GENETIC INVESTIGATION OF IDIOPATHIC LIVER INJURY IN CHILDREN BY WHOLE EXOME SEQUENCING

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE

OF BILKENT UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF

MASTER OF SCIENCE

IN

MOLECULAR BIOLOGY AND GENETICS

By

Aysima Atılgan Lülecioğlu

July 2024

To my family,

for always loving and supporting me

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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July 2024

Childhood liver diseases, caused by multiple etiologies, pose a significant burden globally. Liver injury of unknown causes remains a challenge in pediatric hepatology. For instance, the etiology remains unexplained in nearly half of the pediatric cases with acute liver failure. This ambiguity impedes early diagnosis and the timely consideration of treatment options. Recent studies, utilized by genome-wide approaches such as whole-exome sequencing (WES), reveal that idiopathic liver injury can be due to hitherto silent monogenic liver diseases. In our study, we aimed to investigate the monogenic determinants of idiopathic liver injury in children. We performed WES on 20 pediatric patients presenting with either recurrent elevated liver transaminases (rELT) of unknown etiology or indeterminate acute liver failure (ALF). We searched for potential disease-causing variants in a manually curated panel of 380 genes associated with inherited monogenic diseases with hepatobiliary phenotypes. We identified rare nonsynonymous variants in nine genes in total 6 patients, five rELT patients and one ALF patient. Then, we evaluated the causal concordance between the gene mutated and the clinical phenotype observed in each patient through an in-depth case-level assessment. Overall, we established a genetic diagnosis in four out of 10 rELT patients. We identified two novel mutations in *ACOX2* and *PYGL*, expanding the

spectrum of genetic mutations implicated in monogenic liver diseases. Additionally, we discovered two previously-reported morbid mutations in *ABCB4* and *PHKA2*. Moreover, we identified five variants of uncertain significance (VUS) in *CDAN1*, *JAG1*, *PCK2*, *SLC27A5*, or *VPS33B* in rELT or ALF patients. This study further supports the utility of WES in clinical settings to enhance our understanding and management of idiopathic liver diseases in children, providing early diagnosis and precise treatment. By identifying the genetic variants contributing to liver injury, clinicians can predict disease progression more accurately, provide more personalized treatment strategies, and make decisions on liver transplantation when necessary.

Key words: acute liver failure, children, human genetics, idiopathic liver injury, recurrent elevated transaminases, whole-exome sequencing

ÖZET

ÇOCUKLARDA İDİYOPATİK KARACİĞER HASARININ TÜM EKZOM DİZİLEME İLE GENETİK İNCELEMESİ

Aysima Atılgan Lülecioğlu
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Danışman: Serkan Belkaya
Temmuz 2024

Çocuklarda görülen karaciğer hastalıkları, dünya çapında önemli bir sağlık sorunu oluşturmaktadır. Bu hastalıklar çeşitli nedenlerden dolayı ortaya çıkmakta olup sebebi bilinemeyen karaciğer hasarları, tanı ve tedavi süreçlerini zorlaştırmaktadır. Örneğin, pediyatrik akut karaciğer yetmezliği vakalarının yaklaşık yarısında hastalığın sebebi hala belirlenememiştir. Bu belirsizlik, hastalara erken teşhis konulmasını ve tedavi seçeneklerinin zamanında değerlendirilmesini zorlaştırmaktadır. Tüm ekzom dizilime (WES) gibi genom çapındaki yaklaşımlardan yararlanılan son çalışmalar, idiyopatik karaciğer hasarının şimdiye kadar sessiz kalan monogenik karaciğer hastalıklarından kaynaklanabileceğini göstermiştir. Biz de çalışmamızda çocuklarda karaciğer hasarının monogenik etkenlerini belirlemeyi hedefledik. Çalışmamızda, sebebi bilinmeyen tekrarlayan yüksek transaminaz seviyeleri (rELT) veya belirsiz akut karaciğer yetmezliği (ALF) teşhisi konulan 20 pediyatrik hastaya WES yöntemi uyguladık. Hepatobiliyer semptomlarla ilişkilendirilen 380 geni içerecek şekilde hazırladığımız bir gen paneli kullanarak hastalığa neden olabilecek genetik varyantları inceledik. Analizlerimiz sonucunda, beşi rELT ve biri ALF hastası olan toplam altı hastada, dokuz farklı gende nadir görülen eş anlamlı olmayan mutasyonlar tespit ettik. Ardından, her bir hastada gözlemlenen klinik fenotip ile mutasyona uğrayan gen

arasındaki nedensel uyumu, vaka düzeyinde derinlemesine bir değerlendirme yaparak değerlendirdik. Sonuç olarak, rELT tanılı on hastanın dört tanesinde genetik tanı koyduk. *ACOX2* ve *PYGL* genlerinde bulduğumuz iki yeni mutasyon, monogenik karaciğer hastalıklarında rol oynayan genetik mutasyonların spektrumunu genişletti. Ek olarak, *ABCB4* ve *PHKA2* genlerinde daha önceden hastalık yaptığı bilinen iki mutasyon tespit ettik. Son olarak da rELT veya ALF hastalarında *CDAN1*, *JAG1*, *PCK2*, *SLC27A5* veya *VPS33B* genlerinde belirsiz öneme sahip (VUS) beş varyant belirledik. Çalışmamız, çocuklardaki idiyopatik karaciğer hastalıklarını anlamak ve tedavi etmek için WES kullanımının erken tanı ve kesin tedavi sağladığını desteklemektedir. Bu sayede doktorlar karaciğer hasarına katkıda bulunan genetik varyantları tanımlayarak hastalığın ilerleyişini daha doğru bir şekilde tahmin edebilir, kişiselleştirilmiş tedavi stratejileri sunabilir ve gerektiğinde karaciğer nakli konusunda karar verebilir.

Anahtar kelimeler: akut karaciğer yetmezliği, çocuklar, insan genetiği, idiyopatik karaciğer hasarı, tekrarlayan yüksek transaminazlar, tüm ekzom dizileme

ACKNOWLEDGMENTS

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr. Serkan Belkaya, for his invaluable support and patience. He always challenged my limits, fostering my personal and academic growth. His constant belief in my abilities and limitless support helped me recognize my potential. His profound knowledge, critical insights, and meticulous attention to details have greatly contributed to my academic journey throughout my studies. Without his exceptional guidance and encouragement, completing this thesis would not have been possible.

I am also grateful to my thesis committee members, Dr. Pınar Önal and Dr. Figen Özçay, for their precious time, invaluable feedback and contributions to my thesis.

I would like to express my greatest gratitude to my lab mate, Alperen Baran. Over the past two years, he has been my greatest companion, providing constant support both in my academic journey and my personal life. He has always been there for me, offering a helping hand when I needed it most. I am also very grateful to my friend Tunahan Çalıkoğlu. His cheerful demeanor and persistent positivity have made this journey much more bearable. Their presence and emotional support have given us wonderful and unforgettable memories to cherish. I am also greatly thankful for all the former lab members, Yılmaz Yücehan Yazıcı, Zeynep Güneş Tepe Demir, Umut Tank, and Ladin Işık Köse for their valuable technical support and friendship.

I am profoundly grateful to my family for believing in me wholeheartedly and supporting me through every step of this journey. To my sister, I am eternally grateful for her joyous spirit and uplifting mood that brightened my path whenever I needed. I owe my most heartfelt gratitude to my husband, Mustafa Oğuz Lülecioğlu. Without

his understanding and encouragement, completing this study would have been an insurmountable task. He has been my greatest source of strength regardless of the challenges.

Lastly, I would like to thank The Scientific and Technological Research Council of Turkey (TÜBİTAK) for supporting my master's studies through BIDEB-2210/A scholarship.

ASSOCIATED PUBLICATIONS

1) Lülecioğlu AA, Yazıcı YY, Baran A, et al. Whole-exome sequencing for genetic diagnosis of idiopathic liver injury in children. *J Cell Mol Med*. 2024; 28(11):e18485. doi:10.1111/jcmm.18485

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ABBREVIATIONS

AATD	Alpha-1 antitrypsin deficiency
ACMG	American College of Medical Genetics and Genomics
AD	Autosomal dominant
AD	Allele depth
AF	Allele frequency
AFB	UK Biobank-Allele Frequency Browser
AIH	Autoimmune hepatitis
ALF	Acute liver failure
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMP	Association for Molecular Pathology
AR	Autosomal recessive
AST	Aspartate aminotransferase
BA	Biliary atresia
CADD	Combined annotation dependent depletion
CDAIa	Congenital dyserythropoietic anemia Ia
CLD	Chronic liver disease
CNV	Copy number variation
DILI	Drug-induced liver injury
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DP	Depth of coverage
dPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid

FAH	Fumarylacetoacetate hydrolase
Fwd	Forward
gDNA	Genomic DNA
GGT	Gamma-glutamyl transferase
gnomAD	The genome aggregation database
GQ	Genotype quality
GSD	Glycogen storage diseases
GWAS	Genome-wide association studies
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HELLP	Hemolysis, elevated liver enzymes, low platelet
HEM	Hemizygous
HET	Heterozygous
HLA	Human leukocyte antigen
HOM	Homozygous
HTI	Hereditary tyrosinemia type I
IFN	Interferon
INR	International normalized ratio
LDH	Lactate dehydrogenase
MAFLD	Metabolic dysfunction-associated steatotic liver disease
MQ	Mapping quality
MSC	Mutation significance cutoff
NGS	Next generation sequencing
OAA	Oxaloacetate

OMIM	Online mendelian inheritance in man
PALF	Pediatric acute liver failure
PBC	Primary biliary cholangitis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PFIC	Progressive familial intrahepatic cholestasis
pLOF	Predicted loss of function
PolyPhen-2	Polymorphism phenotyping v2
PSC	Primary sclerosing cholangitis
RBC	Red blood cell
rELT	Recurrent elevated transaminases
Rev	Reverse
RT	Room temperature
SIFT	Sorting intolerant from tolerant
SNPs	Single nucleotide polymorphisms
TBE	Tris-borate-EDTA
TGP	Turkish Genome Project Data Sharing Portal
VUS	Variants of uncertain significance
WES	Whole- exome sequencing
WGS	Whole- genome sequencing
WT	Wild-type
XLR	X-linked recessive

CHAPTER 1

Introduction

1.1 Liver diseases

Liver is a vital organ orchestrating various metabolic functions, including detoxification, synthesis of proteins and lipids, digestion, and formation and excretion of bile[1]. Liver diseases cause a global health burden, with around 2 million annual deaths worldwide[2]. In Europe alone, the estimated number of individuals with chronic liver diseases (CLD) is 29 million per year[3], with approximately 170,000 fatalities resulting from end-stage liver diseases[4]. In US, approximately 4.5 million people are reported with CLD and cirrhosis, corresponding to nearly 2% of the adult population[5].

Liver diseases can result from various causes including drugs and toxins, autoimmunity, infections, and metabolic disorders, or from unknown causes[5]. The severity of livery injury varies greatly, ranging from mild to life-threatening liver failure. The first stage in liver diseases is usually inflammation, known as hepatitis, triggered by various factors, including viral infections and excessive alcohol intake[6]. Ongoing inflammation and liver injury lead to liver fibrosis, the excessive buildup of extracellular matrix proteins due to the liver's attempt to recover itself, resulting in the deposition of scar tissue[7]. Early diagnosis and attempts can prevent further cumulative damage to the liver. In the late-stage of liver disease, cirrhosis defined by the severe scar and permanent hepatic damage, the liver's capability of crucial functions, such as detoxification and synthesis of proteins, is impaired[8]. Hepatobiliary functions can be impaired in liver diseases. The symptoms, laboratory abnormalities, severity of the disease, and treatment options are dependent on the affected part of the hepatobiliary

system[9]. Liver function tests, such as Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline phosphatase (ALP), Gamma-glutamyltransferase (GGT), L-lactate dehydrogenase (LDH), bilirubin, albumin, and the international normalized ratio (INR), are used to check liver function[10,11] (Table 1.1). Overall, these tests are valuable in detecting the presence of any liver damage in healthy individuals and monitoring hepatobiliary functions in patients with various liver disorders[12]. Moreover, the elevation pattern of liver enzymes can guide clinicians in the treatment of potential life-threatening yet treatable conditions[10,13]. If mild or moderate alterations of liver enzymes are not sufficiently investigated, it may impede the early diagnosis of patients[10,13]. Elevated levels of ALT and AST are generally an indicator of hepatocellular injury, which can be associated with various reasons, such as viral hepatitis or drug-induced liver injury (DILI)[11]. For example, metabolic dysfunction-associated steatotic liver disease (MASLD) is a metabolic disease of the liver, which often remains asymptomatic in patients and is diagnosed with abnormal liver enzymes and imaging of liver tissue[14]. Early diagnosis of MASLD enables interventions, such as medical treatment options and changes in lifestyle, which allow the prevention of disease progression and decrease in the risk of morbidity and mortality due to liver failure[15]. Similarly, elevated levels of ALT, AST, GGT, and total bilirubin may result from DILI, which can be caused by exposure to a hepatotoxic drug[16–19]. It is essential to monitor patients with high susceptibility or those using potentially hepatotoxic medications for early identification of any liver injury. This enables the timely discontinuation of the drug and prevents the development of severe liver diseases.

However, despite a comprehensive medical evaluation, a significant proportion of cases presented with recurrent elevated liver transaminases (rELT) , also known as hypertransaminasemia, do not have any identified etiology, are classified as idiopathic,

and need further investigation[20]. Around 10% of chronic hypertransaminasemia in adult cases lack an underlying cause, and the prognosis remains unindentified[21]. Several studies conducted in Turkey have shown that up to 38% of rELT cases in children are idiopathic[22–25].

Table 1.1 Liver function tests that are commonly used to detect liver injury

System	Biomarker	Found in	Function	Interpretation
Hepatocellular	Alanine transaminase (ALT)	Liver, synthesized in hepatocytes	Catalyzes the transfer of amino groups from alanine to α-ketoglutarate and production of pyruvate and L-glutamate	Release of ALT into bloodstream is an indication of hepatocellular injury
	Aspartate transaminase (AST)	Liver, cardiac and skeletal muscle, kidney, brain, pancreas, lung, leukocyte, and erythrocyte	Catalyzes the reversible transaminase reaction between aspartate and glutamate.	Indication of hepatocellular injury
	Albumin	Abundant in human blood, synthesized in hepatocytes	Signiant transporters of various substances, including hormones, pharmaceutical reagents, and fatty acids, they also help maintaining osmotic pressure homeostasis.	Decreased levels are indicator of impaired synthetic function of liver
	Lactate dehydrogenase (LDH)	Almost all cells in the body	Responsible for interconversion of pyruvate to L-lactate during in hypoxia conditions	Indicator of tissue damage, but not specifically for liver
Cholestatic	Alkaline phosphatase (ALP)	Mainly in liver, but also in bone, kidney, intestine	Catalyzes the hydrolysis of phosphate monoesters, such as esters of alcohols, phenols, and amines, as well as inorganic pyrophosphate, found in the extracellular space.	Increased levels may indicate cholestatic liver diseases
	Gamma-glutamyltransferase (GGT)	Mainly in liver, kidney, pancreas, spleen	Catalyzes the transfer of gamma-glutamyl residues to amino acids or small peptides, having role in gamma-glutamyl cycle, particularly synthesis and metabolism of glutathione	Associated with cholestasis and biliary obstruction
Hepatocellular and Cholestatic	Bilirubin		A significant waste product of heme degradation by the heme oxygenase pathway. Unconjugated bilirubin, the end-product of the hemoglobin breakdown, is taken up by the hepatocytes and conjugated with glucuronic acid.	Acute or chronic liver disease, and dysfunction of bilirubin metabolism. Conjugated or unconjugated bilirubin levels determines whether it is intrahepatic or extrahepatic.

This table summarizes the commonly used liver biochemical tests, their respective sites of action, functions, and interpretations of abnormal results[11,26–30].

1.2 Pediatric liver diseases

Liver diseases in children pose a substantial burden on public health, with a huge impact on quality of life and healthcare costs[4]. In the USA, approximately 15,000 children per year are hospitalized due to liver diseases[4], yet the precise incidence of liver diseases in children is not known, partly due to challenges in definitive diagnosis[31].

Pediatric liver diseases include MASLD, Wilson disease, viral hepatitis, autoimmune hepatitis (AIH), and ALF[32]. MASLD is one of the most common liver disorders seen in children, characterized by abnormal fat buildup in hepatocytes, liver inflammation, and injury[33]. Wilson disease is a rare disorder resulting in excessive copper buildup in the different parts of the body, particularly in the brain and liver[34]. Hepatitis, which refers to liver inflammation, can occur due to various reasons. It is primarily caused by viral infections, including Hepatitis A, B, and C, but can also result from autoimmune conditions or other factors, such as drugs, toxins, or inherited metabolic diseases[6,35]. Pediatric ALF (PALF) is a heterogeneous multisystem disorder characterized by severe liver injury in the absence of a preexisting liver disease in children[36]. It is a rare disease of childhood with a high (up to 50%) mortality rate without liver transplantation[37]. The prevalence of PALF is not precisely known; however, 10–15% of liver transplantations performed in children annually were result from ALF in USA[38]. PALF is characterized by hepatocellular injury leading to release of inflammatory mediators, coagulopathy, hepatic encephalopathy and multi-organ failure at the last stage[39–41]. Recurrent episodes of elevated transaminases, specifically ALT and AST, are used to detect the presence and severity of PALF, along with the INR and bilirubin levels[42,43]. The etiology of ALF can differ by age and geographical location[44]. According to a study conducted by a PALF study group, which includes

centers in the UK, US, and Canada, metabolic disorders are commonly seen as the underlying cause of ALF in neonates and infants, while DILI was more frequent in older children[45]. Also, viral hepatitis is the most common reason of PALF in developing countries[44,46,47]. Yet, despite the extensive diagnostic workup, up to %50 of ALF cases in children remain unexplained, complicating the management of disease-specific treatments and decision-making for liver transplantation[44,48–50].

