender difference.

The diagnosis of Wilson's disease still depends primarily on the evaluation of clinical and laboratory evidence of abnormal copper metabolism. The onset of the disease frequently includes manifestations related to the liver (as chronic liver disease or acute liver failure) and neurological symptoms, although it can sometimes be asymptomatic. Despite it being more frequent in young people, WD has been described in all life stages (Ferenci *et al*., 2003). Due to its fatal prognosis, WD should be suspected in all patients with unexplained biochemical liver abnormalities or neurological or psychiatric symptoms. The diagnosis is established with a combination of clinical signs and tests, including the measurement of ceruloplasmin, urinary copper excretion, copper quantification in liver biopsy, or genetic assessment (Ferenci *et al*., 2003). Diagnostic criteria in this study included a blood ceruloplasmin level below 0.2 g/l (normal range 0-2 to 0-6 g/l), an elevated hepatic copper level (>300 ug/g dry liver), and/or a positive biopsy. Demographic and clinical characteristics of each proband were recorded. Steatosis was observed in 85% patients. Cirrhosis was presented in 38% cases, jaundice was seen in 87.5% cases. 80 percent of the patients identified with parental cousin marriage shows that consanguinity is another determinant of the disease pathogenesis, as Pakistan is among those inbred population where rate of consanguinity is more than 50 percent (Iqbal & Van Bokhoven, 2014). 47.5% study participants have family history of WD. 58.75% developed KF ring in their eyes. It was also observed that average age of WD patients is 11.3, showing early age of onset of the disease, previously reported by Walshe *et al* (Walshe *et al.,* 1992). It is also obvious from our clinical data that patients who are diagnosed early with WD, are presented with hepatic anomalies, while those with later diagnosis suffer from neurological abnormalities along with hepatic disorders previously reported by Bandmann in 2015 (Bandmann *et al.,* 2015). Stage of illness is significantly related with the deceased alive status of the cases-means as the stage of the disease progresses it results in the death of the patients. The data shows that there is significant association between stage of illness and age at diagnosis, therapy received (Table 3.1.2).

The mutational pattern in the *ATP7B* gene is highly non-overlapping in different regions of world (Hedera, 2017). Most common mutation found in the mixed European populations like H1069Q responsible for 35 to 45 percent of WD alleles in these populations, and another common mutation i.e., R778L is common mutation type in Asian populations

where it accounts for more than 20 percent of all WD alleles in these populations (Ferenci, 2006). C271X found in our patient was found to be common in Indian patients and Turkish populations (Gupta *et al*., 2007).

Blood samples were collected from patients and their available family members. Direct sequencing of both the forward and reverse directions of the exon 1, 2, 3, 4, 5, 8 & 13 of *ATP7B* gene in selected patients with WD was performed to screen for disease causing variants. The study identified 22 variants in selected exons and intron-exon boundaries, 7 disease causing variants and 15 polymorphisms. Out of **7** Identified disease-causing variant, 3 (p.V761E, p.I976N, p.I774L are novel and 4 (L227Yfs*35, p.D765N, p. A1003A and p.S986T) are reported. Out of 15 Polymorphisms 5 are reported and 10 are novel. L227Yfs*35 mutation, affecting Copper binding domain (Cu2/Cu3) of protein, reducing the biliary excretion of copper and may harbor moderate to severely disabling phenotype with reduced variability. This disease causing variant was previously reported as the most common mutation in Western India has also been observed in exon 2 of one of our patient with frequency of 5% (Aggarwal *et al*., 2013). This mutation was first time reported from India by Aggarwal in 2013. In this study identified patient belonged to Khyber Pakhtunkhwa province, born out of consanguineous marriage was presented with abdominal pain, yellowing of the skin and severe hepatic disabilities, persistently elevated serum aminotransferase activity and cirrhosis (Gul *et al.,* 2022).

The identified novel homozygous non-synonymous variants include (p. I774L, V761E and p. I976N) in 15% cases and 5% cases respectively. Reported Non-synonymous substitutions include (p. D765N and p. S986T) both in 5% of patients. The reported synonymous disease-causing variant p.A1003A identified in 40% cases was predicted to be associated with affecting copper channel domain. The other reported synonymous variant p. A1003A identified in 10% patient was previously reported from Egyptian Population. Pathogenicity of the identified variants were confirmed via Insilico analysis including HOPE, Polyphen 2, Provean, SIFT, Mutalyzer and mutation taster (Table 3.1.4).

