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GENETIC CHARACTERISTICS OF PATIENTS WITH LIVER FIBROSIS AND CIRRHOSIS

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CONTENTS

ABBREVATIONS

HH – hereditary hemochromatosis
SNP – single nucleotide polymorphism
HCV – hepatitis C virus
HBV – hepatitis B virus
NAFLD – non-alcoholic fatty liver disease
ALD – alcoholic liver disease
GWAS – genome-wide association studies
gDNA – genomic DNA
C – concentration
PCR – polymerase chain reaction
RT-PCR – real-time Polymerase chain reaction
OR – odds ratio
CI – confidence interval
NTC – no template controls
RFLP – restriction Fragment Length Polymorphism
aORs – adjusted odds ratios
CI – confidence intervals
PNPLA3 – patatin-like phospholipase domain containing3
ALC – alcoholic liver cirrhosis

INTRODUCTION

Liver diseases are the most common cause of health loss and death worldwide and rates of liver diseases have increased over the past years [1]. The major causes of chronic liver injury include hepatitis C virus (HCV) and hepatitis B virus (HBV) infections, alcohol, non-alcoholic fatty liver disease (NAFLD), autoimmune hepatitis and other rare conditions [2]. Changes in lifestyle predispose the increasing number of patients with alcoholic liver disease (ALD) and NAFLD. According to World Gastroenterology Organisation, prevalence of NAFLD had doubled over the last decades and become a new and major health problem worldwide [3]. Regardless of the cause, chronic inflamation leads to liver fibrosis wich untreated can progress to liver cirrhosis, organ failure and death. Liver cirrhosis is the common end stage of chronic liver diseases which significantly reduces quality of life [4, 5] and is responsible for approximately 2% of all deaths worldwide[6]. The natural course of liver diseases varies considerably between individual patients [2].This inter-individual variability might be related to different confounding factors, including clinical environmental, and host factors [7]. Classical risk factors for progression of liver injury include age, gender, alcohol consumption, and obesity; however, they cannot explain all clinically evident differences observed in patients [8]. Advent of new genomic technologies and decreased costs of genotyping have been followed by multiple studies that point to the importance of genetic predisposition in different liver diseases [9]. The first genetic detection identified the major loci for Mendelian diseases such as haemochromatosis gene *HFE* [9]. C282Y and H63D mutations in *HFE* gene are now recognized as the most common genetic causes in populations of European ancestry. Carriage of *HFE* gene mutations has been linked with increased risk of liver fibrosis or liver cirrhosis; however, published studies report conflicting results [10].Increased contents of iron have been attributed to progression of liver cirrhosis caused by chronic viral hepatitis C infection [11], non-alcoholic fatty liver disease [12] or alcoholic liver disease [13]. A large scale genome wide association study (GWAS) indentified that several single nucleotide polymorphisms (SNPs) to be associated with chronic liver diseases. Patatin-like phospholipase domain containing3 (*PNPLA3*) gene polymorphism rs738409 was associated with non-alcoholic fatty liver disease[14], liver fibrosis progression rate in HCV-infected patients [15] and contributed to the development of alcoholic liver cirrhosis [16]. *RNF7*, also known as sensitive to apoptosis gene *SAG* and proto-oncogene tyrosine-protein kinase MER *(MERTK)*, might influence the development of fibrosis

in chronic hepatitis C [17]. A meta-analysis of five GWAS results revealed association between in the proprotein convertase 7 (*PCSK7*) gene and iron overload, indicated by the serum levels of ferritin and soluble transferrin receptor (sTfR) [18].

The aim of the study

To investigate a role of mutations in *HFE* gene and genetic variants in *PNPLA3, RNF7, MERTK* , *PCSK7* genes in patients with liver fibrosis and liver cirrhosis.

The objectives of the study

1. To determine the association between *HFE* gene C282Y and H63D mutations and liver cirrhosis.
2. To determine association between gender and *HFE* gene C282Y and H63D mutations in liver cirrhosis patients.
3. To investigate associations between key *PNPLA3, RNF7, MERTK* and *PCSK7* gene polymorphisms and liver fibrosis.
4. To investigate associations between key *PNPLA3, RNF7, MERTK* and *PCSK7* gene polymorphisms and liver cirrhosis.
5. To determine association of *PNPLA3, RNF7, MERTK* and *PCSK7* gene polymorphisms with hepatitis C virus – induced and non-hepatitic C virus – induced liver cirrhosis and fibrosis.

Novelty of the study

In the past two decades, our knowledge about the genetic determinants of liver diseases has expanded rapidly [19]. As mentioned before liver cirrhosis is caused by different etiological factors; however, progression of liver injury varies considerably among individuals independently of the cause [20]. Different research groups over the last decade have attempted to identify crucial co-factors that contribute to the development of liver damage [21, 22]. Growing number of studies show that apart from the main underlying causative agents in liver cirrhosis, the process maybe reinforced by confounding factors such as diet, alcohol consumption, etc. [23–25]. Inter-individual variation of time span from normal liver to fibrotic and cirrhotic stages suggested potential influence of congenital variations. Advances in genotyping techniques allowed to identify coexisting genetic alterations associated with liver fibrosis [23] and cirrhosis of different etiologies [25, 26]. To date, several gene polymorphisms have been linked