Moreover, biliary system disorders are also seen in children, including cholestasis, biliary atresia (BA), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). Cholestasis is the impaired production of bile acid[51]. It can cause liver tissue damage and fibrosis due to accumulation of bile in the liver[52]. BA is a congenital obliterative cholangiopathy affecting intrahepatic or extrahepatic bile ducts, and leads to hyperbilirubinemia and jaundice in primarily infants aged below 3 month[53–55]. BA is one of the major reasons of neonatal cholestasis, being responsible for more than 40% of liver transplants in children worldwide[56]. When children with BA do not receive treatment, they are expected to die within the first 2 years of their lives[57]. PSC is a rare inflammatory disease in children, characterized by inflammation, fibrosis, and scarring within the biliary ducts[58]. The most frequent symptoms include hepatomegaly, splenomegaly, and abdominal pain[59]. The prevalence of PSC is 2 cases per 100,000 children. In the first 10 years after the diagnosis, half of the children exhibit clinical manifestations, with 30% of them needing liver transplantation[60].

Providing appropriate medical therapies for patients is only possible with an early and accurate diagnosis. The diagnosis of liver diseases is a complex process involving clinical evaluation, laboratory tests, and liver biopsy if required. Yet, the diagnosis is challenging due to the nonspecific early symptoms, such as abdominal pain, jaundice, and physical changes in urine and stool. The lack of a definitive diagnosis can be

attributed to unknown causes of liver diseases[61]. According to studies from both the USA and Europe, the specific cause of liver conditions is unknown in about half of the cases[62]. This adds complexity to clinical management and critical decision-making, especially in selecting disease-specific treatment options such as transplantation. The lack of definitive underlying causes prevents clinicians from effectively planning a specific treatment, which targets the mechanism triggering patients' condition. For instance, clinicians can induce remission using steroids and azathioprine as treatments in patients with elevated transaminases if they identify the underlying etiology as AIH[63]. Similarly, if a patient has a liver injury caused by Hepatitis B infection, antiviral therapies are prescribed to suppress the infection and inflammation in the liver, aiming to prevent the progression of the liver injury[64]. When the etiology is unknown, broad-spectrum treatments focusing on only symptoms are applied. Furthermore, when the underlying cause is not identified, the course of the disease and the efficiency of the selected treatments cannot be predicted. Particularly, identifying the etiology of the disease is crucial while making decisions about liver transplantation, which is a complex therapy requiring complicated surgery and lifelong use of immunosuppressive medications[65]. Predicting the outcomes is important for selecting the most suitable candidates with a higher chance of survival for liver transplantation to prevent further complications like multiorgan failure. Overall, the uncertainty in diagnosis and treatments affects both the prognosis and outcome of the disease, creating a burden for healthcare providers. Thus, establishing a causal diagnosis is crucial for the management of liver diseases in both children and adults[61].

1.3 Etiology of pediatric liver injury

Precise diagnosis of liver diseases is crucial for effective management and treatment of patients. Etiologies underlying pediatric liver injury can be grouped into metabolic

disorders, infection, autoimmunity, drugs and toxins, vascular diseases, genetics, vascular diseases, and other causes[48,66,67] (Table 1.2).

Table 1.2 Known etiologies of liver diseases

Etiology	Examples
Metabolic	Wilson disease
	Alpha-I antitrypsin deficiency (AATD)
	Metabolic dysfunction-associated steatotic liver disease (MASLD)
	Hereditary tyrosinemia type 1
	Galactosemia
	Crigler-Najjar syndrome
	Fatty acid oxidation disorders
	Hereditary hemochromatosis
	Mitochondrial hepatopathies
	Glycogen storage diseases (GSDs)
	Hereditary fructose intolerance
	Cystic fibrosis
	Urea cycle disorders
	Citrin deficiency
	Gaucher disease
	Niemann-Pick disease
	Respiratory chain disorder
Infection	Hepatitis viruses (A, B, E)
	Herpes simplex virus
	Epstein–Barr Virus
	Herpes simplex virus
	Varicella-zoster virus
	Adenovirus
	Influenza virus
	Chikungunya
	Dengue virus
	Ebola virus
	Parasites
	Zika virus
	Malaria
	Hepatic tuberculosis
Autoimmunity	Autoimmune hepatitis (AIH)
	Primary biliary cholangitis (PBC)
	Primary sclerosing cholangitis (PSC)
Drugs and Toxins	Acetaminophen (Paracetamol)
	Antibiotics (such as amoxicillin-clavulanate, sulfamethoxazole-trimethoprim, ciprofloxacin, isoniazid)
	Anti-fungal drugs
	Steroids
	Nonsteroidal anti-inflammatory drugs
	Anti-epileptic drugs
	Anti-tuberculosis drugs
	Disease-modifying antirheumatic drugs
	Central nervous system-drugs (valproate and phenytoin)
	Chemotherapy drugs
	Antiarrhythmic agents
	Herbal and dietary supplements
	Mushrooms
	Chemicals and solvents
Genetics	Inherited monogenic metabolic diseases (E.g. Hereditary hemochromatosis, Alpha-1 antitrypsin deficiency, Tyrosinemia type I, Progressive familial intrahepatic cholestasis type 3, Wilson's disease, Glycogen storage diseases)
	Polycystic kidney disease
	Alagille syndrome
	Progressive familial intrahepatic cholestasis (PFIC)
	Crigler-Najjar syndrome
Vascular disease	Ischemic hepatitis
	Portal vein thrombosis
	Portal hypertension
	Hepatic vein thrombosis (Budd-Chiari syndrome)
	Hepatic sinusoidal obstruction syndrome (Veno-occlusive disease)
	Hepatic artery thrombosis
Others	Heatstroke
	Excessive alcohol consumption
	Cigarette smoking
	Hypoxic liver injury
	Hepatocellular carcinoma (HCC)
	Cholangiocarcinoma
Unknown	

This table summarizes known causes of liver diseases in children[68–93].

Metabolic disorders impede the liver's function of processing and metabolizing nutrients, and lead to the accumulation of substances within the body[94]. Metabolic culprits for PALF are very common in infants[48]. Two studies from Turkey have demonstrated that metabolic diseases are the most prevalent etiology of ALF in children[46,95]. Furthermore, another study reporting a 14-year experience from a pediatric liver transplantation center in France showed that 42.5% of cases seen in 80 infants with PALF have an underlying inherited metabolic disorder[96]. Wilson disease and disorders related to mitochondria function, known as mitochondrial hepatopathies, are the most common examples of metabolic disorders causing liver injury[96]. Other metabolic disorders leading to liver failure include Tyrosinemia type I, Galactosemia, urea cycle disorders, and Alpha-1 antitrypsin deficiency[97].

Infections, especially viral infections, can lead to liver diseases in children[40]. The incidence of viral hepatitis has decreased in the last years due to advancements in hygiene conditions and improved vaccination status[98]. Yet, viral hepatitis; in particular A, B, and E, is the predominant cause of ALF in developing countries[99]. The estimated number of people infected with HBV worldwide is 2 billion, with around 600,000 deaths every year caused by its hepatic sequelae: cirrhosis and hepatocellular carcinoma (HCC) [100]. According to a review done by Kayaalp in 2014, viral hepatitis, Hepatitis A in children and Hepatitis B in adults, are the most common causes of ALF in Turkey, also being the most prevalent reason for liver transplantation in adults[101,102]. Herpes simplex virus, Epstein-Barr virus, cytomegalovirus, adenovirus, and rubella can also result in liver injury[103–106]. Furthermore, infections with malaria, *Orientia tsutsugamushi*, the causative agent of scrub typhus, and dengue virus have been reported to cause pediatric liver failure in endemic regions[66].

AIH is another significant causative etiology of pediatric ALF[66]. AIH can be due to both genetic and environmental factors, and is characterized by the loss of immunologic tolerance against hepatocyte-specific autoantigens[107]. AIH in children can present with a diverse clinical spectrum, including non-specific symptoms like abdominal pain and jaundice, elevated liver enzymes, acute hepatitis, and liver failure[108]. The incidence of pediatric AIH ranges from 2 to 17 per 100,000 children[90]. It has been reported that up to 5% of patients with AIH have liver failure[48].

Drugs and toxins are another significant factors that contribute to liver disease. Drug-induced hepatotoxicity, one of the major causes of ALF in developed countries, accounts for more than 50% of ALF cases in USA, UK, and Sweden[109]. DILI accounts for nearly 20% of the PALF, being one of the most common causes of liver transplantation in USA[110]. Overdose of acetaminophen, which is one of the most frequently prescribed drugs by pediatricians, was shown to cause pediatric acute hepatic failure[111]. Other examples are anti-epileptic drugs–and antituberculosis drugs[112]. Antituberculosis drugs are the major cause for drug-induced ALF in Turkey, whereas acetaminophen is the first cause of ALF in the UK and US[101]. The severity of DILI can range from mild elevation of liver enzymes to life-threatening conditions[113]. In mild cases, it can be reversed by discontinuation of culprit drugs[114]. Furthermore, toxins such as mushrooms, rat poison, lead, arsenic, and some cleaning products can also cause liver injury in children[115–117]. Mushroom poisoning is the most frequent cause of toxic ALF in both children and adults in Turkey[101].

Other causes underlying liver diseases include hypoxia-induced liver injury, heatstroke, and HELLP (hemolysis, elevated liver enzymes, low platelet) syndrome[118]. Liver metastasis is another rare occurrence associated with ALF[119,120]. Furthermore,

vascular diseases, including Budd-Chiari syndrome, Veno-occlusive disease, and acute portal vein thrombosis, are among the conditions leading to liver failure in children[121].

Inborn errors have been shown to cause pediatric hepatobiliary diseases. Monogenic etiologies of ALF can be categorized based on the affected mechanisms as follows: disorders of metabolic pathways, such as amino acid, carbohydrate, fatty acid, and energy substrate, mitochondrial disorders, vesicular trafficking disorders, aminoacyl-tRNA synthetase deficiencies, Wilson disease, Niemann–Pick type C, disorders of immune system and red cell disorders[122]. Monogenic liver disease, which primarily affects liver and cause tissue damage, can be cured by liver transplantation. Most common examples for this group of diseases are Alagille syndrome, α-1 antitrypsin deficiency, argininosuccinic aciduria, GSD type I, hereditary hemochromatosis, PFIC, tyrosinemia type 1, and Wilson's disease[123]. In addition to monogenic diseases, the progression of several liver diseases, such as MASLD, is determined by both genetic and environmental factors. Although a single-gene mutation is not directly attributed to MASLD, several genetic variants have been associated with an increased risk of MASLD[124].

It is important to note that the underlying cause of liver disease still remains unknown in a significant proportion of pediatric cases[62]. When the physician cannot identify a specific cause of liver dysfunction due to lack of supporting evidence, the final diagnosis is considered as idiopathic[125]. Idiopathic liver diseases emerge as a challenge in both adult and pediatric hepatology. Despite advanced diagnostic methods, approximately 50% of PALF cases remain undiagnosed[62]. According to another study conducted by Ozcay et al, 33% of PALF cases in Turkey had indeterminate etiology[46]. Different studies conducted by various research groups have highlighted current

challenges in diagnosing indeterminate pediatric liver diseases, indicating the need for an in-depth etiological investigation of liver injury[121,126–128].

1.4 Genetic landscape of indeterminate liver diseases

Clinical medicine has been revolutionized by genomics, which integrates the genetic information of patients into their diagnosis and treatment processes[129–131]. Definitive diagnosis can be made utilizing genome-wide approaches, dissecting the genetic variations associated with the disease. In particular, asymptomatic individuals or patients with an atypical clinical presentation can receive accurate and early diagnosis[132]. Furthermore, predicting the outcome and selecting the treatment options are advanced by genomics[132,133]. Several genetic markers may be utilized by clinicians to evaluate the risk of disease progression and tailor the appropriate treatment strategy targeting the disease-related molecular pathways[134]. Overall, clinical decision-making has been enhanced thanks to the advances in genomic medicine[135,135,136].

Next-generation sequencing (NGS) is an advanced technology, which offers parallel and rapid sequencing of millions of DNA fragments. This technology has been widely adapted, with various applications such as whole-genome sequencing (WGS), whole-exome sequencing (WES), and targeted gene sequencing (TGS) by using customized gene panels[137]. NGS technology is now widely used in clinics as a powerful genome-wide scanning tool in the search for genetic variants responsible for the disease of interest[138–140]. Candidate gene approach is employed if a patient presents with a specific phenotype, which can be attributed to a particular inherited disease or genetic defect. TGS has the advantage of high-coverage of selected genes, and it is more time and cost-saving than WES or WGS[141]. TGS has been utilized for various diseases, such as immunodeficiencies, metabolic diseases, and neurological diseases[142–145]. In contrast, WES and WGS are preferred for patients with an uncharacterized disease or

uncommon clinical manifestations of a known disease, particularly when familial history indicates a genetic transmission[141]. WGS is used to sequence both coding and noncoding regions of the genome[146]. It can detect variants that cannot be identified by WES and TGS, which are structural chromosome rearrangements, intronic region variants, and GC-rich regions in the genome[141].

WES is used to sequence 20-30,000 protein-coding genes in the human genome[147]. It has been reported that about 85% of known disease-causing variants are located on the exons[148]. Thus, the reduced cost, time-efficient analysis, and large-scale genetic information offered have contributed to the frequent utilization of WES in clinical diagnostics and translational research studies of various diseases, such as congenital abnormalities, central nervous system diseases, primary immunodeficiencies, cardiovascular diseases, skeletal disorders, and mitochondrial disorders[149–152].

While genetic testing for candidate genes or small gene panels has been employed for certain liver diseases, such as hereditary CLD, WES was not widely utilized until recent years[153]. Yet, recent advances create a remarkable opportunity to dissect the human genetics behind the liver injury with an indeterminate origin. In the past few years, several pediatric and adult cohorts presenting with CLD, idiopathic cholestasis, ALF, elevated transaminases with unknown etiology, non-fatty liver diseases, and hepatic steatosis were evaluated by WES analysis[122,147,149,154–157]. Recent studies demonstrated that approximately 30% of patients with idiopathic liver diseases have a monogenic cause, and about half of the patients with cholestasis of unknown etiology harbor an inherited underlying genetic cause[158]. Furthermore, around 20% of liver transplantations performed in children have a monogenic liver disease, particularly cholestasis[159].

In the study by Stalke et. al. (2017) WES was performed in 135 children presumed to have an inherited liver disease and screened variants in a customized gene panel. Twenty-three of the 135 children (17%) harbored pathogenic monogenic variants[157]. Hakim et al performed WES on 19 patients with idiopathic liver diseases, which were cryptogenic cirrhosis, non-obese MASLD ± nonalcoholic steatohepatitis (NASH), idiopathic cholestasis, HELLP and severe hyperammonemia, and idiopathic non-cirrhotic portal hypertension. They established genetic diagnosis for five patients[149]. In another study by Hegarty et al, patients under the age of 10 with indeterminate ALF were recruited. TGS on a custom ALF gene panel consisting of 64 genes was carried out in 41 children. They also performed WES on additional four patients, and found that overall, 12 out of 45 patients harbor a genetic variant that can be related to their diseases[160]. Liao et al performed WES analysis to investigate the genetic variants in 22 patients with indeterminate ALF, identifying 31 single-nucleotide polymorphisms (SNPs), 11 SNPs within human leukocyte antigen (HLA) Class II genes, showed associations with higher relative risk compared to controls and 5 SNPs were identified for increasing survival probability without transplantation[156]. Xiao-Fei Kong et al focused on the diagnostic utility of WES using a curated gene panel in 758 patients with CLD, 7856 healthy controls, and 2187 patients with chronic kidney disease. They reported that 19.9% of CLD patients harbor pathogenic variants. Following a stringent variant filtering, 5.7% of CLD cases in their cohort had a genetic diagnosis[156]. In another study conducted by Zheng et al, WES analysis of 52 adult patients with indeterminate liver disease, including cholestasis, hepatic steatosis, advanced fibrosis/cirrhosis, elevated transaminases, non-HFE iron overload, and vascular diseases, provided a definitive genetic diagnosis in 17 patients (33%)[147]. Lenz et al conducted a multicenter study from 19 countries, utilizing WES on 260 children with

ALF of unknown etiology. A genetic diagnosis of inherited liver disorder was established in 97 out of 260 patients (37%) received[154].