A reported Polymorphism, c.1544-53A>C is found in very high frequency i.e., 100 percent of our patients. As frequency of this polymorphism was not checked in controls from our

population, so involvement of this variation in disease predisposition could not be established. Laurila & Vihinen suggested the role of non-coding regions in altering the expressions of the coding regions, as there are many reports which suggest the role of UTRs and non-coding regions in disease phenotype (Laurila & Vihinen, 2009). Another previously reported polymorphism i.e., c.1366G>C, is found in 40 percent frequency, in Cu4/Cu5 Binding region of the gene in exon 3. We have found a reported polymorphism c.83C>A, in 5'UTR of 35 percent of our affected samples.

More than 850 mutations in the *ATP7B* gene have been reported worldwide but there is very insignificant data available from Pakistan (Gul *et al*., 2022). Identification of 22 variants adds to the existing knowledge of the Wilson's disease. Our study depicts genetic heterogeneity in our population. Genetic counseling was provided to families explaining neurological and liver manifestations. Early clinical identification and risk stratification in asymptomatic individuals may be possible with the use of genetic counselling and routine follow-up of affected siblings. Disease causing variants were identified in 8 out of 20 families, based on results of our study emphasizes on the necessity of detailed molecular analysis of all exons of *ATP7B* gene, along with their flanking regions for identification prevailing disease-causing variants remaining cases. Detection of shared exonic hotspots will play a fundamental role.

Copper deficiency can be treated with prolonged administration of chelating drugs in conjunction with zinc; however, anemia is often the first symptom of this noncompliance (Brewer, 2001; Mufti *et al*., 2006). If Wilson's disease is diagnosed at initial stages i.e., in asymptomatic individuals only Zn treatment is enough to prevent disease progression, but with latter diagnosis side effects of drug make treatment very complicated. Mostly little disease knowledge is observed in the families of the proband- the reason why patients do not stick to the treatment procedures. Trientine is the most preferred baseline treatment method for WD in Pakistan, based on the possible side effects of the penicillamine.

Quality of life (QoL) is most significant objective patient-reported consequences of treatment of chronic disorders; though, QoL has not been clearly explored in WD. Only 4 studies have been performed, aimed to confirm the Health Survey Questionnaire (SF-36)

and the Brief Questionnaire in Wilson's disease patients (Litwin *et al*., 2018). Main assumption from these studies is that, poor QoL in patients with a long delay without WD treatment (Svetel *et al*., 2011) emphasizing the importance of early diagnosis of WD. It is challenging to approve any QoL measure for use frequently in WD due to the small number of studies, the vast variety of symptoms, and the lack of homogeneity in presentation. It's also important to highlight the fact that huge numbers of WD patients have mental issues like as behavioral, cognitive, or criticism abnormalities, which can make assessments less accurate. There must be more research done, with large number of patient samples.

Wide-ranging genetic studies on WD in local populations are required because of the high suspected incidence of the disease due to consanguinity, the lack of molecular genetics data, the clinical heterogeneity of disease with challenging disease diagnosis, and the high mortality and economic burden of the end-stage disease treatment option, i.e., liver transplant. Research of this nature will aid in the early, precise genetic diagnosis of the affected cases and their asymptomatic family members, which is crucial for the proper care of patients and the provision of genetic counselling. In addition, the information gathered from these studies will allow for genotype-phenotype correlation of the local WD patients, which in turn will raise medical professionals' understanding of the clinical presentation of WD and lessen the likelihood of future morbidity and mortality resulting from a delayed diagnosis.

4.2. Mutational analysis and alinical investigations of patients affected with Glycogen storage disease type 1a (GSD 1a) in Pakistan

Disorders of glycogen breakdown and synthesis, collectively known as glycogen storage diseases (GSDs), are a diverse collection of genetic conditions with similar symptoms. In most cases, children are the ones who are diagnosed with a GSD, and organs most commonly affected are liver, muscles, and heart (Koshy *et al*., 2006). Liver tissue can be examined clinically, biochemically, and enzymatically to distinguish between GSDs. There are 10 primary kinds, distinguished by enzyme deficiencies (Mahmoud *et al*., 2017). The most frequent kind of GSD is GSD I, which was initially characterised by von Gierke (Özen, 2007). Glucose-1,6-disulfide isomerase (G6Pase) deficiency (GSD Ia), glucose-6-