with the progression of liver fibrosis and the development of liver cirrhosis[9]. *HFE*-linked hereditary hemochromatosis (HH) predisposes disease progression to cirrhosis; however, the role of heterozygous C282Y or H63D mutations in the development of cirrhosis in the presence of other etiological factors is still debated. The first part of our study was to determine the association between *HFE* gene C282Y and H63D mutations and liver cirrhosis in Lithuanian population. This study is the first which assesses the prevalence of *HFE* gene mutations in Lithuanian cirrhotic patients and adds additional insights on the impact of *HFE* mutations in development of liver cirrhosis. The second part of our study was dedicated to evaluate the association between single nucleotide polymorphisms (SNPs) in *MERTK* (rs4374383), *PCSK7* (rs236918), *PNPLA3* (rs738409), and *RNF7* (rs16851720) genes and liver fibrosis and cirrhosis. This is the first study which demonstrates that variant in *PNPLA3* (rs738409) gene is associated with liver fibrosis and cirrhosis in an Lithuanian population. To our knowledge, replication analysis of the association between SNP in *RNF7* rs16851720 and liver cirrhosis has also not been reported previously. Furthermore, *MERTK* (rs4374383) and *PCSK7* (rs236918) genetic variant replication studies in patients with liver diseases are scarce.

1. REVIEW OF LITERATURE

1.1. Liver fibrosis and cirrhosis pathway

Liver is made up of: primary parenchymal know as hepatocytes and nonparenchymal cells: hepatic stellate cells (HSCs), Kupffer cells (KCs), and liver sinusoidal endothelial cells (LSECs), forming the walls of liver hepatic sinusoids. In the past decade, our knowledge about the cellular and molecular mechanisms of liver fibrogenesis has advanced. Determinant event in the development of fibrosis and cirrhosis is activation of hepatic stellate cells [27]. In the initiation and progression of liver fibrosis and cirrhosis are involved not only hepatic cells but also many kinds of cytokines (PDGF, TGF-β, TNF-α, interferon, interleukins) and microRNAs [28]. Liver fibrosis is a reversible, dynamic process. Changes in liver architecture during acute injury are transient and reversible whereas in chronic injury are slowly progressive to cirrhosis. Distortion of liver parenchyma and vascular architecture leads to liver failure and death [29]. The most common causes of chronic liver injury are HCV and HBV infections, alcohol, NAFLD, autoimmune hepatitis other rare conditions including inherited diseases such as hemochromatosis and Wilson's disease, primary biliary cirrhosis, primary sclerosing cholangitis [2]. All causes of chronic liver disease may lead to liver fibrosis wich is the precursor of cirrhosis [27,29]. Progression to liver cirrhosis typically is slow, depends on interaction of environmental and genetic factors and varies considerably among individuals[27]. Determination of causative genetic factors may help identify the patients having the highest risk for rapid progression of chronic liver diseases. A recent GWAS identified several gene polymorphisms to be linked with the progression of liver fibrosis and the development of liver cirrhosis [30].

1.2. Genetic predisposition in different liver diseases

In the past decade our knowlegde about genetic background of liver diseases has advanced. Commonly liver diseases are considered to be multifactorial and determined by interaction between genetic and different environmental factors [31]. However, a small portion of rare liver diseases are monogenic. The first liver disease associated genes were identified in monogenic liver disorders, i.e. Wilson's disease (*ATP7B*) and the Haemochromatosis (*HFE*) [9, 31]. Currently, the screening of the known mutations in the disease associated genes are accomplished on the rutine bases in most clinical laboratories. However, in case the patients doesn't carry any of the

known disease associated mutations, gene sequencing is recommended. The development of new genetic methods and technologies has led to multiple new genetic studies that indicated the importance of genetic predisposition in different liver diseases [9]. Currently applied genetic methods in the discovery of the liver diseases associated genes include GWAS, exome sequencing, whole genome sequencing. The aim of GWAS is to determine unknown genetic risk factors contributing to disease susceptibility and progression [19]. GWAS compares frequencies of single nucleotide polymorphisms (frequencies > 1%) between cases and controls [19, 32]. In order to perform statistically powerful GWAS large study and independent replication groups are required [33]. The first GWAS of the hepatobiliary diseases was performed in 2007 and indicated the genetic risk factors for gallstones [31]. Subsequent GWAS have identified genetic assotiations with fatty liver disease, viral hepatitis, chronic cholestatic liver diseases or drug-induced liver injury [31]. Recent GWAS have reported several gene polymorphisms including rs16851720 (*RNF7*), rs9380516 (*TULP1*), rs4374383 (*MERTK*), rs9976971 (*IFNGR2*) and rs8099917 (*IFNL3*) to be linked with the development of fibrosis in chronic HCV disease [34]. Variant in *PNPLA3* (rs738409) has been linked with the development of liver fibrosis and cirrhosis in NAFLD, ALD, chronic HCV infection. Moreover, genetic loci asocciated with iron metabolism, such as transferin (*TF*) transmembrane serine protease 6 (*TMPRSS6) TFR2* and *PCSK7* [35–37] were indetified.

1.2.2. *HFE* gene and liver diseases

Human hereditary hemochromatosis gene was discovered by Feder JN et al. in 1996 [38]. This gene is located on the short arm of chromosome 6,. It contains 7 exons spanning 12 kb and encodes the 343-amino acid glycoprotein – HFE protein [31,38]. *HFE* gene has more than 30 known polymorhisms. The most important of them are three: C282Y (rs1800562), H63D (rs1799945) and S65C (rs1800730).