1.5 Aim of the study

Liver diseases represent a global health problem, with their high morbidity and mortality rates in both children and adults[61,161]. For an effective treatment, timing, and accuracy of diagnosis is crucial. However, childhood liver diseases often lack specific symptoms or exhibit nonspecific clinical manifestations, such as ELT[162]. Despite extensive diagnostic workup and screening, nearly half of the PALF cases remain undiagnosed. Indeterminate ALF and recurrent idiopathic hypertransaminasemia pose significant challenges for pediatric hepatologists, often leading to delayed prognosis and treatment. Recent studies underscored the utilization of WES for the identification of genetic lesions underlying liver diseases of unknown etiology in both pediatric and adult patients. However, genetic studies involving cases with pediatric-onset and, in particular, individuals with different ethnic backgrounds are still limited. The dissection of inborn monogenic causes underlying indeterminate liver diseases is of clinical significance, as it may facilitate familial testing for earlier diagnosis, prognosis prediction, and more precise management in high-risk family members. Genetic diagnosis streamlines treatment modalities and enhances medical management and clinical decision-making for transplantation. We hypothesized that inherited monogenic defects could underlie pediatric liver injury of unknown etiology, at least in some children. Thus, we aimed to identify candidate disease-causing single-gene variants by WES in patients with childhood-onset recurrent idiopathic hypertransaminasemia or with indeterminate pediatric ALF.

This work presented in this thesis was originally published as “Lülecioğlu AA, Yazıcı YY, Baran A, et al. Whole-exome sequencing for genetic diagnosis of idiopathic liver

injury in children. *J Cell Mol Med*. 2024;28(11):e18485. doi:10.1111/jcmm.18485" and reproduced with permission from Wiley (Appendix).

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Buffers

Table 2.1 Buffers

Buffers	**Content**	**Amount**
Agarose gel (2%)	2 g Agarose (Biomax) 100 mL 0.5X TBE Buffer 5 µL SAFE DNA Gel Stain Solution	100 mL
10X TBE Buffer	108 g Tris base 55 g Boric acid 40 mL 0.5M EDTA (pH 8.0) Up to 1 L deionized water	1 L

2.1.2 Chemicals, reagents and enzymes

Table 2.2 Chemicals, reagents and enzymes

Reagent	**Catalog Number**	**Manufacturer**
ClearBand SAFE DNA Gel Stain Solution (20000x)	SDGS1	Ecotech Biotech, Turkey
GeneRuler 1 kb DNA Ladder	SM0311	Thermo Scientific, USA
Orange G Sodium Salt	E783-50G	Amresco, USA
Agarose (Biomax)	BHE500	Prona, USA
RBC Lysis Buffer (10X)	420302	Biolegend, USA
Taq DNA Polymerase, recombinant (5 U/µL)	EP0406	Thermo Scientific, USA

Molecular Biology grade water	01-869-1B	Biological Industries, Israel
TE Solution 1X	809-215 CL	WISENT Inc., Canada
Phosphate Buffered Saline 1X, w/o Calcium & Magnesium	311-010 CL	WISENT Inc., Canada
Absolute Ethanol	920.026.2500	Interlab, Turkey

2.1.3 Kits

Table 2.3 Kits

Kit	**Catalog Number**	**Manufacturer**
WizPrep™ gDNA Mini Kit (Blood)	W71050-100	Wizbiosolutions Inc. Republic of Korea
WizPrep Gel/PCR Purification Mini Kit	W70050-300	Wizbiosolutions Inc. Republic of Korea

2.1.4 Equipment

Table 2.4 Equipment

Equipment	**Model**	**Manufacturer**
Thermal cycler	2720	Applied Biosystems, USA
Thermal cycler	TC-512	Techne, USA
Fluorescent Image Analyzer	Amersham™ Imager 600	GE Healthcare Life Sciences, USA
Centrifuge	Eppendorf 5415 R	Eppendorf, USA
Heat Block	Type 17600 Dri-Bath	Thermolyne, USA
ZX3 Advanced Vortex Mixer	F202A0176	VELP Scientifica, Italy

2.1.5 Primers

Table 2.5 Primers used in this study

Primer	Sequence	Purpose
PCK2_G215D_Fwd	GCTCACTGACTCAGCCTATG	Sanger sequencing (G215D)
PCK2_G215D_Rev	GACTGCTGCTCTGGATCTTG	Sanger sequencing (G215D)
VPS33B_L403F_Fwd	CCCTGTGTCCTGGTGATAATTC	Sanger sequencing (L403F)
VPS33B_L403F_Rev	GCATTTAGAGGTGGGGTCG	Sanger sequencing (L403F)
ACOX2_R225Q_Fwd	CCTAGCCAGTTAGCACCAAG	Sanger sequencing (R225Q)
ACOX2_R225Q_Rev	GACCTGTGCATTGCTTTTCC	Sanger sequencing (R225Q)
PYGL_E394X_Fwd	GGAACACTGTAGCCATCTGT	Sanger sequencing (E394X)
PYGL_E394X_Rev	GCTGACTCCTTCCTTTCCC	Sanger sequencing (E394X)
ABCB4_A984T_Fwd	GCATCATCAGGCATCAGAGAAC	Sanger sequencing (A984T)
ABCB4_A984T_Rev	GGGGAGAAAGGGGATGATTAAG	Sanger sequencing (A984T)
PHKA2_R186C_Fwd	TCCTGATGAAGGGAACCAAATC	Sanger sequencing (R186C)
PHKA2_R186C_Rev	ACTTATGTTTCAGTGTGGCTGC	Sanger sequencing (R186C)
SLC27A5_T308M_Fwd	CCTCAGAAGTGGGCTTACCG	Sanger sequencing (T308M)
SLC27A5_T308M_Rev	TTCAGCCTGTGAACCCAACC	Sanger sequencing (T308M)
CDAN1_R649W_Fwd	CCAAGGAAACCAGTCAGCTT	Sanger sequencing (R649W)
CDAN1_R649W_Rev	ATGCTTGGACCTTTTACTTCCC	Sanger sequencing (R649W)

JAG1_N108H_Fwd	TCTCGCAAGGGATAACAGGG	Sanger sequencing (N108H)
JAG1_N108H_Rev	CGACGAGTGTGACACATACTTC	Sanger sequencing (N108H)

Fwd: Forward, Rev: Reverse

2.2 Methods

2.2.1 Ethics

This study was conducted in accordance with the institutional, local, and national ethical guidelines, and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards and approved by the İhsan Doğramacı Bilkent University Ethics Committee (#2019_11_21_07 and #2020_06_17_01). Clinical history and peripheral blood samples from patients were obtained by the referring physicians. Written informed consent was obtained from each participant enrolled in this study and parents if the participant was a minor.

2.2.2 Patient recruitment

Patient recruitment was carried out together with a network of collaborating physicians in Turkey. The study cohort consisted of children (≤18 years of age) with idiopathic liver injury, involving both prospective and retrospective cases. The final diagnosis and evaluation of the case were completely done by the referring physician's discretion. In cases where a specific cause of liver injury could not be identified by the physician as a result of insufficient evidence, the diagnosis was considered idiopathic. In general, these pediatric cases with unknown etiology were categorized into two diagnostic categories: (i) recurrent elevated liver transaminases (rELT) and (ii) acute liver failure (ALF). The selection criteria for patient recruitment are as in the Table 2.6.

Table 2.6 Patient recruitment criteria for ALF and rELT categories

Categories	Recruitment Criteria
rELT	- At least 3 repeated episodes of elevated liver transaminases: Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST)
	- Liver transaminase levels equal to or more than twice the upper limit of normal range
	- Normalization of liver transaminase levels between crises
ALF	- Biochemical evidence of acute liver injury with no evidence of chronic liver disease
	- Hepatic-based coagulopathy not corrected by vitamin K administration (INR ≥ 1.5 with hepatic encephalopathy or INR ≥ 2.0 without hepatic encephalopathy)

2.2.3 Isolation of genomic DNA (gDNA) from whole blood sample

2.2.3.1 Red blood cell lysis

Blood samples obtained from patients and their family members are collected in Heparin-containing tubes. One mL of blood samples was transferred to a conical centrifuge tube and mixed with 9 mL of 1X red blood cell (RBC) lysis solution, followed by an incubation for 20 min at room temperature (RT). After the incubation, lysed samples were centrifuged at 400 g for 10 min at RT. Next, supernatants were discarded, and the remaining pellets were resuspended in 4 mL of 1X RBC lysis solution. After the incubation for 15 min at RT, the samples were centrifuged at 400 g for 5 min. The supernatants were removed, and pellets were resuspended in approximately 1 mL of 1X RBC Lysis solution and transferred to 1.5 mL microcentrifuge tubes. They were centrifuged at 13,000 rpm for 1 min. After the supernatants were discarded, the remaining pellet was resuspended in 200 μL of 1X Dulbecco's phosphate-buffered saline (dPBS). These mixtures were used for genomic DNA (gDNA) isolation.

2.2.3.2 gDNA extraction

WizPrep™ gDNA Mini Kit (Blood) kit was used for gDNA isolation from leukocyte pellets following the RBC lysis. Cell pellets were resuspended in 200 µL of 1X dPBS in a 1.5 mL microcentrifuge tube. 200 µL of GB Buffer and 20 µL of Proteinase K were added and mixed by vortexing. The mixture was incubated at 56 ℃ for 10 min. Tubes were inverted every 5 min. After the incubation, 200 µL of 100% ethanol was added to sample lysates and mixed by rigorous vortexing. The mixture was transferred to the spin column and centrifuged for 1 min at 13,000 rpm. Flow through in the collection tube was discarded, and the spin column was inserted into a new collection tube. Next, 500 µL of W1 Buffer was added into the spin column and centrifuged for 1 min at 13,000 rpm. Flow through was discarded, and 500 µL of W2 Buffer was added into the spin column, followed by centrifugation for 1 min at 13,000 rpm. Flow through was discarded, and the spin column was centrifuged for 2 min at 13,000 rpm. Then, the spin column was put into a 1.5 mL microcentrifuge tube. 75 µL of Elution Buffer was added to the center of the membrane and incubated for 1 min at RT. Next, the spin column was centrifuged for 1 min at 13,000 rpm, and the eluted gDNA was transferred to a new 1.5 mL microcentrifuge tube. Isolated gDNA samples were stored at -20°C for further use.

2.2.4 Whole-exome sequencing

Whole-exome sequencing (WES) was performed on the gDNA of the participants by WES service providers, Genoks, Turkey, or Macrogen, Europe. Library preparation, collection of raw sequencing data, alignment with the reference human genome, variant calling, and annotations were undertaken by the WES service providers. Exome capturing was performed using Human Comprehensive Exome panel (Twist Bioscience, USA), or SureSelect Human All Exon V6 kit (Agilent Technologies,

USA), utilized by Genoks or Macrogen, respectively. Paired-end sequencing was performed on a NovaSeq 6000 (Illumina, USA) to generate 150-base pair (bp) reads. BCL2Fastq2 v2.20 software was used for FASTQ generation. Alignments with the reference human genome (GRCh37) was done using BWA-MEM software[163]. Downstream processing was performed using Picard Tools, GATK, and SnpEff[164–166]. Publicly available genome databases: gnomAD[167], Bravo[28], and UK Biobank-Allele Frequency Browser (AFB)[168] were used to obtain allele frequency (AF) values of variants.

2.2.5 Variant filtering

WES data variant filtering was done with the help of Yılmaz Yücehan Yazıcı. Variant calls with depth of coverage (DP) <10, mapping quality (MQ) <40, and genotype quality (GQ) <30 were removed. Homozygous or hemizygous variants with allelic depth (AD) to DP ratio <0.9 and heterozygous variants with an AD/DP <0.25 were excluded. Only predicted loss-of-function (pLOF) (frameshift insertion and deletion, stop-gain, and essential splicing [± 2 bp from the exon-intron boundary]), start-loss, stop-loss, in-frame insertion and deletion, and missense variants were retained for further analysis. Given the rare occurrence of pediatric liver injury with unknown etiology, variants with an AF ≥1% in gnomAD v2.1.1, including all subpopulations, and polymorphisms (AF ≥1%) reported in Turkish Genome Project Data Sharing Portal (TGP), consisting of genome data from over 500 healthy individuals in Turkey, were omitted. Moreover, variants identified as blacklist, which consists of pre-calculated non-pathogenic variants frequently reported in exomes of patients but not present in public databases, were filtered out[169].

To predict the damaging impacts of missense variants, publicly available in silico predictions tools, Polymorphism Phenotyping v2 (PolyPhen-2) and Sorting intolerant from tolerant (SIFT) were used. Furthermore, MutationTaster2021 and Combined annotation-dependent depletion (CADD) v1.6 together with Mutation significance Cutoff (MSC) were employed for both single nucleotide variants and indels[170–172]. The variant was considered damaging if its CADD score was higher than the MSC value (95% confidence interval) of the mutated gene. PolyPhen-2, SIFT, and CADD scores were obtained by using the Ensembl Variant Effect Predictor tool (https://www.ensembl.org/info/docs/tools/vep/). The ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) was utilized for the variant significance related to the human health. Additionally, clinical interpretations and classifications of genetic variants were performed manually and using an automated tool such as InterVar, based on the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines[173,174].

2.2.6 Sanger sequencing of gDNA

2.2.6.1 PCR amplification of gDNA

Validation of variants in the patients identified by WES and their familial segregation were performed by Sanger sequencing. Amplification of approximately 250 bp region encompassing the location of selected variants on the gDNA was done by polymerase chain reaction (PCR). Primers used in these PCR reactions are listed in Table 2.7. Sanger sequencing of PCR amplicons was performed by a service provider, Macrogen, Europe. For sequence analysis, SnapGene Viewer software (GSL Biotech LLC, USA) was used.

Table 2.7 PCR reaction setup for amplification of gDNA

Component	**Volume (µL)**
10X Taq Buffer with $(NH4)_2SO_4$	5
$MgCl_2$ (25 mM)	4
Forward Primer (5µM)	2
Reverse Primer (5µM)	2
dNTPs (2 mM each)	1
Taq DNA Polymerase (5 U/µL)	0.25
gDNA (100 ng)	1-5
Molecular Grade Water	up to 50
Total	50

Table 2.8 Thermal cycling conditions for PCR amplification of gDNA

Step	**Temperature (°C)**	**Time (s)**	**Number of Cycle**
Initial denaturation	95	300	1
Denaturation	95	30	35
Annealing	60	30	
Extension	72	30	
Final Extension	72	300	1
Final Hold	4	Hold	-

2.2.6.2 Agarose gel electrophoresis and Purification of PCR amplicons

The expected PCR product lengths were confirmed using agarose gel electrophoresis. Agarose gel (1%) was prepared by dissolving 1 g of Agarose in 100 mL of 0.5X TBE buffer and included 5 µL of SafeView Classic (20,000X). PCR products were mixed with 10X Orange loading dye with a 1:10 ratio and loaded into prepared agarose gel, then run at 120V for 30 min at RT. Image acquisition was done using AmershamTM Imager 600.

WizPrep™ Gel/PCR Purification Mini Kit was used to purify PCR amplicons. PCR products were transferred to a new 1.5 mL microcentrifuge tube, and 5 volumes of GP Buffer was added to 1 volume of PCR product by mixing thoroughly. The mixture was transferred to a spin column connected to a collection tube and centrifuged at 13,000 rpm for 1 min. Flow through was discarded. Next, 700 µL of Wash Buffer was put into the spin column and centrifuged at 13,000 rpm for 1 min. After discarding the flow through, the spin column was centrifuged at 13,000 rpm for 1 min and transferred onto a new 1.5 mL microcentrifuge tube. 30 µL of Elution Buffer was added to the center of the spin column and incubated for 1 min, followed by centrifugation at 13,000 rpm for 1 min. Purified and eluted PCR products were Sanger sequenced by the service provider (Macrogen, Europe).

CHAPTER 3

Results

This work presented in this thesis was originally published as Lülecioğlu AA, Yazıcı YY, Baran A, et al. Whole-exome sequencing for genetic diagnosis of idiopathic liver injury in children. *J Cell Mol Med*. 2024;28(11):e18485. doi:10.1111/jcmm.18485 and reproduced with permission from Wiley (Appendix).

3.1 Characteristics of the study population

A total of 20 patients with liver injury of unknown etiology were recruited for this study, with 10 of them diagnosed with ALF and 10 of them with rELT. The median age of patients was 4 years for the ALF group and 20 months for the rELT group. All cases were sporadic, and none of them had a familial history. Six patients with rELT and two patients with ALF were born to consanguineous parents. Both genders were almost equally represented in each group. Seven ALF patients received liver transplantation, and two of them recovered by medical treatment. Two ALF patients died. All other patients were alive as of conducting this study. WES was performed for all 20 patients, including 13 singletons (five patients with rELT and eight patients with ALF) and seven patient-parent trios (five trio designs with rELT and two trio designs with ALF) (Figure 3.1).