phosphate translocase deficiency (GSD Ib), abnormalities in a putative phosphate transporter (GSD Ic), and flaws in putative glucose transporter (GSD Id) are the four subtypes of GSD I (Chou & Mansfield, 2008). GSD Ia is a metabolic disease that affects around one in every 100,000 newborns (Mahmoud *et al*., 2017). Insufficient activity of the G6PC system, which catalyses the hydrolysis of G6P to phosphate and glucose, is the root cause. Located on 17q21, *G6PC* is a single-copy gene (Lei *et al*., 1993). It is expressed largely in the liver, kidney, and gut, and its five exons cover roughly 12.5 kb of DNA (Lei *et al*., 1993). Glucose 6-phosphatase (*G6PC*) enzyme catalyzes the hydrolysis of glucose-6-phosphate (G6P) to produce inorganic phosphate and glucose in liver and kidney cells. The glucose produced is then transported out of the cell to contribute to maintenance the blood glucose level even during starvation. Disease causing variants in the *G6PC* gene result in defective glucose-6-phosphatase activity causing storage of glycogen in liver and kidney cells leading to glycogen storage disease type Ia (GSD1a). Alternate metabolic pathways are activated by mutations in the *G6PC* gene, causing glycogen to accumulate in the liver and kidneys and so causing progressive hepatomegaly and nephromegaly. Some of the metabolic effects of elevated cytoplasmic G6P levels include hypercholesterolemia, hypertriglyceridemia, hyperuricemia, and lactic acidemia (Janecke *et al*., 2001). GSD1a is a rare but serious metabolic condition that runs in families as an autosomal recessive disorder (Lei *et al*., 1994). Analysis of *G6PC* mutations is required to acquire differential clinical diagnosis (Mahmoud *et al*., 2017). To date, numerous mutations have been reported in *G6PC* gene, including missense (the most prevalent form), nonsense, insertion/deletion and the splicing but there is serious lack of genetic analysis of GSD 1a cases from Pakistan (Ahmed *et al*., 2022).

Autosomal recessive disorders are prevalent in Pakistan because of the high rate of consanguineous marriages (Ullah *et al*., 2017). Incidence of hepatic glycogenesis is unknown for Pakistan, and there have been no case reports or in-depth disease causing variant investigations of GSDs to date. Families at risk of having children with GSD Ia can get genetic counselling through the investigation of disease-causing variants in their family tree. In addition, early diagnosis improves quality of life by allowing for proper metabolic management techniques and therapies to be implemented before issues arise (Mogahed *et al*., 2015).

The focus of this study was on clinical and genetic analysis of GSD 1a cases from Pakistan. Clinical analysis was performed on 40 GSD 1a cases, with presenting symptoms of seizures, increased respiratory rate caused by hypoglycaemia, irritability and hyperlactacidaemia as well as hepatomegaly was predominantly present (Table 3.2.1). The study identified that males (65%) were more affected as compared to females (35%), although there is no such finding previously previously reported. 70% of the patients belonged to the age group 0-10 years in which the disease appeared during early ages. 87.5% cases in this study belonged to consanguineous families. 57.5% patients could not survive disease severity due to lack of proper disease management resulting in complications including severity of hypoglycaemic events leading to high mortality rate as reported previously by Ai *et al*., 2020 (Ai *et al*., 2020). Seizures and delayed motor development were observed in 25% and 12.5% cases respectively, as observed in previous studies (Ellingwood & Cheng, 2018). Three patients were observed with cushingoid appearance and osteopenia was found in 9 cases. Cushingoid appearance, delayed motor development and osteopenia are attributed to untreated GSD1a (Ellingwood & Cheng, 2018). These complications are attributed to hypothalamic-pituitary-adrenal (HPA) axis stimulation due to chronic hypoglycaemic stress causing elevated glucocorticoid secretion (Hannah *et al*., 2022). In our study cohort, inflammatory bowel disease (IBD) was present in 15 patients (37.5 %), that is higher as studies have reported an occasional presence of IBD in GSD1a cases (Ai *et al*., 2020). .

Disease causing variant analysis of GSD1a in 20 selected cases identified four novel missense variants i.e., p.Ala37Pro, p.Asp69Asn, p.Gln82Lys and p.Thr108Pro in homozygous condition (Table 3.2.2). All these variants were predicted to be disease causing according to Polyphen 2 and SIFT prediction. The identified polymorphisms are reported in table 3.2.3. In addition, we also identified three missense variants including p.Gln24Pro, p.Val45Leu and p.Cys284Tyr in heterozygous conditions in GSD1a affected cases. All these disease-causing variants have not been reported yet. Variants identified in coding sequence of exon 1 include p.Gln24Pro, p.Ala37Pro, p.Val45Leu and p.Asp69Asn respectively. An insertion disease causing variant also identified in 5'UTR g.50_51insT. The variant in coding sequence of exon 2 lead to protein changes p.Thr108Pro (replacement of a hydrophilic with hydrophobic amino acid) and p.Gln82Lys (replacement