1.2.2.1. *HFE* mutations

Discovery of *HFE* gene has helped identify hereditary haemochromatosis (HH) associated mutations: c.845G > A (p.Cys282Tyr, C282Y, rs1800562) c.187C > G (p.His63Asp, H63D, rs1799945). C282Y and H63D are the most common mutations causing HH in Caucasians [39]. The prevalence of *HFE* C282Y mutations varies significantly across Europe, with highest estimated frequencies in Ireland (>10%), intermediate frequencies

(2.7%–7%) in Latvia [40] and Poland [41], and very low rates of (0%–2%) in Mediterranean areas [42]. *HFE* H63D mutation also occurs at different frequencies in separate regions. Carriage of heterozygous (*HFE*) gene mutations has been attributed as the risk factor for iron overload and liver damage, but equivocal conclusion on the role of these mutations has not been achieved [10, 43]. The rationale that suggested iron as a susceptible hepatotoxic factor is based on the ability of this metal to induce oxidative stress by stimulating free radical formation in liver tissue [12, 44]. The presence of the C282Y mutation has been associated with more advanced degrees of fibrosis or cirrhosis [11, 45], but these findings were not confirmed in other studies [12, 46].

1.2.2.2. *HFE* hemochromatosis

According to meta-analysis by *Ellervik* et al., homozygosity for the C282Y mutation is associated with an increased risk (4–11-fold) of liver disease [47]. Homozygosity is responsible for 82% to 90% and compound heterozygosity (i.e.C282Y/H63D) for 4% for hemochromatosis cases in Europeans [48, 49]. These mutations have low penetration and vary widely from 1% to 28% [50]. Mutations alone cannot be responsible for the development of HH and environmental factors such as gender, alcohol consumption, physiological or pathological blood losses and additional genetic factors play important role in the progression liver injury in symptomatic haemochromatosis [50]. Symptomatic haemochromatosis in homozygotes C282Y male occurs more often than in female as they have protective regular iron loss during menstruation [50]. Alcohol and iron has cumulative effects. *Fletcher* and colleagues described that more than 60g. of alcohol per day is associated with higher levels of ferritin in C282Y homozygotes and leads to progression of liver cihhrosis [51]. Hepatic steatosis has often been linked to metabolic syndrome, alcohol abuse, co-existent viral C hepatitis infection are also an important cofactors in liver injury in HH [50]. HH is characterized by elevated serum ferritin and transferrin saturation and progressive iron overload in the liver, heart and endocrine glands [52]. Affected individuals have a high risk of different complications such as: liver cirrhosis, cardiomyopathy, diabetes, arthritis, and skin pigmentation, that are completely preventable with phlebotomy [53]. There are four type of haemochromatosis:

1. **Type1:** *HFE*-related-haemochromatosis.

Type1a: C282Y homozygosity.

Type1b: compound C282Y / H63D heterozygosity.

Type1c: other *HFE* mutations, e.g. Ser65Cys, etc. These mutations do not substantially affect the phenotype.

2. **Type2**: Non-HFE-related – haemochromatosis.

Type2a: haemojuvelin mutations related juvenile haemochromatosis.

Type2b: hepcidin mutations related juvenile haemochromatosis.

3. **Type3**: TFR2 transferrin receptor2 (*TFR2*) related hemochromatosis.
4. **Type4**: Ferroportin 1 gene related haemochromatosis.

Homozygotes for C282Y according to stage of liver injury can be divided into several groups:

1. Genetic predisposition without abnormalities.
2. Iron overload without symptoms.
3. Iron overload with symptoms.
4. Iron overload with organ damage.

Delayed diagnosis leads to decompensated liver cirrhosis, hepatocellular carcinoma (HCC) and death. Due to available testing for *HFE* mutations, HH is now diagnosed at an early stage and patients could be treated before the development of symptomatic disease.Typically hemochromatosis occurs in C282Y homozygotes adults. To develop clinical HH, homozigocity are necessary but not a sufficient condition. Other modifier genes are involved in the process and different study groups identify such modifier genes [24, 37, 50].

1.2.2.3. *HFE* mutations in other liver diseases

Carriage of heterozygous hemochromatosis gene mutations has been attributed as the risk factor for iron overload and liver damage. Increased contents of iron have been attributed to progression of liver cirrhosis caused by HCV infection [11], NAFLD [12] or ALD [13]. Individuals with chronic liver diseases may have mild to moderate iron overload but the mechanism is not fully understood [54]. Studies in different populations examining the relationship between *HFE* mutations and chronic liver diseases have produced varying outcomes. According to meta – analysis by *Qing Ye*, et al, *HFE* C282Y and *HFE* H63D may be linked to the risk of NAFLD, especially in the Caucasian population, [30] but significant difference could not be found between *HFE* C282Y and H63D and liver cirrhosis risk [30]. Study in non-European population has suggested that iron overload and *HFE* gene mutations do not play a primary role in cryptogenic cirrhosis in the south Iranian population[55]. A study from Italy including 587 patients with NAFLD and 184 control subjects did not find a link between *HFE* mutations and hepatocellular iron accumulation[12]. Australian study by

Osborne at al. has shown that homozygosity for Cys282 has the risk of colorectal and breast cancer [56]. Scottish study has shown that carriage of *HFE* mutations does not have any role in the accumulation of iron or the progression of liver disease in HCV infection [57]. *Tung and colleagues* found that the presence of *HFE* mutations was associated with iron loading and advanced fibrosis in patients with chronic HCV infection [58].