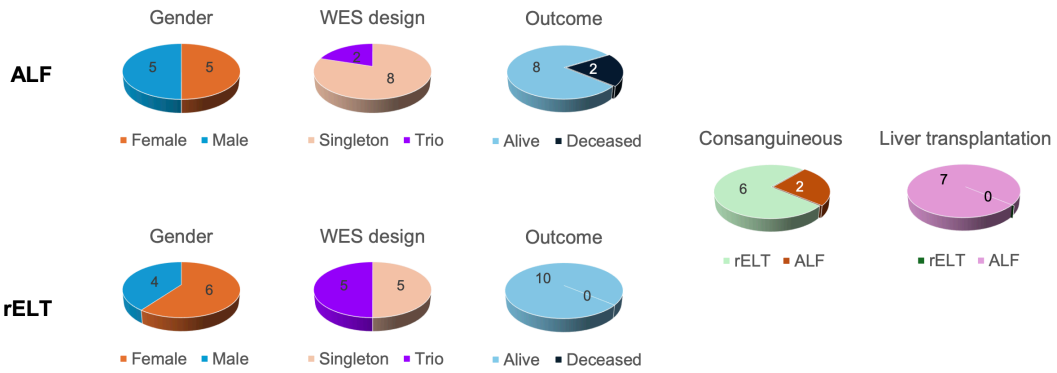


Figure 3.1 Characteristics of the study population

3.2 Searching for genetic variants in genes predisposing to hereditary diseases involving liver phenotypes

We undertook a biased WES analysis to identify candidate disease-causing variants in genes known to be associated with inherited diseases with liver involvement. Thus, we manually curated a liver gene panel containing 380 genes associated with a hepatobiliary phenotype or finding of elevated liver enzymes in the OMIM database (https://www.omim.org/) (Table 3.1). We analyzed the WES data of all patients and searched for (i) biallelic (homozygous and compound heterozygous) variants with AF <1% in liver panel genes linked with autosomal recessive (AR) or X-linked recessive (XLR) modes of inheritance and (ii) monoallelic (heterozygous and hemizygous) variants with AF <0.01% in liver panel genes linked with autosomal dominant (AD), X-linked dominant, or XLR modes of inheritance, respectively. We removed the variants listed as benign in the ClinVar. Also, missense variants, which were predicted to be benign by all in silico prediction algorithms, CADD, MutationTaster, PolyPhen-2, and SIFT, were excluded. Based on the retained variants, we performed a case-level assessment to determine the association between the pathogenicity of the variant and the clinical manifestations of the affected patient. Overall, we found that six (one ALF

and five rELT) out of 20 patients had rare nonsynonymous variants in nine genes from the liver panel (Table 3.2). The clinical and laboratory details for these six patients are given in Table 3.3. The amino acid residues mutated were conserved across different species, and all these variants were predicted as damaging by at least three in silico prediction tools tested (CADD, SIFT, PolyPhen-2 and MutationTaster) (Figure 3.2-3.8).

Table 3.1 Liver panel genes

#	Gene	Phenotype MIM	Inheritance	#	Gene	Phenotype MIM	Inheritance	#	Gene	Phenotype MIM	Inheritance	#	Gene	Phenotype MIM	Inheritance
1	ABCA1	205400	AR	46	BAAT	619232	AR	95	DPAGT1	608093	AR	144	HADH	231530	AR
2	ABCB11	601847; 605479	AR	47	BBS1	209900	AR	96	DPM1	608799	AR	145	HADHA	609016; 609015	AR
		614972	AD, AR	48	BCAP31	300475	XLR	97	DYNC2H1	613091	AR	146	HADHB	609015	AR
3	ABCB4	602347	AR	49	BCS1L	603358; 124000	AR	98	EARS2	614924	AR	147	HAMP	613313	AR
		600803	AD, AR	50	BLVRA	614156	AD, AR	99	EFL1	617941	AR	148	HFE	235200; 176100	AR
4	ABCC2	237500	AR	51	BMP2	235200	AD, AR	100	EIF2AK3	226980	AR	149	HGSNAT	252930	AR
5	ABCD3	616278	AR	52	BOLA3	614299	AR	101	ENG	187300	AD	150	HJV	602390	AR
6	ACAD9	611126	AR	53	BSCL2	615924; 269700	AR	102	EPM2A	254780	AR	151	HMGCL	246450	AR
7	ACADM	201450	AR	54	BTD	253260	AR	103	ERCC4	615272; 610965	AR	152	HMGCR	620375	AR
8	ACADVL	201475	AR	55	C2ORF69	619423	AR	104	ETFA	231680	AR	153	HMGCS2	605911	AR
9	ACOX1	264470	AR	56	CARS2	616672	AR	105	ETFDH	231680	AR	154	HNF1B	137920	AD
10	ACOX2	617308	AR	57	CASR	239200	AD, AR	106	F5	600880	AD, AR	155	HNF4A	616026	AD
11	ACSF3	614265	AR	58	CAV1	612526	AR	107	FADD	613759	AR	156	HSD17B13	620116	AD
12	ACVRL1	600376	AD	59	CAVIN1	613327	AR	108	FAH	276700	AR	157	HSD17B4	261515	AR
13	ADA	102700	AR	60	CC2D2A	619111; 612284	AR	109	FAN1	614817	AR	158	HSD3B7	607765	AR
14	ADAMTSL	231050	AR	61	CCDC115	616828	AR	110	FARS2	614946	AR	159	IARS1	617093	AR
15	ADK	614300	AR	62	CCDC47	618268	AR	111	FARSA	619013	AR	160	IDS	309900	XLR
16	AGA	208400	AR	63	CDAN1	224120	AR	112	FARSB	613658	AR	161	IDUA	607014; 607015	AR
17	AGL	232400	AR	64	CEP164	614845	AR	113	FAS	601859	AD	162	IER3IP1	614231	AR
18	AGPAT2	608594	AR	65	CEP19	615703	AR	114	FBP1	229700	AR	163	IFIH1	615846	AD
19	AKR1D1	235555	AR	66	CEP41	614464	AR	115	FBXL4	615471	AR	164	IFT172	615630	AR
20	ALAS2	300752	XL	67	CFTR	219700	AD, AR	116	FECH	177000	AR	165	IFT56	619534	AR
21	ALDOA	611881	AR	68	CIDEC	615238	AR	117	FH	606812	AR	166	IL18BP	618549	AR
22	ALDOB	229600	AR	69	CIITA	209920	AR	118	FOCAD	619991	AR	167	IL1RN	612852	AR
23	ALG1	608540	AR	70	CLDN1	607626	AR	119	FUCA1	230000	AR	168	IL21R	615207	AR
24	ALG13	300884	XL	71	COG2	617395	AR	120	G6PC	232200	AR	169	INPP5E	213300	AR
25	ALG2	607906	AR	72	COG4	613489	AR	121	GAA	232300	AR	170	INSR	246200	AR
26	ALG6	603147	AR	73	COG5	613612	AR	122	GALE	230350	AR	171	ITCH	613385	AR
27	ALG8	608104	AR	74	COG6	614576	AR	123	GALM	618881	AR	172	JAG1	118450	AD
28	ALG9	608776; 263210	AR	75	COG7	608779	AR	124	GALNS	253000	AR	173	JAK1	618999	AD
29	ALMS1	203800	AR	76	COQ2	607426	AR	125	GALT	230400	AR	174	JAK2	600880	AR
30	AMACR	214950	AR	77	COX16	619355	AR	126	GANAB	600666	AD	175	KARS1	619147	AR
31	ANKS6	615382	AR	78	COX5A	619064	AR	127	GATA6	600001	AD	176	KIF12	619662	AR
32	AP1B1	242150	AR	79	CPE	619326	AR	128	GATC	618839	AR	177	KIF20A	619433	AR
33	AP1S1	609313	AR	80	CPOX	618892	AD, AR	129	GBA1	608013; 230800; 230900; 231000; 231005	AR	178	KRT18	215600	AR
34	APOC2	207750	AR	81	CPT1A	255120	AR	130	GBE1	232500	AR	179	LARS1	615438	AR
35	ARCN1	617164	AD	82	CPT2	600649; 608836	AR	131	GCDH	231670	AR	180	LARS2	617021	AR
36	ARSB	253200	AR	83	CREB3L3	619324	AD	132	GFM1	609060	AR	181	LBR	613471	AD
37	ASAH1	228000	AR	84	CSPP1	615636	AR	133	GIMAP5	619463	AR			215140	AR
38	ASL	207900	AR	85	CTNS	219800	AR	134	GLB1	230600	AR	182	LIPA	278000	AR
39	ASS1	215700	AR	86	CYC1	615453	AR	135	GLIS3	610199	AR	183	LIPE	615980	AR
40	ATIC	608688	AR	87	CYP7B1	613812	AR	136	GNE	269921	AD	184	LIPT1	616299	AR
41	ATP6AP1	300972	XLR	88	DCDC2	616217; 617394	AR	137	GNMT	606664	AR	185	LMNA	151660	AD
42	ATP6AP2	301045	XLR	89	DDOST	614507	AR	138	GNPTAB	252500	AR			144250	AD
43	ATP7B	277900	AR	90	DGUOK	251880; 617068	AR	139	GNS	252940	AR	186	LPL	238600	AR
		147480	AD	91	DHDDS	613861	AR	140	GPC3	312870	XLR	187	LRPPRC	220111	AR
44	ATP8B1	211600	AR	92	DKC1	305000	XLD, XLR	141	GPD1	614480	AR	188	LYN	620376	AD
		243300	AR	93	DLD	246900	AR	142	GPIHBP1	615947	AR	189	LYRM4	615595	AR
45	B4GALT1	607091	AR	94	DNASE2	619858	AR	143	GUSB	253220	AR	190	MAN1B1	614202	AR

Table 3.1 Liver panel genes (continued)

#	Gene	Phenotype MIM	Inheritance	#	Gene	Phenotype MIM	Inheritance	#	Gene	Phenotype MIM	Inheritance	#	Gene	Phenotype MIM	Inheritance
191	MAN2B1	248500	AR	239	PC	266150	AR	285	RBCK1	615895	AR	333	SPTB	616649	AD
192	MARS1	615486	AR	240	PCCA	606054	AR	286	RFT1	612015	AR	334	SQOR	619221	AR
193	MCCC2	210210	AR	241	PCCB	606054	AR	287	RFX5	209920	AR	335	SRD5A3	612379	AR
194	MED12	301068	XLD	242	PCK1	261680	AR	288	RFX6	615710	AR	336	STAT2	618886	AR
195	MICOS13	618329	AR	243	PCK2	261650	AR	289	RFXANK	209920	AR	337	STT3B	615597	AR
196	MICU1	615673	AR	244	PCYT1A	620680	AR	290	RFXAP	209920	AR	338	STX5	620454	AR
197	MKS1	249000	AR	245	PEPD	170100	AR	291	RINT1	618641	AR	339	STXBP2	613101	AR
198	MMAA	251100	AR	246	PEX1	214100; 601539	AR	292	RMND1	614922	AR	340	SUMF1	272200	AR
199	MMAB	251110	AR	247	PEX10	614870	AR	293	RNASEH2A	610333	AR	341	TALDO1	606003	AR
200	MMUT	251000	AR	248	PEX12	266510	AR	294	RNF220	619688	AR	342	TANGO2	616878	AR
201	MOGS	606056	AR	249	PEX13	614883	AR	295	RPGRIP1L	619113; 611561	AR	343	TEFM	620451	AR
202	MPI	602579	AR	250	PEX14	614887	AR	296	RRAGC	620609	AD			613989	AD, AR
203	MPV17	618400; 256810	AR	251	PEX16	614876	AR	297	RTEL1	616373	AD, AR	344	TERT	614742	AD
204	MRM2	618567	AR	252	PEX19	614886	AR	298	SBDS	260400	AR	345	TFAM	617156	AR
205	MRPL3	614582	AR	253	PEX2	614866; 614867	AR	299	SC5D	607330	AR	346	TFR2	604250	AR
206	MRPL44	615395	AR	254	PEX26	614872	AR	300	SCO1	619048	AR	347	TJP2	615878; 607748	AR
207	MRPS16	610498	AR	255	PEX3	614882	AR	301	SCYL1	616719	AR	348	TKFC	618805	AR
208	MRPS23	618952	AR	256	PEX5	214110	AR	302	SDHD	619167	AR	349	TMEM107	617562	AR
209	MRPS28	618958	AR	257	PEX6	614863	AD, AR	303	SEC23B	224100	AR	350	TMEM165	614727	AR
210	MRPS7	617872	AR			614862	AR	304	SEC63	617004	AD	351	TMEM199	616829	AR
211	MTM1	310400	XLR	258	PFKM	232800	AR	305	SEMA7A	619874	AR	352	TMEM67	216360; 610688; 607361; 613550	AR
212	MVK	610377	AR	259	PGM1	614921	AR	306	SERAC1	614739	AR	353	TRAPPC11	615356	AR
213	MYO5B	619868; 251850	AR	260	PHKA2	306000	XLR	307	SERPINA1	613490	AR	354	TREX1	192315	AD
214	NAF1	620365	AD	261	PHKB	261750	AR	308	SGSH	252900	AR			225750	AD, AR
215	NAGLU	252920	AR	262	PHKG2	613027	AR	309	SH2D1A	308240	XLR	355	TRIM37	253250	AR
216	NBAS	616483	AR	263	PIBF1	617767	AR	310	SKIC2	614602	AR	356	TRMT10C	616974	AR
217	NCF2	233710	AR	264	PIGA	300868	XLR	311	SKIC3	222470	AR	357	TRMU	613070	AR
218	NCKAP1L	618982	AR	265	PKHD1	263200	AR	312	SLC10A1	619256	AR	358	TTC26	619534	AR
219	NDUFS4	252010	AR	266	PLIN1	613877	AD	313	SLC17A5	269920	AR	359	TTC7A	243150	AR
220	NEK8	615415	AR	267	PMM2	212065	AR	314	SLC22A5	212140	AR	360	TULP3	619902	AR
221	NEU1	256550	AR	268	PNPLA2	610717	AR	315	SLC25A1	615182	AR	361	TWNK	271245	AR
222	NFKB1	616576	AD	269	POLD1	615381	AD	316	SLC25A13	603471; 605814	AR	362	UBR1	243800	AR
223	NFS1	619386	AR	270	POLG	203700; 613662	AR	317	SLC25A15	238970	AR	363	UNC13D	608898	AR
224	NGLY1	615273	AR			619425	AR	318	SLC25A20	212138	AR	364	UNC45A	619377	AR
225	NHLRC1	254780	AR	271	POLG2	610131	AD	319	SLC27A5	619232	AR	365	UQCRB	615158	AR
226	NHLRC2	618278	AR			618528	AR	320	SLC2A1	608885	AD	366	UQCRC2	615160	AR
227	NOP10	620400	AD	272	POMC	609734	AR	321	SLC2A2	227810	AR	367	UROD	176100	AD, AR
		224230	AR	273	POT1	620367	AD, AR	322	SLC30A10	613280	AR	368	USP18	617397	AR
228	NOS3	189800	AD	274	PPARG	604367	AD	323	SLC37A4	619525	AD	369	USP53	619658	AR
229	NOTCH2	610205	AD	275	PRF1	603553	AR			232220	AR	370	VIPAS39	613404	AR
230	NPC1	257220	AR	276	PRKCSH	174050	AD	324	SLC39A4	201100	AR	371	VPS33B	208085; 620010; 620009	AR
231	NPC2	607625	AR	277	PSAP	611721	AR	325	SLC39A7	619693	AR	372	VPS50	619685	AR
232	NPHP3	267010; 604387; 208540	AR	278	PSMB4	617591	AR	326	SLC40A1	606069	AD	373	VPS51	618606	AR
233	NR1H4	617049	AR	279	PSMB8	256040	AR	327	SLC44A1	618868	AR	374	WDR19	614378; 614377; 616307; 614376	AR
234	OCLN	251290	AR	280	PSMB9	617591	AR	328	SLC51A	619484	AR	375	WDR35	613610; 614091	AR
235	OFD1	311200	XLD	281	PSMC1	620071	AR	329	SLC51B	619481	AR	376	XIAP	300635	XLR
236	OSTM1	259720	AR	282	PTF1A	615935	AR	330	SLC7A7	222700	AR	377	YARS1	619418	AD, AR
237	OTULIN	617099	AD, AR	283	PYGL	232700	AR	331	SMPD1	257200; 607616	AR	378	YARS2	613561	AR
238	PARS2	618437	AR	284	QRSL1	618835	AR	332	SOCS1	619375	AD	379	YRDC	619609	AR
												380	ZNFX1	619644	AR

We manually curated this panel by searching for genes annotated with hepatic and/or biliary phenotype or laboratory finding of elevated liver enzymes/transaminases in the OMIM database (https://www.omim.org/). We only included protein coding genes in the panel. Genes associated with somatic morbid mutations were eliminated.

Table 3.2 Patients with rare nonsynonymous variants in liver panel genes.