of acidic with basic amino acid) respectively were predicted to be damaging with Polyphen score of 1 and 0.907. A missense damaging variant with amino acid changes p.Cys284Tyr (replacement of sulfur containing with aromatic amino acid) is identified in coding sequence of exon 5 which is predicted to be damaging with Polyphen score of 0.771 and SIFT score 0.08. Sequencing of exon 1 of GSD 5 and GSD 11 identified homozygous disease-causing variants i.e., p.Asp69Asn & p.Ala37Pro and heterozygous i.e. p.Val45Leu & p.Gln24Pro . Identified homozygous variants in both patients were predicted to be probably damaging according to Polyphen 2 prediction, while both heterozygous variants were benign signifying the presence of other heterozygous variants in non-coding region of the exon.

Glucose 6 phosphatase is anchored in ER membrane by nine transmembrane helix structures, amino terminus lies in the lumen of ER while carboxy terminal in cellular cytoplasm (Chou & Mansfield, 2008) and all of our identified missense disease-causing variants in transmembrane helix structures of *G6PC*. Shieh and Angaroni., 2003 have suggested that majority of helical missense variants cause decreased stability of *G6PC* protein compared to the wild-type enzyme (Shieh *et al*., 2003). Although the functional studies could not be performed to confirm damages caused by these mutations but laboratory and bioinformatic analysis demonstrated these mutations to be pathogenic GSDIa associated variants. Hence identification of homozygous missense disease-causing variants in five cases and heterozygous variants in three cases provide molecular genetic basis of clinical manifestations of the GSDIa in these patients.

In fourteen cases showing clinical symptoms of GSDIa, no disease-causing variants in coding regions was found, which highlights the ratio of *G6PC* disease causing variants in our study to be 30%, however there is still need of further molecular studies since due to overlapping clinical presentations of Glycogen storage disease types (Scriver, 2001).. There is a possibility of identification of disease-causing variants in genes reported for other subtypes, as molecular analysis is a definitive tool to differentially diagnose GSD (Iijima *et al*., 2021).

Despite many advances at molecular genetics level, there are yet a number of inconsistencies in GSD 1a that remained unresolved, i.e etiology of the renal and liver disease in GSD-Ia remains unclear, phenotypic heterogeneity and the lack of a stringent genotype-phenotype in GSD-Ia (Cassiman *et al*., 2010).. It is necessary to conduct extensive genetic studies in the local population due to a high suspected incidence of disease, a lack of molecular genetic data, and the clinical heterogeneity of GSD with the challenging disease diagnosis and the high death rate and economic burden of the end-stage disease treatment, such as liver transplantation. These tests will be able to assist in the early genetic identification of patients as well as their asymptomatic family members. Additionally, they will enable frequent follow-ups, which will improve patient treatment as well as genetic counselling. It is necessary to conduct research on the genotype-phenotype correlation of local GSD1a patients in order to assist our health care providers in better understanding the clinical presentation of GSD. This will help reduce the likelihood of morbidity and mortality in the future that will be caused by a delayed diagnosis.

4.3. Enrolment of Intrahepatic cases of pregnancy cases, their clinical features and molecular analysis

Pruritus, abnormal liver function test results, and an increased bile serum level are all symptoms of intrahepatic cholestasis (ICP), a liver condition that only manifests during pregnancy. These symptoms resolve implications on fetal development and cause fetal complication (Glantz *et al*., 2004). The etiology of ICP is undoubtedly multifactorial, with genetic, environmental, and the hormonal factors having important roles (Kreek,1987). Hepatocytes produce and exude bile acids, which travel through the biliary tree to the gallbladder. Intrahepatic cholestasis (ICP) occurs when pregnancy hormones cause the bile ducts to constrict, leading to hepatic inflammation and a rise in blood bile acid output (Wikström Shemer & Marschall, 2010). Possible adverse outcomes for the developing foetus include preterm birth, meconium staining of the amniotic fluid, respiratory difficulty, and even intrauterine death (Williamson & Geenes, 2014). The genetic component of ICP has been investigated, and it has revealed that mutations in several ATP-

binding cassette (ABC) transporter, including *ABCB11*, *ABCB4*, and a P-type ATPase, *ATP8B1* may predispose to ICP (Dixon *et al*., 2017).