1.2.3. *PCSK7* gene in liver diseases

GWAS studies have identified genetic loci asocciated with iron metabolism (*TF, TMPRSS6, PCSK7, TFR2*) but their correlation with clinical manifestation is not clearly understood [24]. *Tayrac* and colleagues showed that rs3811647 polymorphism in the *TF* (transferrin) gene can affect iron metabolism through serum transferrin and iron levels in C282Y homozygotes. Transferrin receptor 2(*TFR2)* is the only gene in which mutations is associated with total iron levels and is the cause of hemochromatosis type 3 [50]. Association study performed by *Stickel* and colleagues analyzed association between *TF, TMPRSS6, PCSK7, TFR2* genes and advanced fibrosis and cirrhosis in *HFE* C282Y homozygotes and identified association only between SNP in *PCSK7* (rs236918) and risk for cirrhosis in homozygous Cys282 patients from Central-North Europe [24]. *Pelucchi* and colleagues observed that *PCSK7* rs236918 is asocciated with cihhrosis development in Italian *HFE* C282Y homozygotes [59]. Hypothesizing that this SNP might contribute to the development of liver damage in other chronic liver diseases, we genotyped *PCSK7* rs236918 in our cohort of patients with liver fibrosis and cirrhosis.

1.2.4. *PNPLA3* gene in liver diseases

As mentioned earlier, GWAS reported that several gene polymorphisms have been linked with the progression of liver fibrosis and the development of liver cirrhosis. One of them is patatin-like phospholipase domain containing 3 (*PNPL3*) gene polymorphism rs738409(M148I). The *PNPLA3* gene encodes transmembrane protein adiponutrin in huma which is highly expressed in the liver and adipose tissues. In humans, *PNPLA3*, a 481-residue protein, is observed in various tissues but is mostly expressed in the liver and the retina and is a major predictor of hepatic fat conten [60]. *PNPLA3* rs738409 is the first locus that has been strongly associated with steatosis, fibrosis and liver cirrhosis in various chronic liver diseases such as: NAFLD, ALD and HCV infection [61]. The exact mechanisms on how *PNPLA3* contributes to fibrogenesis are still under investigation [61]. It is

known that *PNPLA3* as lipase is responsible for triglycerides in hepatocytes and retinyl-palmitate hydrolysis in hepatic stellate cells in humans [62,63]. Increased intracellular synthesis of triglycerides in the liver has decreased hepatic secretion of very low-density lipoprotein whereas decreased lipolysis might lead to increased hepatic lipid accumulation in hepatocytes [63]. Studies have shown that *PNPLA3* I148M variant (rs738409) is relevant for the retinyl-palmitate content in human liver [62] and influences circulating retinol levels in adults [64]. Individuals with the *PNPLA3* rs738409 are predisposed to rapid progression of chronic liver diseases, betimes detection of individuals who are at-risk of severe liver demage is crucial to prevent the progression of liver disease.

1.2.4.1. *PNPLA3* gene in non-alcoholic liver disease

Non-alcoholic fatty liver disease (NAFLD) is a common cause of liver disease due to high prevelance of obesity, diabetes, metabolic sindrome worldwide [65]. Clinical manifestation varies between individuals from asymptomatic liver staetosis, which is benign condition in patients with NAFLD, to non-alcocholic steatohepatitis (NASH) which can lead to liver cirrhosis and deterioration of liver function and hepatocellular carcinoma [66]. In Lithuanian patients NAFLD cirrhosis is rare in comparison to the Western countries and the reasons for that are still not completely understood. GWAS has demonstrated that the *PNPLA3* gene polymorphism rs738409 is associated with NAFLD [67] and affects the activity of liver enzymes in plasma [68]. Results have been summarized in several meta-analysis by R. *Xu et al*. [69], *B. Salameh et al*, [70], *L. Zhang* et al. [71].

1.2.4.2. *PNPLA3* gene in alcoholic liver diseases

Alcoholic liver disease (ALD) is the leading cause of chronic liver disease in Europe [72]. ALD can range from fatty liver, acute alcoholic hepatitis, alcohol-induced hepatic fibrosis, and cirrhosis (ALC) [73]. Only 10%–20% heavy drinkers develop cirrhosis [74]. Interactions of genetic and environmental factors play important role in the risk of developing ALD in heavy drinkers. Several studies have reported an association between ALD, ALC and SNP in *PNPLA3* (rs738409) gene. The first GWAS in ALD reported that *PNPLA3* rs738409 was associated with advanced disease course [75]. According to *Chamorro et al.* meta-analysis *PNPLA3* is associated with ALC [76] and with shorter time to hepatic decompensation [77].

1.2.4.3. *PNPLA3* gene in Hepatitis C infection

It is known that prevalance of HCV infections is about three percent of the worldwide population [78]. Clinical progression and severity of liver disease depends on enviromental factors such as age, heavy alcohol consuption, HCV genotype 3, male gender and higher body mass index [79]. Some patients with chronic HCV infection may develop different stage liver fibrosis, but not all patients develop liver cirrhosis [80]. Enviromental factors allone cannot be responsible for the progression of liver damage, genetic factors are involved. According to *Jia-Hao Fan* et al meta-analysis genotype GG of *PNPLA3* rs738409 was associated with the risk of advanced liver fibrosis and steatosis in Caucasian patients [79]. Subgroup analysis by HCV genotype didn't show any significant differences. In order to confirm the asocciation between *PNPLA3* rs738409 polymorphism and HCV genotype in HCV disease progression, more studies are needed [79].