Patient	Disease	Gene	Variation					
			Type	Change	Status	AF	ACMG-AMP	
							Classification	Criteria
P1	rELT	*ACOX2*	Missense	NM_003500.4:c.674G > A:p.Arg225Gln	Hom	5.14E-05	VUS	PM1, PP3, PM5
P2	rELT	*PYGL*	Nonsense	NM_002863.5:c.1180G > T:p.Glu394*	Hom	-	P	PVS1, PM2, PP3
		SLC27A5	Missense	NM_012254.3:c.923C > T:p.Thr308Met	Hom	1.86E-05	VUS	PM1, PM2, PP3
P3	rELT	*PHKA2*	Missense	NM_000292.3:c.556C > T:p.Arg186Cys	Hem	9.26E-07	P	PS1, PM1, PM2, PP3, PP5
P4	rELT	*ABCB4*	Missense	NM_000443.4:c.2950G > A:p.Ala984Thr	Hom	1.37E-06	LP	PS1, PM2, PP3, BP1
P5	ALF	*CDAN1*	Missense	NM_138477.2:c.1945C > T:p.Arg649Trp	Hom	3.28E-05	LP	PS1, PM2, PP3
P6	rELT	*JAG1*	Missense	NM_000214.3:c.322A > C:p.Asn108His	Het	2.05E-06	VUS	PM2, PP3, BP1
		PCK2	Missense	NM_004563.4:c.644G > A:p.Gly215Asp	Hom	2.01E-04	VUS	PM1, PM2, PP3, BP1
		VPS33B	Missense	NM_018668.5:c.1209G > T:p.Leu403Phe	Hom	1.37E-06	VUS	PM1, PM2, PP3, BP1

AF: overall allele frequency in gnomAD v4.1.0 (as of May 08, 2024); Hom, Homozygous, Hem, Hemizygous, Het: Heterozygous, VUS, variant of uncertain significance, P: Pathogenic, LP: Likely pathogenic. PS1: Pathogenic Strong 1, PM1: Pathogenic Moderate 1, PM2: Pathogenic Moderate 2, PM5: Pathogenic Moderate 5, PP3: Pathogenic Supporting 3, PP5: Pathogenic Supporting 5, PVS1: Pathogenic Very Strong 1, BP1: Benign Supporting 1. Adapted from Lülecioğlu AA et al., 2024[175]

Table 3.3 Clinical and laboratory findings of six patients with rare nonsynonymous variants in liver panel genes

		P1	P2	P3	P4	P5	P6
Sex		Female	Male	Male	Female	Female	Male
Consanguinity		Yes	Yes	No	Yes	Yes	Yes
Delayed growth or development		No	No	No	No	No	No
Age of disease onset		7 months	8 months	12 months	13 years	13 months	7 years
Clinical symptoms at admission		Asymptomatic	Asymptomatic	Asymptomatic	Abdominal pain	Vomiting, fever, diarrhea	Asymptomatic
ELT episodes	**Encephalopathy**	No	No	No	No	No	No
	ALT (U/L) (min-max)	86-885	80-570	93-1121	105-174	6530	92-169
	AST (U/L) (min-max)	95-1226	90-500	82-2067	108-181	8890	72-140
	ALP (U/L)	Normal	Normal	Normal	Normal	Normal	Normal
	GGT (U/L) (min-max)	Normal	Variable (Elevated range: 76-109)	Variable (Elevated range: 216-280)	Elevated (70-154)	Normal	Mostly normal (Elevated: 38)
	PT (sec) / INR	Normal / Normal	Normal / Normal	Mostly normal (Elevated: 15 / 1.3)	Variable (Elevated range: 15.1-16.5 / 1.26-1.43)	34 / 3.22	Normal / Normal
	Direct / Indirect bilirubin (mg/dL)	Normal	Normal	Normal	Normal	Normal	Normal
Organomegaly		No	Hepatomegaly	Hepatomegaly	Hepatosplenomegaly	No	No
Autoantibodies*		Negative	Negative	Negative	ANA (+), others: negative	Negative	Negative
Cytopenia		No	No	No	No	No	No
Infectious diseases		No	No	No	No	No	No
Metabolic workup		Normal	Hyperlipidemia	Normal	Normal	Normal	Normal
Extrahepatic abnormalities		No	No	No	No	No	No
Abdominal ultrasound exam		Liver size, surface, and echogenicity were normal.	Liver was 98 mm in the midclavicular line and showed mildly increased parenchymal echogenicity (hepatosteatosis). The right kidney was normal in size and echogenicity. Left renal pelvis anteroposterior diameter was 28 mm, with Grade-4 hydronephrosis (mild parenchymal loss).	Liver parenchymal echogenicity and surface were normal. The size of the liver was 15 cm.	Liver was 15 cm and its left lobe was hypertrophic. Increased liver parenchymal echogenicity (grade 1-2). Lymphadenopathy (20x10 mm) in the liver hilum. Spleen was 20 cm. Two calculi, each measuring 4 and 9 mm, were seen in the gallbladder lumen.	Liver size, surface, and echogenicity were normal	Liver size, surface, and echogenicity were normal
Liver biopsy		NA	NA	NA	NA	NA	Ground glass appearance of hepatocytes with eosinophilic inclusions were seen.
Treatment		UDCA	No	No	UDCA	Medical treatment	UDCA
Outcome		Alive	Alive	Alive	Alive	Alive	Alive

Abbreviations: ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, GGT: gamma-glutamyl transferase, PT: prothrombin time, INR: international normalized ratio, NA: Not available, UDCA: Ursodeoxycholic acid

Reference ranges: ALT: <40 U/L for P1-P3, P5, P6; <27 U/L for P4, AST: <40 U/L for P1-P3; <47 U/L for P4; <30 U/L for P5 and P6, GGT: <40 U/L for P1-P3; <23 U/L for P4; <21 U/L for P5 and P6, ALP: 0-300 U/L for P1-P3; 83-382 U/L for P4; 30-120 U/L for P5 and P6, PT: 9.9-11.8 sec for P1-P3; 10-14.7 sec for P4; 10-14.5 sec for P5 and P6, INR: 0.8-1.2 for P1-P6, Direct bilirubin: 0-0.2 mg/dL for P1-P3; 0-0.5 mg/dL for P4-P6, Indirect bilirubin: 0-0.9 mg/dL for P1-P3; 0-1.5 mg/dL for P4; 0-0.7 mg/dL for P5 and P6.

*Autoantibody screening: antinuclear antibodies (ANA), extractable nuclear antigen panel, anti-smooth muscle antibodies, antimitochondrial antibodies, antibodies to liver-kidney microsome type-1/2, antibodies to soluble liver antigen, antineutrophil cytoplasmic antibodies, Anti-Saccharomyces cerevisiae antibodies, Anti-transglutaminase IgA/IgG, Antigliadin IgA/IgG.

Adapted from Lülecioğlu AA et al., 2024[175]

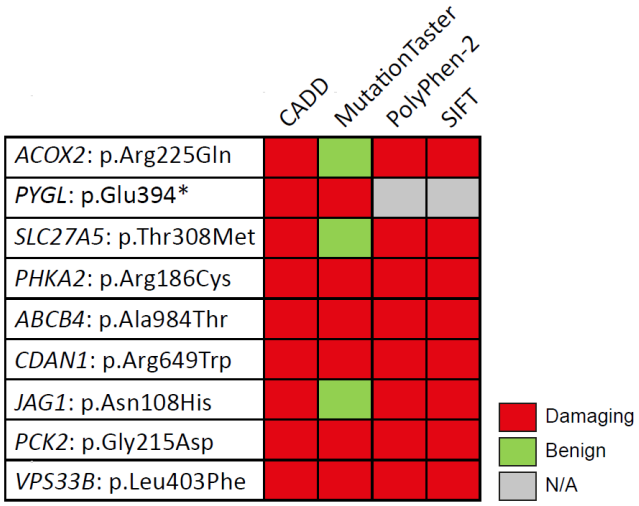


Figure 3.2 Variant effect predictions. The predicted impact of variants using four algorithms, CADD, MutationTaster, PolyPhen-2 and SIFT, is shown. Red color indicates damaging, whereas green color is benign. N/A, Not applicable. The CADD and MSC scores for each variant are as follows: *ACOX2* (31, 26.9), *PYGL* (42, 19), *SLC27A5* (22.2, 13.1), *PHKA2* (32, 16.4), *ABCB4* (26.8, 16.3), *CDAN1* (29.4, 15.2), *JAG1* (25, 19.3), *PCK2* (27.6, 21.8), *VPS33B* (29.8, 19), where the first number is the CADD score of the variant and the second number is the MSC score of the gene mutated. Adapted from Lülecioğlu AA et al., 2024[175]

3.2.1 A homozygous missense variant in *ACOX2* in P1

P1 had a homozygous p.Arg225Gln variant in Acyl CoA Oxidase 2 (*ACOX2*) (Table 3.2). The patient's mother was heterozygote confirmed by Sanger sequencing; however, the patient's father's status was not assessed as his gDNA sample was not available (Figure 3.3.A-B). Biallelic mutations in *ACOX2* were linked with congenital bile acid synthesis defect (MIM number: 617308), characterized by elevated liver enzymes and other liver phenotypes such as cholestasis and fibrosis. ACOX2 is involved in the degradation of branched-chain fatty acids and bile acid intermediates within peroxisomes and converting the C27 bile acid intermediates, which are 3a,7a-dihydroxycholestanoic acid and 3a,7a,12a-trihydroxycholestanoic acid, into C24 bile acids in the β-oxidation pathway[176]. Previously, a homozygous premature stop-gain mutation, p.Y69* in *ACOX2*, was identified in an 8-year-old Turkish boy with liver fibrosis, mild ataxia, rELT, and cognitive impairment[177]. Moreover, Monte et al

identified a different homozygous mutation at the same position, p.Arg225Trp, in an adolescent with persistent hypertransaminasemia and accumulated levels of C27 intermediates with decreased C24 bile acids[176]. Yet, the p.Arg225Gln was not associated with any disease or reported in ClinVar. The p.R225 is an evolutionarily conserved residue across various species (Figure 3.3.C). The p.Arg225Gln, which is very rare (Figure 3.3.D), was predicted as damaging by CADD, PolyPhen-2, and SIFT (Figure 3.2). However, it was listed as benign by the MutationTaster and classified as a variant of uncertain significance (VUS) according to the ACMG-AMP variant classification guidelines (Figure 3.2 and Table 3.2). Yet, P1 presented with rELT, low to normal levels of GGT, and a positive response to ursodeoxycholic acid treatment, indicating a bile acid synthesis defect, which can be attributed to the p.Arg225Gln variant (Table 3.3).

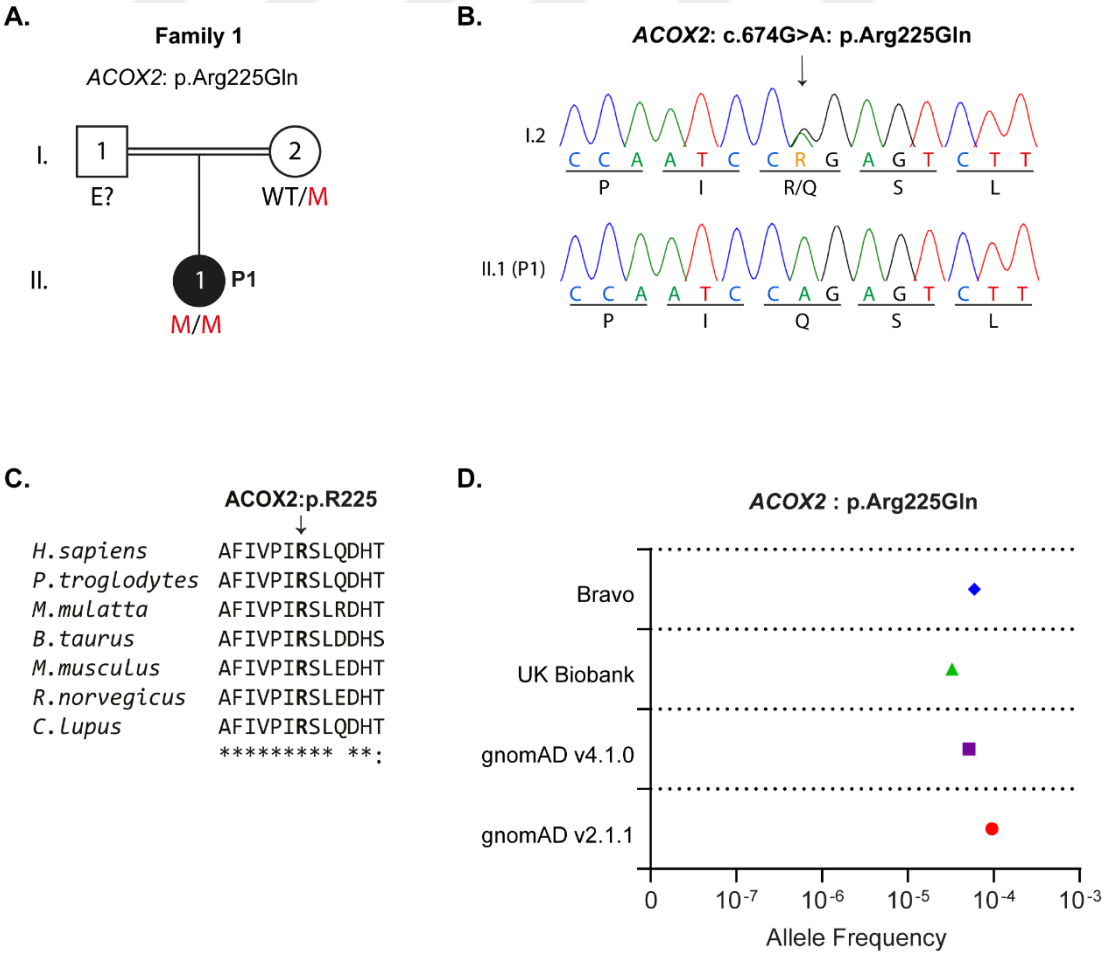


Figure 3.3 A homozygous p.Arg225Gln in *ACOX2* in P1 **(A)** Pedigree of the Family 1 affected by rELT. The patient is shown in black, whereas healthy individuals are shown in white. *ACOX2* mutation (NM_003500.4: c.674G > A:p.Arg225Gln) status is indicated in red. WT, wild type, M, mutation. **(B)** Sanger confirmation of p.Arg225Gln variant in P1 and her mother. **(C)** Conservation of p. Arg225Gln residue across different species. Asterisk (*), colon (:), and period (.) indicate fully conserved, strongly similar, and weakly similar sites, respectively. Source: Clustal Omega. **(D)** Graph showing the allele frequencies of p.Arg225Gln in Bravo (blue diamond), UK Biobank (green triangle), gnomAD v4.1.0 (purple square), and gnomAD v2.1.1 (red circle). Adapted from Lülecioğlu AA et al., 2024[175]

3.2.2 A homozygous nonsense variation in *PYGL* and a homozygous missense mutation in *SLC27A5* in P2

A homozygous p.Glu394* in Glycogen phosphorylase L (*PYGL)* was found in P2 with rELT (Table 3.2). The patient's mother was heterozygous for this allele, whereas his father's and his siblings' genotypes could not be determined since their gDNA samples were not available (Figure 3.4.A-B). PYGL is one of the key enzymes of glycogen metabolism in the liver. PYGL catalyzes the phosphorolysis of α-1, 4-glycosidic bonds in the glycogen molecules and releases glucose-1-phosphate from the liver[178]. Biallelic mutations in *PYGL* have been associated with Glycogen storage disease VI (GSD6) (MIM number: 232700), also known as Hers disease, characterized by hepatomegaly and increased liver glycogen. This variant was located on a highly conserved amino acid residue across various species (Figure 3.4.C). The p.Glu394* was not found in any public database or previously associated with any disease (Figure 3.4.D). MutationTaster and CADD predicted this variant as damaging (Figure 3.2). As it is a nonsense mutation, SIFT and PolyPhen-2 predictions were not applicable (Figure 3.2). This variant was classified as pathogenic based on ACMG-AMP guidelines (Table 3.2). P2 had elevated liver enzymes, hyperlipidemia, and hepatosteatosis, which are the characteristics of GSD6[179] (Table 3.3). Therefore, patient's phenotype can be explained by the homozygous nonsense mutation in *PYGL*. Additionally, P2 had a

homozygous p.Thr308Met in Solute Carrier Family 27 Member 5 (*SLC27A5)*, which encodes for a bile acyl CoA synthetase (Table 3.2). The patient's mother was heterozygous for p.Thr308Met. The patient's father's and two siblings' status could not be assessed as their gDNA samples were not available (Figure 3.4.A-B). SLC27A5 is mainly expressed in the liver and is responsible for the conjugation of taurine and glycine to bile acids[180]. Biallelic mutations in *SLC27A5* have been associated with Bile acid conjugation defect 1 (MIM number: 619232). Chong et al identified a homozygous p.His338Tyr mutation in *SLC27A5* in a child with cholestasis and fibrosis, but with no experimental evidence for causality[180]. Furthermore, Slc27a5 deficiency in mice causes activation of hepatic stellate cells and liver fibrosis due to the accumulation of unconjugated bile acids[181]. The p.Thr308Met was not reported in ClinVar or associated with any disease. This variant affected amino acid residue that is highly conserved among different species (Figure 3.4.C) and is found to be very rare (Figure 3.4.D). CADD, PolyPhen-2, and SIFT predicted that this variant was damaging (Figure 3.2). It was predicted as benign by MutationTaster and classified as VUS by ACMG-AMP guidelines (Figure 3.2 and Table 3.2).