The prevalence of ICP varies worldwide due to multifactorial etiology. In Europe ICP occurs in 0.2%- 2% pregnancies (Williamson and Victoria., 2014). The Pakistani and Indian groupings saw a far greater ICP prevalence than whites with incident rate of 1.46%, 1.24% and 0.62% respectively (Abedin *et al*., 1999). In an observational study conducted in Kharyan Pakistan, the occurrence rate of ICP was reported to be 3.1 % (Noor *et al*., 2021). The mean age of cases was 29.8±4.76 (Hafeez *et al.,* 2016). In a study of Caucasian region mean age of ICP cases was 30 years (Bacq *et al*., 2009). The significant high difference in prevalence demands further investigation of ICP in Pakistani population as this disease has role in fetal morbidity and mortality.

A higher prevalence of genetic disorders is linked to high rates of consanguinity. Consanguinity increases the incidence of familial progressive intrahepatic Cholestasis (PFIC), which develops when heterozygous mutations that cause ICP segregate into homozygous form (Jacquemin & Hadchouel, 1999). The Pakistan Demographic and Health Survey (PDHS) estimates that 61.2% of Pakistanis are consanguineous, with first and second cousins being the inclusion criterion. Furthermore, consanguineous marriages also had higher rates of child mortality and morbidity (Afzal *et al*., 2020).

ICP-related foetal problems are thought to have an elevated flow of bile acids into foetal circulation, as shown by raised levels in amniotic fluid. The exact cause of this increased flux of bile acids into the foetal circulation is unknown. Vectorial transfer of the bile acids from fetus to the mother is hindered in ICP, and this is due to lower efficacy of ATP-independent transport, according to in vitro investigations of isolated placental vesicles. When considered as a whole, these data imply that bile acids build up in foetal portion and are hence likely to increase foetal risk (Geenes & Williamson, 2009).

There is evidence that the disease is triggered by the presence of different steroid hormones. Symptoms often appear toward the end of pregnancy when progesterone and oestrogen levels are at their peak (Wasmuth *et al*., 2019). In present study onset of ICP in all cases was in third trimester. Additionally, when hormone levels have returned to normal,

postpartum symptoms go away. When using combination oral contraceptive pills, cholestasis recurrence in women with a history of ICP and a greater incidence of twin gestations have been observed (Noor *et al*., 2021).

For the statistical and molecular analysis of intrahepatic cholestasis of pregnancy (ICP) 50 patients and 50 control samples were recruited from Wah General Hospital and Izzat Ali shah Hospital Wah cantt after informed consent. SPSS 21.0. was used for the descriptive analysis i.e., Frequency and percentages of non-genetic risk factors.

Clinical analysis showed that mostly patients belonged to positive blood group types with B+ blood group observed in 42% cases. Rh factor may contribute to the disorder's etiology or be affected by it (Noor *et al*., 2021). A sizable cohort research is needed to verify this theory. This clinical characteristic has not before been evaluated, therefore it may add a new perspective to our understanding of the etiology of this illness. B+ is predominant type of blood group (Batool *et al.,* 2017), therefore occurrence of ICP in B+ women may not have any contribution. Most of the cases belonged to middle class families. The observed factors in our patients, that might contribute to the disease onset include parental cousin marriage, being pregnant with multiple babies, multigravida, family history of intra-hepatic cholestasis, intake of folic acids and being overweight (Ovadia et al., 2019). Pruritis stillbirths' history of miscarriages and jaundice were the predominant disease outcomes observed in maximum cases, relating with the disease pathology.

Evidence from reported familial instances showed that heritable risk factors contribute to the genesis of ICP (Jacquemin & Hadchouel, 1999). ICP follows a strictly sex-based autosomal dominant pattern of inheritance (Jacquemin & Hadchouel, 1999). 60% of those who participated in the present study said they had a history of ICP in their families, whereas the other 40% were isolated incidents. 32 (or 64%) of the 50 ICP cases tested positive for paternal consanguinity. *ABCB4* gene missense mutation in an inbred family was linked to varying degrees of liver disease, from intrahepatic cholestasis (ICP) to cirrhosis, suggesting that parental consanguinity may play a role in the development of ICP (Gotthardt *et al*., 2008). Therefore, a range of cholestatic diseases with varied degrees of severity are associated with *ABCB4* mutations and variants. Previously reported

polymorphic variants c.504 C>T and c.1686 A>G were reported to be associated with ICP in a study by Noor *et al* (Noor *et al*., 2021).