1.2.5. *RNF7, MERKT* genes in liver diseases

As mention befor GWAS identified to SNPs in *RNF* (rs16851720) and *MERTK* (rs4374383) genes which are associated with the development of liver fibrosis in chronic hepatitis C [80]. *RNF7* gene also known as sensitive to apoptosis gene (*SAG*). SNP rs16851720 is located in the first intron of *RNF7*. *RNF7* encodes an antioxidant that induced reactive oxygen species (ROS) and protects against apoptosis [81]. HCV inducts ROS production and leads to hepatocyte apoptosis and may lead to development of liver fibrosis.[82]

MERTK gene encodes enzyme proto-oncogene tyrosine-protein kinase MER. The initial association between *MERTK* rs4374383 and development of fibrosis in chronic HCV disease was observed in a GWAS among HCV patients who received blood transfusions [17]. In a later study the *MERTK* SNP was significantly associated with increased fibrosis progression rate in the Swiss Hepatitis C Cohort Study [15]. Replication studies on *MERTK* and *RNF7*is scare. So we genotyped *MERTK* rs4374383 and *RNF* rs16851720 SNPs in our cohort of patients with diferent etiology chronic liver fibrosis and cirrhosis.

Genetics is a rapidly advancing area of medicine. Molecular genetics, however, is never going to replace clinical medicine. Clinical symptoms and laboratory data are frequently overlapping, thus rendering a differential diagnosis difficult. A chronic liver involvement that can predispose a cirrhosis may be observed in a number of genetic diseases with a different penetrance, age at onset, and outcome. Disease-genes identification may

help to identify individuals with the highest risk of developing severe liver disease also who are at risk for rapid progression of chronic liver diseases to initiate timely treatment, before the development of symptomatic disease.

2. METHODS

2.1. Ethics

The study was approved by the Lithuanian Bioethics Committee (Protocol No. 2/2008) and Kaunas Regional Ethics Committee of Biomedical Surveys (Protocol No. BE-2-10). All patients and controls gaved their informed consent to take part in this study.

2.2. Design of the study

2.2.1. Patients

Our study included patients with chronic parenchymal liver disease regardless of aetiology, who were recruited at the Department of Gastroenterology, Hospital of Lithuanian University of Health Sciences, during the period 2012–2015. The study included consecutive patients with liver cirrhosis, patients with liver fibrosis and healthy control individuals. The study consists of two parts. In the first part of our study 209 patients with liver cirrhosis and 1005 controls (581 men and 423 women) were investigated to determine the association between *HFE* gene C282Y and H63D mutations and non- HH liver cirrhosis. In the second part of our study 317 patients with liver cirrhosis, 154 patients with liver fibrosis and 498 controls were used in order to determine the association between SNPs in *MERTK* (rs4374383), *PCSK7* (rs236918), *PNPLA3* (rs738409), *RNF7 (*rs16851720) genes and the risk for liver fibrosis and liver cirrhosis. Control groups consisted of voluntary, unrelated Lithuanian blood donors from the National Blood Center collected during the period 2008 to 2009. Genetic data of control samples for the fist part of the study was acquired from our previous study on the prevalence of *HFE* mutations in the Lithuanian population [83]. The diagnosis and etiology of chronic liver disease was confirmed by laboratory tests, clinical features, radiological imaging and liver biopsy. ALD was confirmed when daily consumption of alcohol was > 30g/20g/day for males/females, respectively, as confirmed by at least 1 family member of affected individual. The patients in liver fibrosis group underwent percutaneous liver biopsy and were included in the study if their stages of fibrosis from 1 to 3 were documented by histological evaluation using METAVIR score [84]. The patients in liver cirrhosis group underwent percutaneous or tranjugular liver biopsy. For patients who had significant coagulation disorders, diagnosis of liver cirrhosis was confirmed by laboratory tests, clinical features, and radiological imaging.

2.2.2. DNA extraction

After recruitment blood samples were collected in EDTA-containing vacutainer tubes and stored at –80°C until procedure. Genomic DNA (gDNA) from samples was isolated from whole blood mononuclear cells by using salting-out method. Samples were stored at −20°C until analysis.

Equipment:

1. Automatic pipettes “Eppendorf research” 100 µl – 1000 µl (Eppendorf AG, Germany).
2. Automatic dispenser “Ependorf Multipette plius” 500 µl – 10 ml (Eppendorf AG, Germany).
3. Centrifuge “Eppendorf Centrifuge 5424” (Eppendorf AG, Germany)
4. 4.Mikrocentrifuge “Eppendorf Centrifuge 5810R” (Eppendorf AG, Germany).
5. Moving, warming water bath “GFL 1083” (GFL, Germany).
6. Plastic centrifuge tubes with caps, 50 ml.
7. Eppendorf type tubes, 1.5 ml–2 ml.
8. Fume hood “ESCO AURSTREAM“ (Esco, Singapore).
9. Thimble 10 ul –100 ul, 100 ul –1000 ul.
10. Termomixer “Eppendorf Thermomixer comfort” (Eppendorf AG, Germany).
11. Mixer “IKA MS 3 basic” (IKA, USA).
12. Magnetic mixer MMS-3000 (Biosan, Latvia).
13. pH meter METTLER TOLEDO (FiveEasy, Germany).
14. Electronic scales KERN 440-35N (KERN, Germany).