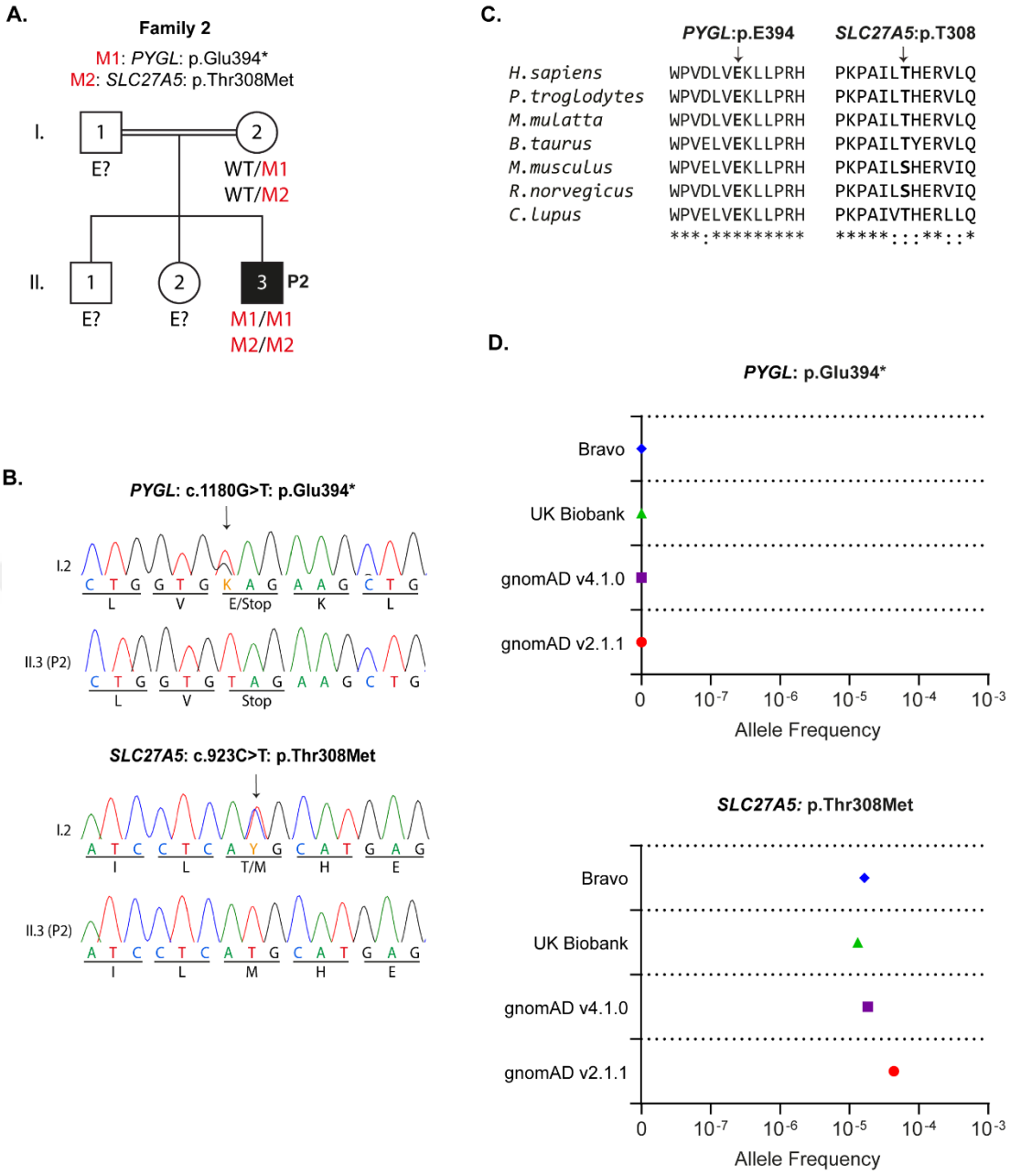


Figure 3.4 A homozygous nonsense variation in *PYGL* and a homozygous missense mutation in *SLC27A5* in P2. **(A)** Pedigree of the Family 2 affected by rELT. The patient is shown in black, whereas healthy individuals are shown in white. *PYGL* and *SLC27A5* mutations (NM_002863.5: c.1180G > T: p.Glu394*, NM_012254.3: c.923C > T: p.Thr308Met) statuses are indicated in red. WT, wild type, M, mutation. **(B)** Sanger confirmation of the mutations in P2 and his mother. **(C)** Conservation of the residues mutated, p.Glu394 in *PYGL* and p.T308 in *SLC27A5*, across different species. Asterisk (*), colon (:), and period (.) indicate fully conserved, strongly similar, and weakly similar sites, respectively. Source: Clustal Omega **(D)** Graph displaying the allele frequencies of both variants in Bravo (blue diamond), UK Biobank (green triangle), gnomAD v4.1.0 (purple square), and gnomAD v2.1.1 (red circle). Adapted from Lülecioğlu AA et al., 2024[175]

3.2.3 A hemizygous mutation in *PHKA2* in P3

We identified a hemizygous p.Arg186Cys variant in Phosphorylase kinase regulatory subunit alpha 2 *(PHKA2)* in P3 with rELT (Table 3.2). The patient's father had WT allele, while his mother was heterozygous, which is consistent with the X-linked recessive (XLR) pattern of inheritance (Figure 3.5.A-B). PHKA2 is responsible for activating the glycogen phosphorylase, initiating the glycogen breakdown in the liver[182]. The p.R186C is located in a highly conserved residue among various species (Figure 3.5.C). This variant, which is very rare (Figure 3.5.D), was predicted as damaging by CADD, MutationTaster, PolyPhen-2 and SIFT (Figure 3.2). It is also classified as pathogenic based on the ACMG-AMP classification (Table 3.2). Mutations in *PHKA2* have been associated with Glycogen storage disease (MIM number: 306000), characterized by elevated liver enzymes, hepatomegaly, and abnormal distension of hepatocytes with glycogen. The p.Arg186Cys variant was reported as likely pathogenic in ClinVar (VCV000010535.8). It was identified in a patient with X-linked liver glycogenosis type II (XLGII)[183,184]. Thus, p.Arg186Cys in *PHKA2* was strongly considered as the monogenic cause underlying rELT in P3 (Table 3.3).

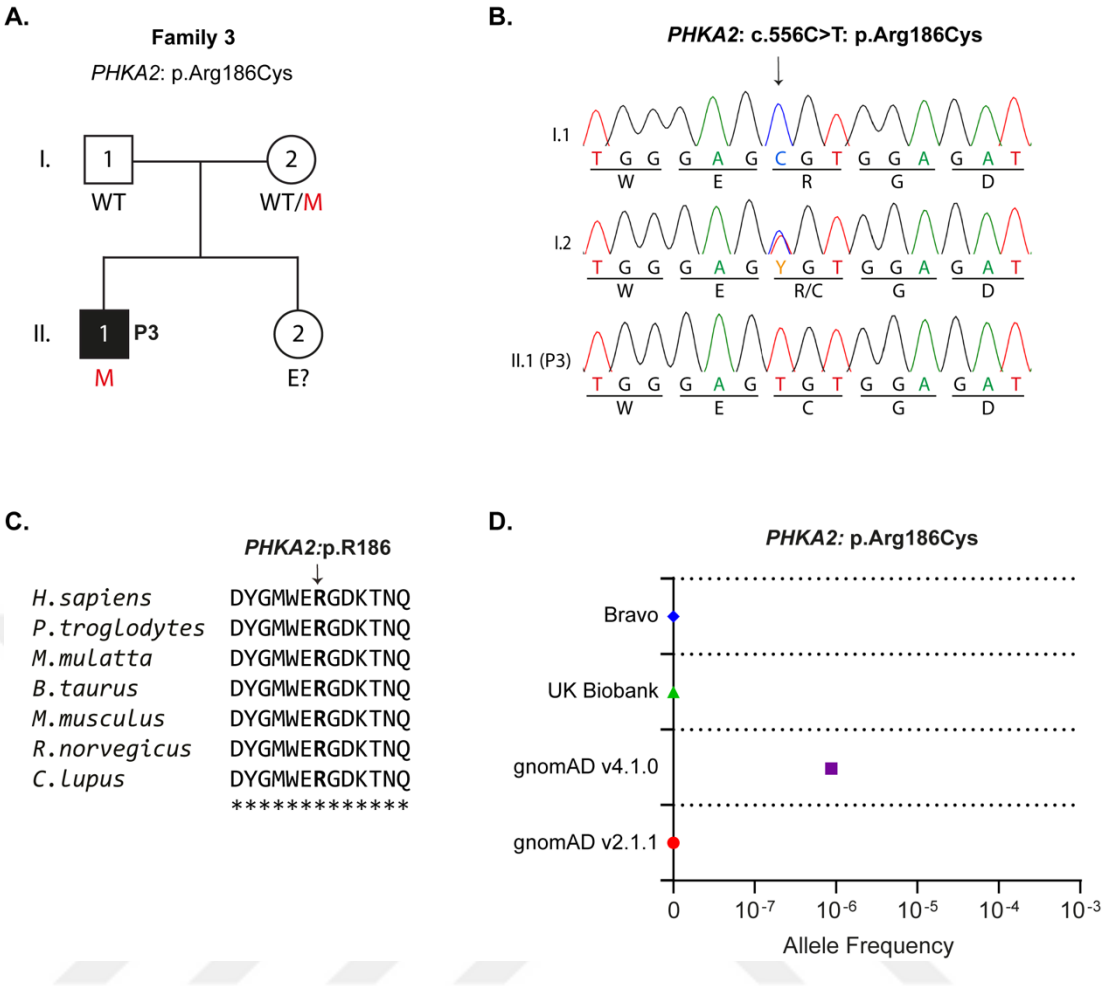


Figure 3.5 A hemizygous p.Arg186Cys in *PHKA2* in P3 **(A)** Pedigree of the Family 3 affected by rELT. The patient is shown in black, whereas healthy individuals are shown in white. *PHKA2* mutation (NM_000292.3: c.556C>T: p.Arg186Cys) status is indicated in red. WT, wild type, M, mutation. **(B)** Sanger confirmation of p.Arg186Cys variant in P3 and his parents. **(C)** Conservation of the p.Arg186 residue across various species. Asterisk (*), colon (:), and period (.) indicate fully conserved, strongly similar, and weakly similar sites, respectively. Source: Clustal Omega **(D)** Graph showing the allele frequencies of the variant found in Bravo (blue diamond), UK Biobank (green triangle), gnomAD v4.1.0 (purple square), and gnomAD v2.1.1 (red circle). Adapted from Lülecioğlu AA et al., 2024[175]

3.2.4 A homozygous missense mutation in *ABCB4* in P4

A homozygous missense mutation, p.Ala984Thr, in ATP binding cassette subfamily B member 4 (*ABCB4*) was found in P4 with rELT (Table 3.2). Sanger sequencing showed that both parents were heterozygous for this allele, whereas siblings of P4 did not carry the mutation (Figure 3.6.A-B). ABCD4, also known as MDR3, facilitates the

transportation of phosphatidylcholine from the inner to the outer side of the canalicular membrane of the hepatocyte[185,186]. Once there, phosphatidylcholine is secreted into the bile and forms micelles with bile salts, thereby reducing the detergent activity of bile salts to prevent damage to canalicular membranes and biliary tract[185,186]. Mutations in *ABCB4* have been associated with Gallbladder disease 1 (MIM number: 600803) and progressive familial intrahepatic cholestasis type 3 (PFIC3) (MIM number: 602347) characterized by cholestasis, abnormal liver function tests, and elevated GGT. Previously, a heterozygous p.Ala984Thr variant was reported in a patient with PFIC3, however, there was no experimental evidence provided to support the causality. The p.Ala984Thr affected an amino acid residue that is highly conserved among different species (Figure 3.6.C). This variant was listed as very rare (Figure 3.6.D) and predicted to be damaging by CADD, MutationTaster, PolyPhen-2, and SIFT (Figure 3.2). Moreover, it is classified as likely pathogenic by the ACMG-AMP guidelines (Table 3.2). Overall, P4's clinical manifestations, including elevated GGT level, hepatosplenomegaly, and cholelithiasis, can be attributed to the homozygous p.Ala984Thr mutation in *ABCB4* (Table 3.3).

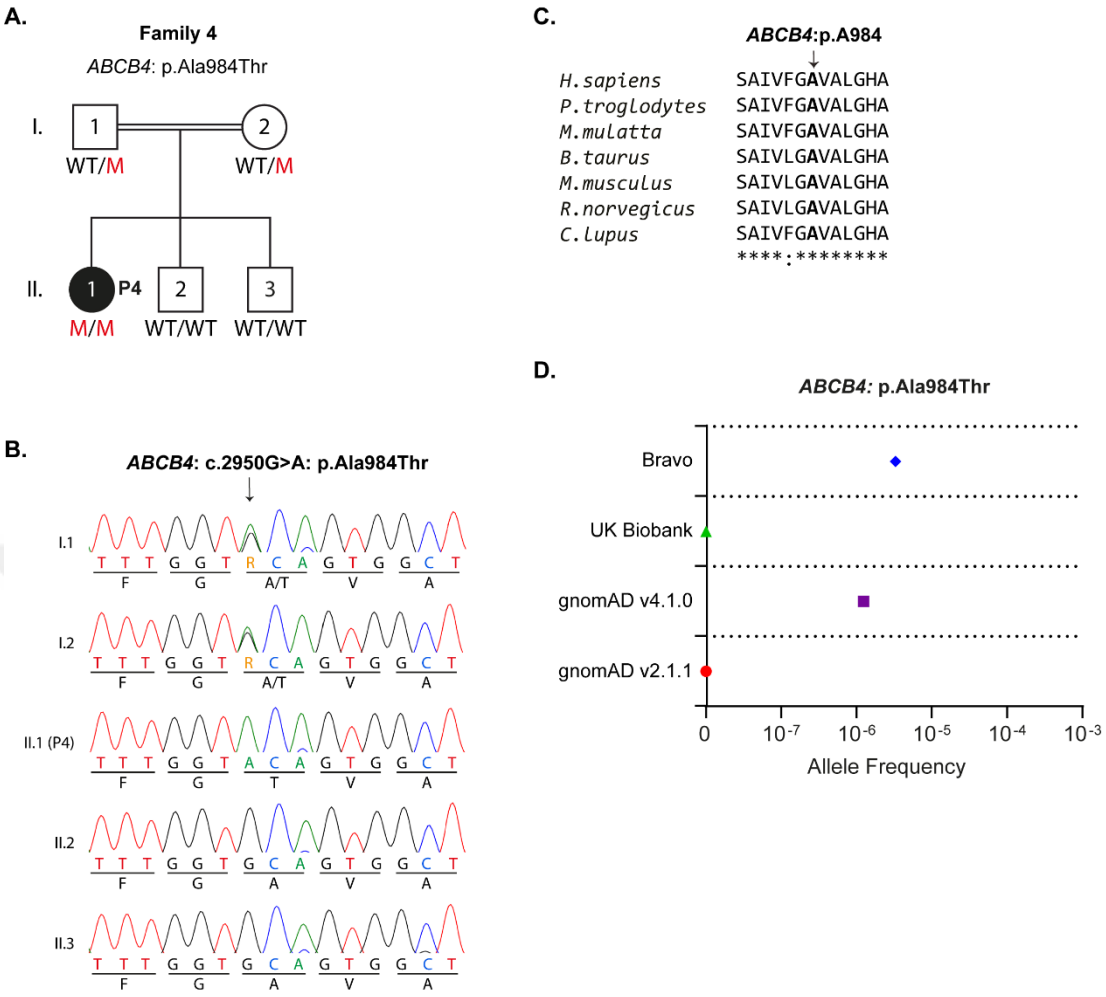


Figure 3.6 A homozygous P. Ala984Thr in *ABCB4* in P4 **(A)** Pedigree of the Family 4 affected by rELT. The patient is shown in black, whereas healthy individuals are shown in white. *ABCB4* mutation (NM_000443.4: c.2950G >A: p.Ala984Thr) status is indicated in red. WT, wild type, M, mutation. **(B)** Sanger confirmation of p.Ala984Thr variant in Family 4. **(C)** Conservation of p.Ala984 residue across different species. Asterisk (*), colon (:), and period (.) indicate fully conserved, strongly similar, and weakly similar sites, respectively. Source: Clustal Omega **(D)** Graph showing the allele frequencies of p.Ala984Thr in Bravo (blue diamond), UK Biobank (green triangle), gnomAD v4.1.0 (purple square), and gnomAD v2.1.1 (red circle). Adapted from Lülecioğlu AA et al., 2024[175]

3.2.5 A homozygous missense variant in *CDAN1* in P5

We found a homozygous missense variant, p.Arg649Trp, in Codanin 1 (*CDAN1)* in P5 with ALF (Table 3.2). Mutation status of family members could not be assessed as their gDNA samples were not available (Figure 3.7.A-B). The function of *CDAN1* is

not fully explored. It is a heterochromatin protein that regulates the incorporation of histones into newly synthesized DNA during cellular replication, DNA organization, and replication during erythroid maturation[187,188]. Biallelic mutations in *CDAN1* have been associated with congenital dyserythropoietic anemia Ia (CDAIa) (MIM number: 224120), which is a rare red blood cell disorder characterized with macrocytic/normocytic anemia, impaired erythropoiesis, secondary hemochromatosis, iron overload, and jaundice. Also, liver injury, such as ALF, was reported in some cases of CDAIa[189,190]. The p.Arg649Trp was found on a highly conserved amino acid residue among different species (Figure 3.7.C) and listed as very rare in public databases (Figure 3.7.D). It was predicted to be damaging by CADD, MutationTaster, PolyPhen-2, and SIFT (Figure 3.2). The p.Arg649Trp was not listed in the ClinVar, yet, p.Arg649Trp and p.Arg397Trp variants, found in compound heterozygosity, were identified in a patient with CDAIa[191]. This variant was classified as likely pathogenic according to ACMG-AMP classification (Table 3.2). However, clinical manifestations of P5 were not consistent with the phenotypic characteristics of human CDAN1 deficiency (Table 3.3).