In this study ARMS PCR for molecular analysis of genetic variants of ICP in 50 cases and 50 controls for the identification of known variants c.504 C>T and c.1686 A>G in exon 6 and 14 of *ABCB4* gene showed significant association of c.504 C>T, whereas c.1686 A>G was non-significantly associated with the disease. Educating obstetric care providers and pregnant women about disease incidence in different parts of the country is important for early presentation, accurate diagnosis and management of disease to decrease unfavorable outcomes. Present study identified the known variant c.504 C>T in homozygous condition and heterozygous in 6 cases and 11 cases respectively. Gendrot *et al.* found c.504 C>T in cases with ICP who were of various racial and ethnic backgrounds, including Caucasians and Japanese (Gendrot *et al*., 2003). The influence of variant on the splicing mechanism can be analyzed with a number of in silico analysis techniques. The exonic variation c.504 C>T was expected to trigger breakage of the Exonic Splicing Enhancers (ESEs) and the development of a novel ESE site 3 nucleotide in this study by using HSF3.0 in silico analysis. Changes in the putative exonic splicing enhancer binding location may have an effect on wild-type pre-mRNA splicing(Diken *et al*., 2014). The exon-intron junctions on the pre-mRNAs, as well as exonic and intronic splicing enhancers/silencers, are all recognized by the nuclear spliceosome for accurate exon recognition (Chen *et al*., 2009). The exon/intron regions contain long sections of altered sequences that serve as binding sites for trans-acting RNA-binding proteins that can either promote or inhibit the splicing of pre-mRNA (Chen *et al*., 2009).

Six individuals were found to be heterozygous for the reported synonymous change c.1686A>G in *ABCB4*'s exon 14, and two patients were homozygous for the variant. Although c.1686A>G was predicted to be pathogenic by the mutation taster, it was also found in 16 healthy controls. According to HSF3.0 prediction, the c.1686A>G mutation might alter *ABCB4* pre-mRNA splicing by creating a probable new donor site or by encouraging a new Exonic Splicing Silencer site (ESSs). Splice-regulating factors (ESSs) bind members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family that play a

negative role in splicing by preventing exon inclusion and promoting intron retention (Chen *et al*., 2009).

Identification of c.504C>T variant in 34% cases and c.1686A>G in 16% cases, and high percentages of both mutant alleles as compared to controls suggested a hereditary predisposition to disease in the local population, highlighting the need to inquire about a progressive personal or family history of ICP/PFIC3 from each pregnant woman at her first gynecological visit and to implement a screening test for ICP at the beginning of the third trimester.

4.1. Conclusion and future perspectives

Clinical and genetic analysis of Wilson's disease in Pakistani population showed diverse clinical heterogeneity. Improper diagnosis, lack of the disease knowledge, unable to undergo liver transplant due to economic burden are the main causes of mortality due to Wilson's disease in Pakistan. Screening of *ATP7B* gene in local population resulted in identification of novel and known disease causing variants from Pakistan. Genetic screening of whole *ATP7B* gene will reveal novel disease-causing synonymous variants involved in underlying protein defects. Several mutations in G6Pase gene of the affected individuals that completely inactivate enzyme have been identified. Owing to the recessive inheritance pattern of GSD1a, its incidence is higher in the populations with conventional consanguineous marriages like Pakistan, necessitating comprehensive clinical and the genetic studies on this disease from our local population. In present study, the clinical and molecular characterization of the GSD 1a in Pakistani cases presented at tertiary care hospitals, identified the clinical heterogeneity and novel disease-causing variants in *G6PC* gene. These results establish the molecular basis of this disease and open the way for future gene therapy. Identification of disease-causing variants in the ICP cases of Pakistan adds to the existing knowledge of the disease. The management of these individuals is of the utmost significance in order to decrease the morbidity and mortality of feto-maternal conditions. The most important contributions of this study are to raise awareness about the prevalence of this condition in our nation and to pave the path for more research to be conducted. This might open up new areas for research into the reasons of many stillbirths

and fatalities that occur within the uterus that are unexplained. Education of the affected people as well as healthcare providers should be the goal to improve the health of pregnant women and children. This could be a cause of foetal distress and sudden unexplained intrauterine deaths. More research has to be done in various sections of the nation in order to determine the precise prevalence of the condition, since this will allow us to avoid issues for both the fetus and the mother if we are aware of the disease's exact burden. In addition to this, there is a pressing need to make bile acid assays readily available for early and consistent diagnosis, as well as to make the procedure more cost-effective.