Reagents:

1. Ammonium chloride (NH4Cl) M=53.49 g/mol (Carl Roth GmbH + Co,Germany).
2. Potassium bicarbonate (KHCO3) M=100.12 g/mol (Sigma-ALORICH Chemie GmbH, Germany).
3. Etilendiaminum tetraacetatatum (EDTA) (C10H16N2O8) M=292.25 g/mol (Carl Roth GmbH + Co,Germany).
4. Three-hydrogen chloride (Three HCl) (C4H11NO3HCl) M=157.6 g/mol (Carl Roth GmbH + Co, Germany).
5. Sodium chloride (NaCl) M=58.44 g/mol (Merck KGaA, Germany).
6. Proteinase K 934 u/ml, C=18.5 mg/ml (Fermentas, Lithuania).
7. 96% ir 70% ethanol (A.B. Stumbras, Lithania).
8. Sodium lauryl sulfate (SDS) (C12H25NaO4S) M=288.38 g/mol (AppliChem, Germany).

9. Three – EDTA buffer “Roti stoch” (Carl Roth GmbH + Co, Germany).
10. Caustic soda (NaOH) (Merck KGaA, Germany).
11. 99.9 % chloroform (Scharlau Chemie S.A., Spain).

DNA extraction Protocol:

Day – 1

1. The blood is cooled down in order to maintain as low temperature as possible (4°C). The cooled blood is transfused to the centrifugal test-tube of 50 ml volume with a cap.
2. 20 ml Lysis I buffer (composition: 155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4) is poured to the blood and the blood is incubated for 30 minutes at 4°C temperature.
3. The sample is centrifuged for 15 minutes at 4°C temperature using the 2500 g – 3000 g power.
4. The top layer is poured off carefully without getting the sediment mixed. 20 ml – 30 ml cold Lysis I buffer is poured on the remaining sediment. The centrifugation is repeated. Washing is repeated for 2–3 times or as many times as necessary in order for sediment and upper layer to lose red colour.
5. 6 ml of Lysis II buffer is poured to each test-tube containing sediment from white blood cells and cellular membranes. The mixture is well mixed to get it homogeneous and then 400 μl 10% SDS solution and 30 μl proteinase K solution are added.
6. The test-tube is covered, well mixed and incubated in moving water bath for 16 hours at 37°C temperature.

Day – 2

1. The samples are incubated for 1 h at 55°C temperature. 2 ml NaCl solution is added, well mixed and centrifuged at 3000 g. speed for 15 minutes in the centrifuge at 16°C temperature.
2. 2 ml chloroform is added to the sample in the laminar of vertical air flow and then mixed. The sample is centrifuged at 3000 g. speed for 20 minutes in the centrifuge at 16°C temperature.
3. The upper layer is transfered carefully to the clean 50 ml centrifugal test-tube. The upper layer is mixed with 20 ml of cold 96% isopropanol solution. The test-tube is rotated until DNA threads become evident.
4. The precipitated DNA is taken with the help of tip and moved to the 1.5 ml test-tube.
5. 1 ml of 70% ethanol solution is poured in order to rinse DNA. It is carefully shaken and centrifuged in the micro-centrifuge.

6. Ethanol is poured off carefully. The test-tube containing DNA sediment is carefully turned over, air dried until ethanol evaporated.
7. DNA is melted in 500 µl 1 × TE buffer.
8. DNA sample is kept in the refrigerator at 4°C temperature; it can be frozen for longer periods in the –20°C freezer.

2.2.3. Evaluation of DNA concentration and purity by spectrophotometer

Nucleic acids (DNA and RNA) absorb ultraviolet light at a wavelength of 260 nanometres (nm). The concentration of DNA can be determined by measuring the absorbance in a spectrophotometer. To identify the concentration of DNA (C), using the following calculation: CDNA = 50 µg/ml × D260 × dilution factor [85].

Equipment:

1. NanoDrop™ 2000 Spectrophotometers (Thermo Scientific™)
2. Vortex mixer "IKA MS 3 basic" (IKA, USA)
3. Automatic pipettes "Eppendorf research" 1 µl – 10 µl (Eppendorf AG, Germany),
4. Pipette tips 10 µl – 100 µl

Reagents:

1. 1 × TE buffer Roti stock (Carl Roth GmbH + Co, Germany)

Method:

1. DNA sample is vortexed and 2 µl are pipetted directly on to the pedestal of spectrophotometer (Fig. 2.2.3.1). The upper pedestal arm is lowered to form a liquid column and the measurement is done. The device's readings are recorded.

Fig. 2.2.3.1. *Evaluation of DNA concentration and purity by spectrophotometer „Nanodrop 2000"*
(Isogen Life Science, 2012)

2.2.4. Plate design

For genotyping, 93 DNA samples were arranged in a 96 wells plate layout. Patients with the same diagnosis were kept on the same plate. Three empty (no template controls) wells were used for quality control, to reveal potential contaminations. Each plate was labeled with a unique plate name for database storage (Table 2.2.4.1).