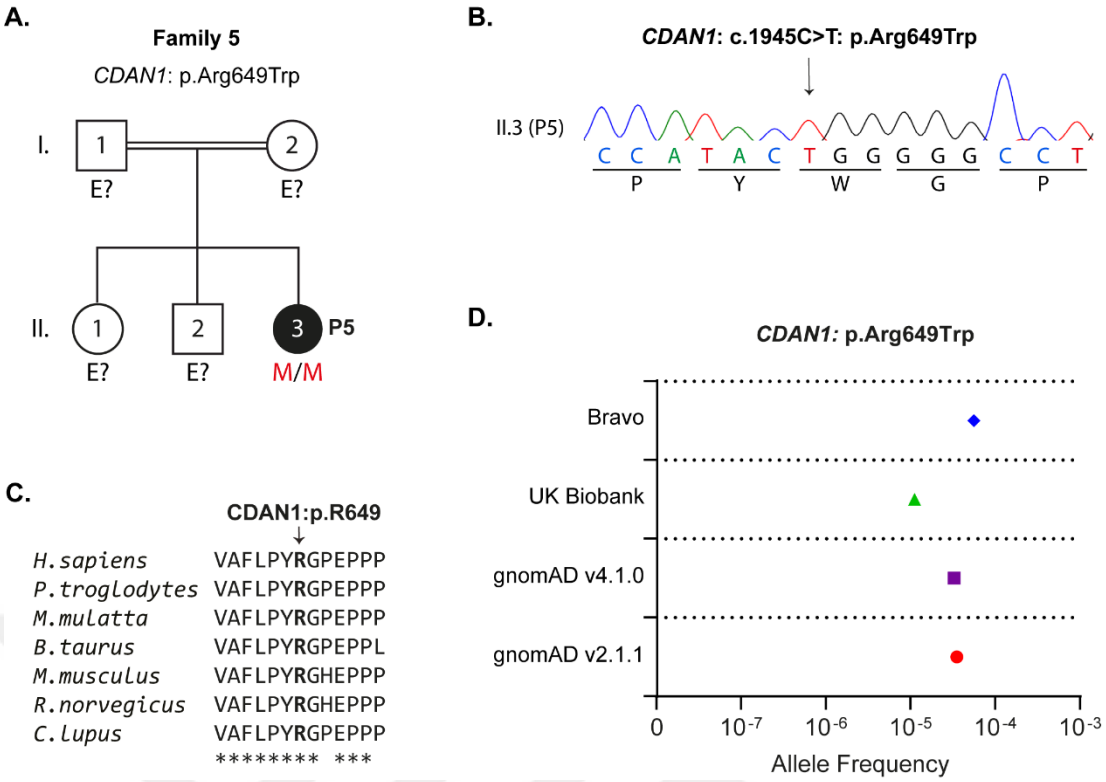


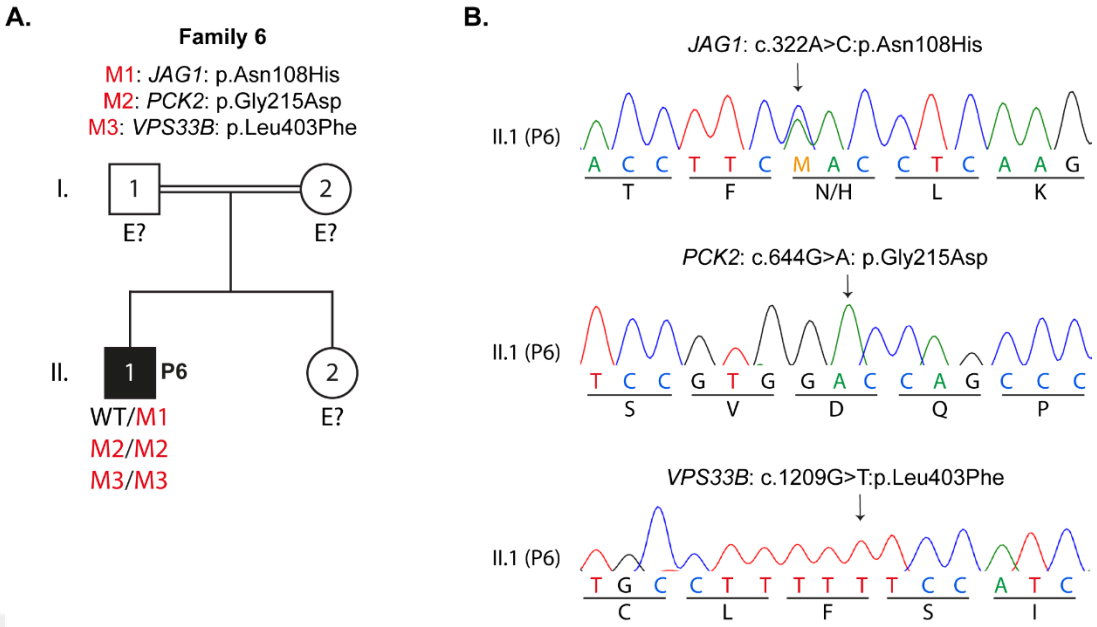
Figure 3.7 A homozygous p.Arg649Trp in *CDAN1* in P5 **(A)** Pedigree of the Family 5 affected by ALF. The patient is shown in black, whereas healthy individuals are shown in white. *CDAN1* mutation (NM_138477.2: c.1945C>T: p.Arg649Trp) status is indicated in red. WT, wild type, M, mutation. **(B)** Sanger sequencing of p.Arg649Trp variant in P5. **(C)** Conservation of p.Arg649 residue across different species. Asterisk (*), colon (:), and period (.) indicate fully conserved, strongly similar, and weakly similar sites, respectively. Source: Clustal Omega **(D)** Graph demonstrating the allele frequencies of p.Arg649Trp in Bravo (blue diamond), UK Biobank (green triangle), gnomAD v4.1.0 (purple square), and gnomAD v2.1.1 (red circle). Adapted from Lülecioğlu AA et al., 2024[175]

3.2.6 Missense variations in *JAG1*, *PCK2*, and *VPS33B* in P6

Last, P6 with rELT was found to harbor three missense variants in three different liver panel genes, which are heterozygous p.Asn108His in Jagged canonical Notch ligand 1 (*JAG1)*, homozygous p.Gly215Asp in Phosphoenolpyruvate carboxykinase 2 (*PCK2)* and homozygous p.Leu403Phe in Vacuolar protein sorting 33B, late endosome and lysosome-associated (*VPS33B)* (Table 3.2). Familial segregation of these variants was not evaluated since the gDNA of the patient's parents was not available (Figure 3.8.A-B). All variants were located on highly conserved amino acid residues across

various species (Figure 3.8.C) and listed as rare (Figure 3.8.D). JAG1 interacts with Notch receptor, involving signaling cascades important for cell differentiation and development of various organs[192]. Heterozygous mutations in *JAG1* have been shown to cause Alagille syndrome (MIM number: 118450), characterized by liver phenotypes including cholestasis, intrahepatic duct deficiency, and cirrhosis, and abnormalities in laboratory tests such as ELT, increased conjugated bilirubin, hypercholesterolemia, and hypertriglyceridemia. This variant was predicted as damaging by CADD, PolyPhen-2, and SIFT, whereas classified as benign by MutationTaster (Figure 3.2). It was listed as VUS in the ClinVar (VCV001020741.7), and similarly classified as VUS based on the ACMG-AMP guidelines (Table 3.2). Additionally, PCK2 (also known as PEPCK-M), catalyzes the reaction of oxaloacetate (OAA) conversion to phosphoenolpyruvate (PEP) by utilizing GTP as energy source[193]. This enzyme has a key role in gluconeogenesis in the liver and is responsible for the conversion of non-carbohydrate substrates to glucose[194]. Biallelic mutations in *PCK2* have been linked to mitochondrial phosphoenolpyruvate carboxykinase deficiency (MIM number: 261650), characterized by hypoglycemia, fatty liver, and liver failure. *PCK2*:p.Gly215Asp was predicted as damaging by CADD, MutationTaster, PolyPhen-2, and SIFT (Figure 3.2). However, it was reported as VUS in the ClinVar (VCV002713587.2) and similarly classified according to the ACMG-AMP guidelines (Table 3.2). Finally, *VPS33B* is responsible for protein trafficking to lysosomal compartments and vesicular membrane fusion[195]. This protein has been shown to be important for hepatocyte polarity to maintain the structure and function of the liver[196]. Biallelic mutations in *VPS33B* have been linked to arthrogryposis, renal dysfunction, and cholestasis (MIM: 208085), progressive familial intrahepatic cholestasis (MIM: 620010), and keratoderma-ichthyosis-deafness syndrome (MIM: 620009),

demonstrating liver phenotypes across all of them. The p.Leu403Phe was not listed in the ClinVar. It was predicted as damaging by CADD, MutationTaster, PolyPhen-2 and SIFT (Figure 3.2); however, classified as VUS based on ACMG-AMP guidelines (Table 3.2). Nonetheless, none of these three genetic variants of uncertain significance matched with the patient's clinical phenotype (Table 3.3).



C.

	JAG1:p.N108	PCK2:p.G215	VPS33B:p.L403
H.sapiens	IGGNTF**N**LKASRG	KCLHSV**G**QPLTGQ	LRLMCL**L**SITENG
P.troglodytes	IGGNTF**N**LKASRG	KCLHSV**G**QPLTGQ	LRLMCL**L**SITENG
M.mulatta	IGGNTF**N**LKASRG	KCLHSV**G**QPLTGQ	LRLMCL**L**SITENG
B.taurus	IGGNTF**D**LKASRG	KCLHSV**G**QPLTGQ	LRLMCL**L**SITENG
M.musculus	IGGNTF**N**LKASRG	KCLHSV**G**QPLTGH	LRLMCL**L**SITENG
R.norvegicus	IGGNTF**N**LKASRG	KCLHSV**G**QPLTGH	LRLMCL**L**SITENG
C.lupus	LGGNTF**N**LKAGRG	KCLHSV**G**QPLTGQ	LRLMCL**L**SITENG
	:*****:***.**	***********:	**********

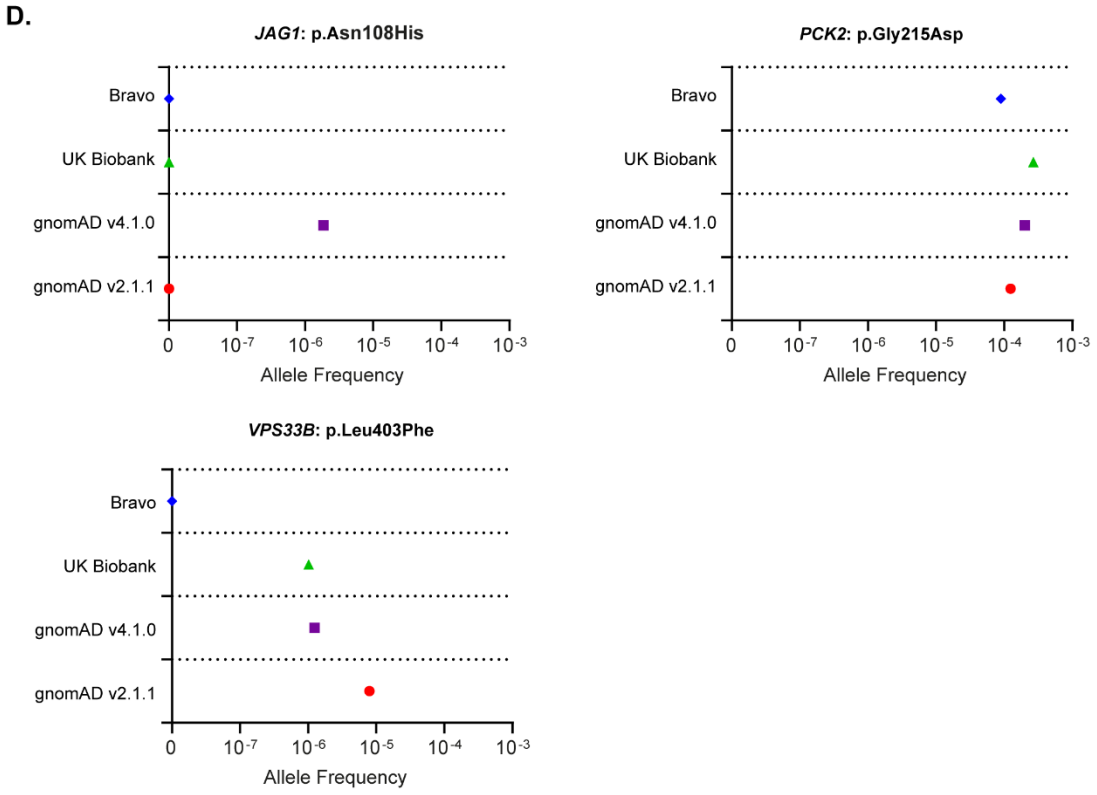


Figure 3.8 Heterozygous p.Asn108His in *JAG1*, homozygous p.Gly215Asp in *PCK2*, and homozygous p.Leu403Phe in *VPS33B* in P6 **(A)** Pedigree of the Family 6 affected by rELT. The patient is shown in black, whereas healthy individuals are shown in white. Mutation (NM_000214.3: c.322A>C: p.Asn108His, NM_004563.4: c.644G>A: p.Gly215Asp, NM_018668.5: c.1209G>T: p.Leu403Phe) statuses are indicated in red. WT, wild type, M, mutation. **(B)** Sanger confirmation of variants identified in P6. **(C)** Conservation of p.N108 in *JAG1*, p.G215 in *PCK2* and p.L403 in *VPS33B* residues across different species. Asterisk (*), colon (:), and period (.) indicate fully conserved, strongly similar, and weakly similar sites, respectively. Source: Clustal Omega **(D)** Graph showing the allele frequencies of three variants identified in in Bravo (blue diamond), UK Biobank (green triangle), gnomAD v4.1.0 (purple square), and gnomAD v2.1.1 (red circle). Adapted from Lülecioğlu AA et al., 2024[175]

CHAPTER 4

Discussion

Liver diseases are a broad spectrum of diseases, which can affect the life quality of affected individuals and be life-threatening. They become a significantly growing health concern worldwide, contributing to increased morbidity and mortality rates in all age groups and accounting for 1 in every 25 deaths globally[161]. The exact incidence of pediatric liver diseases is unknown, but it is estimated that 1 in every 2,500 infants suffer from a liver disease[197]. In 2021, pediatric cases accounted for 13% of liver transplantations performed in USA[198]. Underlying causes of liver injury are various, including metabolic conditions, genetic factors, viruses, autoimmunity, drugs and toxins, and other culprits. Identifying the definitive etiology is crucial for understanding the pathophysiology of liver disease and its effective clinical management. However, liver injury of indeterminate etiology still remains a daunting problem in pediatric hepatology. Despite recent advancements and comprehensive diagnostic strategies, almost half of the ALF cases still lack an identified etiology[62]. Recent studies demonstrated that a significant proportion of liver diseases with unknown causes have underlying hitherto inherited diseases[122,149,154–157,199].

In this study, we investigated 20 patients with pediatric-onset unexplained liver injury: 10 with rELT and 10 with ALF. We hypothesized that inborn monogenic defects could account for idiopathic liver injury in otherwise healthy children. We performed WES on gDNA isolated from peripheral blood samples of recruited patients. We searched for candidate disease-causing variants in a liver panel consisting of 380 genes, which we manually curated from the OMIM database. We identified rare nonsynonymous

variants in nine different genes in six unrelated patients, among whom five were diagnosed with rELT and one had ALF. We could establish a genetic diagnosis in 40% of patients with rELT by assessing the causal concordance between the gene mutated and the clinical symptoms of the affected patient.