Sequencing of whole *ATP7B* gene in all available cases and their suspected siblings should be done, and their parents should be screened for segregation of the recessive allele in their family tree for understanding the inheritance pattern. Better clinical diagnosis for GSD subtypes should be focused for better understanding of the diagnostic criteria and disease onset. Sequencing of involved genes like *G6PC* should be done in all available patients and suspects from consanguineous populations like Pakistan. *ABCB4* gene for ICP cases in Pakistan is not fully screened for disease causing variants. ICP being multigenic disorder needs screening of other genes for variant identification, that might also be playing role in the pathophysiology of the disorder. The frequency of metabolic liver disorders is increasing in consanguineous populations. Early detection and screening, dietary modifications, cost effective drugs, better treatment methods and genetic testing and timely picking-up of asymptomatic potential cases and the genetic counselling of the parents for describing the mode of inheritance of disease and early detection of cases are much needed in the developing countries like Pakistan for disease management and control.

CHAPTER 5
5. REFERENCES

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ANNEXURE 1

ETHICAL APPROVALS

QUAID-I-AZAM UNIVERSITY
OFFICE OF THE DEAN FACULTY OF BIOLOGICAL SCIENCES

No.DFBS/2016-371 Dated: April 25, 2016

Dr. Sabika Firasat
Assistant Professor
Department of Animal Sciences
Quaid-i-Azam University
Islamabad.

SUBJECT: **"MOLECULAR GENETIC ANALYSIS OF METABOLIC LIVER DISORDERS IN LOCAL POPULATION: TO ESTABLISH MOLECULAR GENETICS DIAGNOSTIC TESTING "**

Dear Dr. Sabika Firasat,

We wish to inform you that your subject research study has been reviewed and is hereby granted approval for implementation by Bio-Ethical Committee (BEC) of Quaid-i-Azam University. Your study has been assigned protocol # BEC-FBS-QAU-37.

While the study is in progress, please inform us of any adverse events or new, relevant information about risks associated with the research. In case changes have to be made to the study procedure, the informed consent form and/ or informed consent process, the BEC must review and approve any of these changes prior to implementation.

Sincerely,

25/4/16

Prof. Dr. Wasim Ahmad
Dean Faculty of Biological Sciences
Chairperson, BEC
Quaid-i-Azam University.

Prof. Dr. Wasim Ahmad

Office of the Dean Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, 45230 Pakistan
Tel No: 92-51-90643073 92-51-90643101 & 92-51-90643003 Fax: 92-51- 90643170

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PROF. DR. ALI JAWA
Secretary
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MD (USA), MPH (USA), FACE (USA)
Diplomate
ABIM- Endocrinology & Diabetes
Diplomate
American Board of Internal Medicine,
Diplomate
American Board of Physician Nutrition Specialists
Professor of Endocrinology
Shaheed Zulfiqar Ali Bhutto Medical University, PIMS, Islamabad, Pakistan

No. F. 1-1/2015/ERB/SZABMU/ Dated : 17-11-2016

Dr. Sabika Firasat
Assistant Professor
Department of Animal Sciences
QAU, Islamabad.

Subject: **Molecular Genetics Analysis of Metabolic Liver Disorders in Local Population: To Establish Molecular Genetics Testing.**

Thank you for submitting your research proposal to the Ethical Review Board. After evaluation of your project, an unconditional permission is given to proceed with this project.

However, the committee reserves the right to discontinue the research study if reports are received regarding causation of undue risks/hazards to study subjects.

Prof. Dr. Ali Jawa
Secretary

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Ref. No 24/CH/ICH

Date 26/09/2017

CERTIFICATE OF ETHICAL APPROVAL

After reviewing all the aspects of the project titled **"Molecular genetic analysis of metabolic liver disorders (Wilson) in local population: to establish molecular genetic diagnostic testing"** submitted by **BUSHRA GUL,** Quaid-e-Azam University, Islamabad, Institutional Review Board (IRB)/ Ethical Committee has no objection on further proceeding of this project.

The approval is subject to the understanding that the researcher would abide by the ethical principles for medical research involving human subjects as adopted by the World Medical Association Declaration of Helsinki (DoH/Oct2008).

The IRB may monitor the progress of the study anytime at its discretion/need.

PROF. SAJID MAQBOOL
Professor of Emeritus
Chairperson Institutional Review Board (IRB)
The Children's Hospital & ICH, Lahore

A copy is forwarded for information to;
1. The Dean, The Children's Hospital & ICH, Lahore.
2. The Medical Director, The Children's Hospital & ICH, Lahore.