Table 2.2.4.1. *Plate layout Wells H3 A6 and H12 were used as negative controls*

	1	2	3	4	5	6
A	GAP0726	GAP0688	GAP0733	GAP0556	GAP0745	NTC
B	GAP0722	GAP0684	GAP0734	GAP0653	GAP142	GAP0467
C	GAP0723	GAP0683	GAP0004	GAP0419	GAP0470	GAP0516
D	GAP0721	GAP0667	GAP0005	GAP0566	GAP0471	GAP0515
E	GAP0716	GAP0729	GAP0024	GAP0565	GAP0472	GAP0512
F	GAP0714	GAP0732	GAP0046	GAP0577	GAP0481	GAP0510
G	GAP0711	GAP0730	GAP0056	GAP0690	GAP0464	GAP0509
H	GAP0705	GAP0731	NTC	GAP0735	GAP0468	GAP0508
	7	8	9	10	11	12
A	GAP0507	GAP0492	GAP0485	GAP0463	GAP0458	GAP0482
B	GAP0506	GAP0494	GAP0473	GAP0464	GAP0459	GAP0435
C	GAP0502	GAP0496	GAP0474	GAP0465	GAP0443	GAP0436
D	GAP0501	GAP0498	GAP0475	GAP0466	GAP0444	GAP0438
E	GAP0500	GAP0499	GAP0479	GAP0454	GAP0447	GAP0439
F	GAP0491	GAP0452	GAP0480	GAP0461	GAP0448	GAP0440
G	GAP0487	GAP0483	GAP0460	GAP0455	GAP0449	GAP0441
H	GAP0493	GAP0484	GAP0462	GAP0457	GAP0450	NTC

NTC – no template controls.

2.2.5. Genotyping

In this study two genotyping methods were used:

1. RFLP method – to determine *HFE* gene C282Y and H63D mutations.
2. TaqMan® method – for genotyping *PNPLA3* C>G (rs738409), *RNF7* A>C (rs16851720), *MERTK* A>G (rs4374383) and *PCSK7* C>G (rs236918).

2.2.5.1. PCR

The polymerase chain reaction (PCR) is necessary to copy DNA. The reaction consists of six stages: initiation, denaturation, hybridisation, extension, final extension, final hold. Three of them get repeated and the DNA amplification is carried out cyclically. Multiple cycles are required to amplify DNA. The sufficient amount of PCR product is received after 35 reactions and it is possible to analyze it. The reaction's stages are the following:

1. Initiation – During this stage the solution of PCR reaction consisting of PCR primers, DNA polymerase, nucleotides, 10× reaction buffer, and tested DNA is heated up to 94°C–96°C. This stage is necessary for denaturation of primary two-chain DNA and for activation of thermally stable DNA polymerase. The reaction's duration is 5 minutes.
2. Denaturation – During this stage the solution of PCR reaction is heated up to 94°C–98°C for 20–30 seconds. Two complementary DNA chains are separated during this reaction.
3. Hybridisation – When the temperature falls down to 50°C –65°C, the primers connect to the tested DNA for 20–40 seconds. The connections are stable only when the segments of primers and DNA are complementary.
4. Extension. The temperature is raised to 72°C and left for 1–2 minutes. It is an optimal temperature for DNA polymerase. It connects further nucleotides to the newly synthesized DNA coils.

Steps 2–4 are reapeated 35 times in order to multiply the gene region of interest.

5. Final extension. During the last PCR cycle the samples are kept at 70°C–74°C for 5–15 minutes so that no one-chain DNA fragments would be left in the samples.
6. Final hold. The received product is stored at 4°C–15°C temperature for unlimited period.

In this study the amplification of HFE gene 2 and 4 exons was performed by the PCR. The sequences of exons 2 and 4 of HFE gene were verified and the sequencing was carried out in "Thermo Fisher Scientific Inc." (former "Fermentas").

2.2.5.1.1. Amplification reaction of HFE exon 2 using PCR

Reagents:

1. Nuclease free water
2. PCR reaction buffer "10×DreamTaq™ Green buffer" (former "Fermentas", Lithuania)
3. Magnesium chloride ($MgCl_2$) 25 mM (former "Fermentas", Lithuania)
4. 2 mM nucleotide: A, G, T, C solution "dNTP Mix" (2mM each nucleotide) (former "Fermentas", Lithuania)
5. Thermostable DNA polymerase "DreamTaq™ Polymerase" (former "Fermentas", Lithuania).
6. 10 μM *HFE*-2 F foward primer: 5'- TGT GGA GCC TCA ACA TCC (IDT, USA)
7. 10 μM *HFE*-2 R reverse primer: 5'- TGA AAA GCT CTG ACA ACC (IDT, USA)

Table 2.2.5.1.1.1. *HFE exon 2 PCR protocol*

Reagents	***Final concentration***	***Volume 17× reactions***
Nuclease free water	–	327.25 μl
25 mM Magnesium chloride	4 mM	17 μl
10xDreamTaq™ buffer	1×	42.5 μl
Primer (F) HFE2	0.2 μM	8.5 μl
Primer (R) HFE2	0.2 μM	8.5 μl
dNTPworking solution	0.2 mM k each nucleotide	17 μl
DreamTaq™ polymerase	2.5 U	4.25 μl
DNA solution	100 ng/1μl	25 μl total

Method:

1. The master mix is made from the components of PCR reaction: nuclease free water, 25 mM magnesium chloride solution, 10× DreamTaq™ buffer, *HFE*-2 F primer, *HFE*-2 R primer, 2 mM dNTP Mix, according to the protocol presented in the Table 2.2.5.1.1.1.
2. Finally 4.25 µl 5U/µl DreamTaq™ polymerase is added to the mixed solution.
3. 0.2 ml PCR stripes are put to ice thermal block and 25 µl of master mix is pipetted in each test-tube.
4. 100 ng of tested genomic DNA is added to each test-tube. No DNA sample is added to the negative control tube.
5. PCR reaction was performed in the thermal cycler "Eppendorf Master cycler gradient S".
6. *HFE* 2 exon amplification reaction. The following settings are entered in the programme of thermal cycler:
   - 1st stage – 5 minutes at 95°C temperature,
   - 2nd stage – 30 seconds at 95°C temperature,
   - 3rd stage – 30 seconds at 56°C temperature,
   - 4th stage – 1 minute at 72°C temperature.