We discovered a novel homozygous missense mutation (p. Arg225Gln) in *ACOX2* in P1. Although another missense mutation at the same amino acid residue was shown to be pathogenic, the p. Arg225Gln has not been previously associated with any disease. Clinical presentation of P1, including rELT, low to normal GGT levels, and positive response to UDCA treatment, indicated bile acid synthesis defects likely caused by the *ACOX2*:p.Arg225Gly variant. P2 harbored two homozygous variants, *PYGL*: p.Glu394* and *SLC27A5*:p.Thr308Met. Biallelic mutations in *PYGL* have been associated with Glycogen storage disease type VI (GSD6), and various nonsense variants of *PYGL* have been reported as pathogenic in the ClinVar. Patients with GSD6 have been reported to have ELT[179,200,201]. P2's clinical manifestations, which included hyperlipidemia, hepatosteatosis, and elevated liver enzymes, are consistent with those of GSD6, which is high likely attributed to the *PYGL*:p.Glu394* mutation. SLC27A5 has a role in the transportation and activation of long-chain fatty acids in the liver, lipid metabolism, and bile acid conjugation[181,202]. Mutations in *SLC27A5* have been associated with bile acid conjugation defect represented with elevated liver enzymes and increased serum levels of unconjugated bile acids[180]. Previously-reported p.His338Tyr variant in *SLC27A5* has been associated with bile acid defects in an individual. Although the pathogenic impact of *SLC27A5*:p.Thr308Met in P2 is unclear, both of these mutations could contribute to the rELT in P2. P3 had a hemizygous *PHKA2*:p.Arg186Cys variant, which has been previously-reported in the ClinVar as pathogenic and linked to glycogen storage disease. This makes *PHKA2*:p.Arg186Cys

is the most likely genetic cause underlying rELT in P3. We found a homozygous p.Ala984Thr in *ABCB4* in P4. Mutations in *ABCB4* have been associated with Gallbladder disease and progressive familial intrahepatic cholestasis type 3 (PFIC3). The p.Ala984Thr mutation was previously-reported at heterozygosity in a patient with PFIC3. P4's clinical presentation, including elevated GGT level, hepatosplenomegaly, and cholelithiasis, was consistent with PFIC3, indicating that homozygosity of the *ABCB4*:p.Ala984Thr can explain the clinical phenotypes of the patient. P5 had a homozygous missense variant (p.Arg649Trp) in *CDAN1*. Mutations in *CDAN1* have been linked to congenital dyserythropoietic anemia Ia (CDAIa), which can cause liver injury, in particular ALF. Although p.Arg649Trp and p.Arg397Trp variants have been previously-reported in a patient with CDAIa at compound heterozygosity, clinical symptoms of P5 were not concordant with the human CDAN1 deficiency. We found that P6 carried heterozygous p.Asn108His in *JAG1*, which has been associated with Alagille syndrome, homozygous p.Gly215Asp in *PCK2*, which has been linked to mitochondrial phosphoenolpyruvate carboxykinase deficiency, and homozygous p.Leu403Phe in *VPS33B*, which has been associated with arthrogryposis, renal dysfunction, and cholestasis, progressive familial intrahepatic cholestasis, and keratoderma-ichthyosis-deafness syndrome. Inherited defects of all these three genes in humans have liver hepatobiliary involvement; however, all three mutations were classified as VUS, and the phenotype of P6 was not consistent with any of the clinical conditions related to these mutated genes.

Our findings support the hypothesis that inborn errors may underlie pediatric-onset idiopathic liver injury, at least in some children. Our findings underscore the importance of utilizing WES for molecular diagnosis of pediatric liver diseases of unknown origin in clinical settings. It enables early diagnosis and treatment, especially

in patients who present with nonspecific findings such as hypertransaminasemia. Indeterminate liver injury still remains as an important concern in pediatric hepatology, as the lack of a causative etiology complicates the establishment of a definitive clinical diagnosis and management strategy. Various factors contribute to this diagnostic challenge. Firstly, some microbial infections causing liver injury cannot be detected and diagnosed until the severe clinical manifestations become evident[61]. For instance, patients infected with viruses, such as cytomegalovirus, herpes simplex virus, and adenovirus, can remain asymptomatic or exhibit mild and non-specific symptoms upon infection, such as loss of appetite, muscle and joint pain, fever, and fatigue[203]. These viruses are not routinely screened for in many hepatology clinics, although they are known to cause hepatitis and even lead to liver failure[204–206]. Additionally, a very small proportion of children infected with hepatitis viruses, particularly HAV and HBV, can develop fulminant viral hepatitis (FVH), which leads to life-threatening ALF[207]. Recent studies show that inborn errors of immunity predispose FVH[207–210]. For instance, Interleukin-18 binding protein (IL-18BP) deficiency in a child with previously no known liver disease led to uncontrolled IL-18 activity and immune-mediated hepatotoxicity upon HAV infection, resulting in fulminant hepatic failure[207]. Moreover, Interleukin-10 receptor subunit beta (IL-10RB) deficiency leads to the disruption of IL-10 response pathway. IL-10 is an antagonist of IFN-γ in phagocytes[210]. Excessive IFN-γ activity during HAV infections in the liver may underlie the molecular basis of FVH in patients with IL-18BP or IL-10RB deficiency[207,210]. Thus, identifying the genetic culprits underlying viral hepatitis contributes to a better understanding of the disease mechanism and management of the disease. Another reason for the diagnostic challenge for idiopathic liver injury might be due to novel liver diseases with unknown clinical characteristics and unidentified

etiologies[61]. In the recent years, advancements in genomics and human genetics have helped to discover novel liver diseases[158,211]. Additionally, some liver diseases can also have polygenic causes. Genome-wide association studies (GWAS), which require thousands of patients, can identify common variants and increased risk factors for liver diseases[61,212]. Chen et al conducted a meta-analysis of GWAS of serum liver enzymes using UK BioBank and BioBank Japan, identifying 160 novel ALT, 190 AST, and 199 ALP genome-wide significant associations[213]. Additionally, there are several GWAS that lead to the identification of genetic risk variants for fatty liver diseases, especially in *PNPLA3* and *TM6SF2*[213–217]. Also, NGS technologies have enabled the discovery of new infectious agents, including both known and novel viruses[218]. Viral transcripts can be identified at frequencies lower than 1 in 1,000,000[219]. It is used to determine the genetic sequences of viruses, enabling the identification of the viruses in the clinical sample and characterization of the viruses identified[218]. It is also effective in detecting various strains and mutations in the viruses, such as hepatitis B and C[68,218,220,221]. NGS enhances our understanding of viral evolution, disease epidemiology, and surveillance[222,223]. Sequencing the viral genome provides a comprehensive understanding of virus types that can cause liver injury and lead to the development of effective antiviral treatments. Thus, it can reveal novel causes for unexplained liver diseases, especially viral infections.

The use of WES in a clinical context offers numerous advantages for both the patients and clinicians. Most importantly, it allows for the early and definitive diagnosis of liver injury with unknown causes. Early diagnosis is particularly crucial in ALF cases as it is a rapidly progressive disease that requires timely management and decision-making regarding treatment options, in particular, liver transplantation. Identifying the genetic lesion underlying ALF can facilitate disease-specific targeted therapies and decrease

the rates of comorbidity and mortality in affected individuals. Zheng et al showed that establishing a diagnosis of monogenic liver disease changed the treatment of patients, highlighting the significance of early and definitive diagnosis in clinical decision-making[147]. Furthermore, uncovering the underlying genetic mechanism of hypertransaminasemia can enable early detection of liver injury and treatment to prevent further tissue damage. In addition, patients' genetic information can be used to create personalized therapies based on which molecules or pathways are defective. Drug selection or targeted therapies can be more effectively used for these patients. Another benefit of utilizing WES in clinical settings is that identifying the genetic cause of the liver injury can provide a more accurate prediction of disease progression and outcome, which is particularly crucial during the donor selection for liver transplantation. Several monogenic liver diseases, including inherited cholestatic disorders and alpha-1 antitrypsin deficiency, can be treated by liver transplantation. Thus, early diagnosis of monogenic liver diseases using WES can facilitate timely decision-making for liver transplantation[123]. Finally, discovering disease-causing inborn defects in patients can provide genetic counseling for the family, facilitating carrier screening and evaluation of genetic risks for future generations. Dissecting genetic disposition in family members, who are asymptomatic at the time of genetic testing, can suggest prophylactic treatments to be started before the onset of symptoms, thus impeding disease progression. Additionally, from a broader perspective, discovering novel candidate genes and morbid variants can enhance our understanding of underlying molecular mechanisms in liver diseases, leading to the discovery of new biomarkers that can be used for early diagnosis of liver disorders and the development of targeted therapies. It will also provide new evidences for genotype-phenotype correlations. Overall, the implementation of WES into practice in hepatology can

improve clinical management, positively affecting the disease outcome and life quality of patients. These discoveries can widen the genetic and phenotypic spectrum of inherited liver diseases and enhance our current understanding of their pathogenesis.

By performing a biased WES analysis of 20 individuals with pediatric-onset idiopathic liver injury, we could establish a genetic diagnosis in four patients with rELT, but there were no candidate pathogenic variants in genes predisposing to diseases with liver involvement in the other 16 patients. This can be explained by several reasons. First, we focused solely on exonic mutations. WGS could be performed, providing an increased yield of genetic diagnosis as it covers up to 98% of the whole human genome[224]. WGS allows the identification of the variants in both coding and non-coding regions of the genome, including exons, introns, regulatory regions, and intergenic regions. Also, it can detect structural variants (such as large insertions and deletions, duplications, inversions, and translocations)[225,226]. A significant proportion of patients with unexplained diseases have been shown to have disease-causing variants in non-coding regions of their genome[227]. Mutations affecting regulatory elements, such as enhancers and insulators, can lead to congenital disorders changing the gene expression[227–229]. Also, structural variations and variants in chromatin boundary elements can disturb the three-dimensional structure of chromatin and normal folding, leading to pathological conditions[227,229]. Exploring the potential disease-causing variants in these non-coding regions could identify novel mutations and uncover the roles of regulatory elements in the hepatobiliary system. Thus, utilizing WGS could offer a more comprehensive understanding of how regulatory elements in genes related to liver phenotypes contribute to disease onset and progression. Additionally, the patients for whom an underlying genetic defect was not identified in liver panel genes could harbor variants in novel genes that are not yet

associated with a liver disease. Our manually curated liver panel consists of genes, which have been previously associated with a hepatobiliary involvement and/or an abnormal laboratory finding of liver enzymes in the OMIM database. A recent comprehensive study utilizing WES in a total of 260 children with indeterminate ALF revealed a genetic diagnosis in ~37% of the cases, corresponding to 36 different previously known disease-causing genes. Of note, six of these 36 genes, which are *MRPS5*, *SUCLG1*, *AP4M1*, *CACNA1E*, *NSD1*, and STAT3, were not included in our panel of 380 genes. However, there were not any candidate morbid variations in those six genes in ALF patients identified in our study. Manually curated gene panels should be regularly updated for clinical use, as recent genetic discoveries result in the addition of new liver-related genes to such databases. It is estimated that approximately 250 new disease-causing genes are reported each year[230]. Therefore, re-analyzing the WES data of patients without a genetic diagnosis by using an updated liver gene panel can be performed to search for defects in newly discovered morbid genes. Besides utilizing a liver panel, unbiased WES analysis could be conducted to identify potential disease-causing variants across all genes in the genome. Variants identified in genes whose function is not known or well-characterized or in genes that are not associated with any human disease could be investigated. Approximately 75% of human genes have not been associated with a human phenotype[231]. Thus, often, re-evaluation of the rare variants in the patients following an unbiased approach may increase the diagnostic yield as new information arises regarding gene functions. In addition to the genetics, epigenetic, transcriptomic, metabolomic, and proteomic analyses can be integrated to further dissect underlying etiologies. Another factor that should be considered is that WES on gDNA isolated from whole peripheral blood may not help to detect mosaic genetic disorders. Genetic mosaicism is a condition, in which a single individual,

arising from a single zygote, harbors two or more genetically different cell lineages[232]. Hereditary tyrosinemia type I (HTI), characterized by fumarylacetoacetate hydrolase (FAH) deficiency, is an example of mosaicism in the liver, where some hepatocytes have the wild-type *FAH* allele while others harbor mutated *FAH* allele[233]. Furthermore, there might be cases, in which peripheral blood cells of patients possess wild-type alleles but their hepatocytes have the mutated allele, which can be explained by tissue-specific mosaicism. Thus, genetic diagnosis could differ depending on the cell or tissue types, from which gDNA is isolated. Also, we focused solely on germline mutations in our pediatric patient cohort. However, it should be noted that somatic mutations are also implicated in liver pathogenesis in adults, including CLD and hepatocellular carcinoma, highlighting the importance of somatic mutations in the genetic diagnosis of the patients[234–236]. Finally, there are non-genetic factors contributing to rELT and ALF in both children and adults, such as environmental factors, including poor dietary habits, obesity and environmental toxins, drugs, alcohol, and smoking[237,238]. They can also contribute to liver injury regardless of the genetic background of the individuals.

Our cohort consists of the patients recruited in Turkey, representing the first study in Turkey employing WES for genetic investigation of childhood-onset idiopathic liver injury. Despite the relatively small number of patients in our cohort, we identified two novel mutations in *ACOX2* and *PYGL*, expanding the genetic spectrum of clinical symptoms associated with these genes. Including more patients could reveal more novel mutations, expanding our understanding of the genetics of liver diseases. Additionally, recruiting more patients from different ethnic backgrounds can demonstrate the genetic diversity in broader and more diverse populations. In our study, WES analysis was not performed as a trio design, which involves sequencing both the affected children and their parents in all patients. Trio design could allow the

detection of all true genetic variations, including de novo and compound heterozygote mutations. Nevertheless, our overall genetic diagnostic yield was comparable to the previous similar studies and supported the clinical utility of WES for the diagnosis of idiopathic liver diseases[122,147,149,154,156,157,199].

CHAPTER 5

Conclusion and Future Perspectives

In this study, we performed WES analysis on the gDNA from 20 patients with pediatric-onset idiopathic rELT or indeterminate ALF. We aimed to identify the disease-causing variants underlying liver injury in these otherwise healthy individuals. We utilized a manually curated liver gene panel consisting of 380 genes linked to a hepatobiliary phenotype in the OMIM database and searched for rare variants in these genes. In conclusion, we identified nine rare nonsynonymous variants in six patients, two of which were novel mutations in *ACOX2* and *PYGL*, and two of them were previously reported variants in *ABCB4* and *PHKA2*. We also identified five rare variants of uncertain clinical significance in *CDAN1*, *JAG1*, *PCK2*, *SLC27A5*, or *VPS33B* in our patients. Our findings support that utilizing WES in clinical settings can increase the diagnosis yield, facilitate precision medicine and accurate management in pediatric cases of indeterminate ALF and rELT. Further research can focus on conducting WGS in larger and more diverse cohorts of patients with idiopathic liver injury. WGS analysis could reveal potential disease-causing variants in non-coding regions of the genome and structural variants as well. Performing trio designs for all patients could enable the discovery of all true genetic variants. In particular, an unbiased approach can be utilized to identify novel disease-causing variants across the whole genome. Furthermore, digenic and multigenic inheritance patterns can be investigated to establish genetic diagnosis in patients. Noteworthy, the integration of multi-omics approaches, such as transcriptomics and metabolomics, can further help in the dissection of disease-causing factors. Functional studies should also

be conducted to validate the pathogenicity of novel variants in unknown genes, enabling the development of targeted therapies and providing insights into the role of genes with unknown functions. Overall, NGS can provide pediatric hepatologists with early identification of the disease etiology, efficient medical intervention, and timely decision for liver transplantation when needed.

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Order Date	17-Jul-2024	Type of Use	Republish in a thesis/dissertation
Order License ID	1505988-1		
ISSN	1582-4934	Publisher	JOURNAL OF CELLULAR AND MOLECULAR MEDICINE
		Portion	Chapter/article

LICENSED CONTENT

Publication Title	Journal of Cellular and Molecular Medicine	Publication Type	e-Journal
		Start Page	n/a
Article Title	Whole-exome sequencing for genetic diagnosis of idiopathic liver injury in children	End Page	n/a
		Issue	11
		Volume	28
Date	01/01/2001	URL	http://www.jcmm.org
Language	English		
Country	Romania		
Rightsholder	John Wiley & Sons - Books		

REQUEST DETAILS

Portion Type	Chapter/article	Rights Requested	Main product
Page Range(s)	1-9	Distribution	Worldwide
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Lifetime Unit Quantity	Up to 499		

NEW WORK DETAILS

Title	GENETIC INVESTIGATION OF IDIOPATHIC LIVER INJURY IN CHILDREN BY WHOLE EXOME SEQUENCING	Institution Name	Bilkent University
		Expected Presentation Date	2024-07-30
Instructor Name	Aysima Atılgan Lülecioğlu		

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REQUESTED CONTENT DETAILS

Title, Description or Numeric Reference of the Portion(s)	whole article	Title of the Article / Chapter the Portion Is From	Whole-exome sequencing for genetic diagnosis of idiopathic liver injury in children
Editor of Portion(s)	Lülecioğlu, Aysima Atılgan; Yazıcı, Yılmaz Yücehan; Baran, Alperen; Warasnhe, Khaled; Beyaz, Şengül; Aytekin, Caner; Özçay, Figen; Aydemir, Yusuf; Barış, Zeren; Belkaya, Serkan	Author of Portion(s)	Lülecioğlu, Aysima Atılgan; Yazıcı, Yılmaz Yücehan; Baran, Alperen; Warasnhe, Khaled; Beyaz, Şengül; Aytekin, Caner; Özçay, Figen; Aydemir, Yusuf; Barış, Zeren; Belkaya, Serkan
Volume / Edition	28		
Page or Page Range of Portion	n/a-n/a	Publication Date of Portion	2024-06-12

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Last updated October 2022