ANNEXURE II

QUESTIONAIRES

Disease: Glycogen storage Type 1a
Patient Name_____ Father Name_____
Age:_____ Gender_____
Age at Diagnosis_____
Guardians contact_____
Consanguinity?_____
Number of affected individuals_____

Clinicals: **Family pedigree**

Hyperlipidemia
Hyperuricemia
Hypoglycemia
Cognitive impairment
Hypotonia
Recurrent infections
Short stature
Seizures
Xanthomatosis
Decreased muscle tone
Delayed puberty
Intermittent diarrhoea
Lactic acidosis
Nephrolithiasis
Abnormal bleeding
Doll like facial features
Any other clinical symptoms

Guardian signatures **Researcher signatures**

ANNEXURE III

PUBLICATIONS

PLOS ONE

RESEARCH ARTICLE

Analysis of Wilson disease mutations in copper binding domain of *ATP7B* gene

Bushra Gul[1,2]*, Sabika Firasat[1]*, Raeesa Tehreem[1], Tayyaba Shan[1], Kiran Afshan[1]

1 Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan,
2 Department of Biosciences, Faculty of Basic Sciences, University of Wah, Wah Cantt., Pakistan

* bushra.gul@uow.edu.pk (BG); sabika.firasat@qau.edu.pk (SF)

Abstract

Wilson's disease (WD) is an autosomal recessive disorder, resulting from variations in *ATP7B* gene. Clinical heterogeneity, including neuropsychiatric and hepatic manifestations over a large range of age groups make diagnosis difficult. Most of WD patients suffer severe disabilities and even die. So, overall goal of proposed study is the genetic and clinical characterization of Wilson's disease cases from Pakistani population. Clinical data was collected, and patients were investigated for variations in selected *ATP7B* exons using PCR based Sanger sequencing. Pathogenic effect predictions for detected variants were carried out using PROVEAN, MutationTaster2, and HSF software's. Clinical heterogeneity was observed in patients including reduced serum ceruloplasmin, signs of chronic liver damage and raised 24 h urinary copper excretion. Mean age of onset was 11.3 years. Kayser-Fleischer rings were present in 75% of cases. About 82.5% patients belonged to inbred families. Patients having neurological disorder were above 12 years of age. Total ten variants in analyzed region of *ATP7B* gene, including a reported variation (p. L227Yfs*35) were found in patients. The study also identified 4 putative novel synonymous variants (c.251A>C, c.15T>A, c.6T>C, c.238C>T) and 5 reported polymorphisms (c.83C>A, c.39_40insCGGCG, p.V456L, c.39_40insCGCCG and c.1544-53A>C). Reliable understanding of clinical presentations and genotype-phenotype correlation provide insight to function and structure of *ATP7B* and may assist in disease prognosis and family counseling. The study revealed clinical presentation of Pakistani WD cases and identification of sequence variants in screened region of *ATP7B*.

Check for updates

OPEN ACCESS

Citation: Gul B, Firasat S, Tehreem R, Shan T, Afshan K (2022) Analysis of Wilson disease mutations in copper binding domain of *ATP7B* gene. PLoS ONE 17(6): e0269833. https://doi.org/10.1371/journal.pone.0269833

Editor: Jen-Tsung Chen, National University of Kaohsiung, TAIWAN

Received: March 20, 2022

Accepted: May 30, 2022

Published: June 28, 2022

PONE-D-22-33240R4
Mutational Analysis and Clinical Investigations of Medically Diagnosed GSD 1a Patients from Pakistan

Dear Dr. Firasat:

I'm pleased to inform you that your manuscript has been deemed suitable for publication in PLOS ONE. Congratulations! Your manuscript is now with our production department.

If your institution or institutions have a press office, please let them know about your upcoming paper now to help maximize its impact. If they'll be preparing press materials, please inform our press team within the next 48 hours. Your manuscript will remain under strict press embargo until 2 pm Eastern Time on the date of publication. For more information please contact onepress@plos.org.

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Kind regards,
PLOS ONE Editorial Office Staff

› J Pak Med Assoc. 2021 Jun;71(6):1633-1638. doi: 10.47391/JPMA.420.

Clinical profile and screening of exon 6 and 14 of ABCB4 gene in obstetric cholestasis patients at a tertiary care hospital in Rawalpindi, Pakistan

Nuzhat Noor [1], Sabika Firasat [1], Naheed Bano [2], Kiran Afshan [1], Bushra Gul [1], Haiba Kaul [3]

Affiliations + expand

PMID: 34111087 DOI: 10.47391/JPMA.420

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