   The 2nd, 3rd and 4th stages are repeated for 30 cycles, then follows the
   - 5th stage – 10 minutes at 72°C temperature,
   - 6th stage – unlimited period at 4°C temperature.

2.2.5.1.2. Amplification reaction of HFE exon 4 using PCR

Reagents:

1. Nuclease free water
2. PCR reaction buffer "10×DreamTaq™ Green buffer" (former "Fermentas", Lithuania)
3. 2 mM of nucleotides: A, G, T, C solution "dNTP Mix" (2mM each nucleotide) (former "Fermentas", Lithuania)
4. Thermostable DNA polymerase "DreamTaq™ Polymerase (former "Fermentas", Lithuania)
5. 10 µM *HFE*-4 F Foward primer: 5'- TCC AGT CTT CTT GGC AA- (IDT, USA)
6. 10 µM *HFE*-4 R Reverse primer: 5'- TTC TAG CTC CTG GCT CTC (IDT, USA)

Table 2.2.5.1.2.1. *HFE exon 4 PCR protocol*

Reagents	Final concentration	Volume17× reactions
Nuclease free water	–	344.25 µl
10×DreamTaq™ buffer	1×	42.5 µl
Primer (F) HFE4	0.2 µM	8.5 µl
Primer (R**)** HFE4	0.2 µM	8.5 µl
dNT working solution	0.2mM each nucleotide	17 µl
DreamTaqTM polimerase	2.5 U	4.25 µl
DNR solution	100 ng/1µl	25 µl total

Method:

1. The master mix is made from the components of PCR reaction: nuclease free water, 10×DreamTaqTM buffer, *HFE*-4 F primer, *HFE*-4 R primer, 2 mM dNTP Mix, according to the protocol presented in the Table 2.2.5.1.2.1.
2. Finally 4.25 µl 5U/µl DreamTaq™ polymerase is added to the mixed solution.
3. 0.2 ml PCR stripes are put to ice thermal block and 25 µl of master mix is pipetted in each test-tube.
4. 100 ng of tested genomic DNA is added to each test-tube. No DNA sample is added to the negative control tube
5. PCR reaction was performed in the thermal cycler "Eppendorf Master cycler gradient S".
6. *HFE* 4 exon amplification reaction. The following settings are entered in the programme of thermal cycler:
   - 1st stage – 5 minutes at 95°C temperature,
   - 2nd stage – 30 seconds at 95°C temperature,
   - 3rd stage – 30 seconds at 56°C temperature,
   - 4th stage – 1 minute at 72°C temperature.

   The 2nd, 3rd and 4th stages are repeated for 30 cycles, then follows the:
   - 5th stage – 10 minutes at 72°C temperature,
   - 6th stage – unlimited period at 4°C temperature.

2.2.6. Agarose gel electrophoresis

Method of agarose gel electrophoresis is used to separate DNA molecules by size. After restriction of PCR product with the restriction enzymes the product is separated in agarose gel according to their size and analyzed.

2.2.6.1. *HFE* exons 2 and 4, analysis in agarose gel

Reagents:

1. Agarose "SeaKem LE Agarose" (Lonza, USA).
2. 10× TBE buffer "Rotiphorese" (Carl Roth GmbH + Co, Germany).
3. Ethidium bromide 10 mg/ml (Carl Roth GmbH + Co, Germany).
4. DNA dye "6×DNA Loading Dye" (former "Fermentas", Lithuania).
5. DNA ladder "pUC 19 DNA Mspl (HpaCl)" (former "Fermentas", Lithuania).

Equipment:

1. Horizontal gel electrophoresis system (Bio-Rad, USA).
2. Gel electrophoresis power supply (Bio-Rad, Singapore).
3. Microwave "Samsung G2712NR"(Samsung, Malaysia).
4. Automatic pipettes "Eppendorf research" 0.5 µl – 10 µl. (Eppendorf AG, Germany).
5. Gel documentation system "Molecular Imager® Gel Doc™ XR System" (Bio-Rad, USA).
6. Glass flask.
7. Pipette tips 0.5 µl – 10 µl ; 10 µl – 100 µl.

Method:

1. 1.5% agarose gel is needed for analysis of *HFE* 2 and 4 exon PCR products.
2. 2.25 g of agarose is mixed with 150 ml 1 × TBE buffer in glass flask. 1 ul of 10 mg/ml ethidium bromide solution is added.
3. Agarose is heated in microwave oven in order to melt it and acquire transparent solution.
4. The melted agarose is cooled under the flow of cold water for 30 seconds and poured carefully (trying to avoid bubbles) to the gel-casting frame that has 4 gel combs of 30 wells each (120 wells).
5. When the agarose gel sets, the combs are removed carefully and gel is put into the horizontal gel electrophoresis device filled with 1× TBE buffer so that the gel would be submerged in the buffer. 3 µl DNA fragment ladder "pUC 19 DNA Mspl (HpaCl)" is loaded on the gel (first well of each row).

6. Mix four volumes of DNA with one volume of gel loading buffer containing dye and load the samples on the gel.
7. Electrophoresis is carried out at 110 V for 60 min.
8. The acquired results are documented using Bio-Rad gel documentation system.
9. Data analysis. The tested DNA fragments are compared with the fragments of DNA fragment marker "pUC 19 DNA Mspl (HpaCl)". The size of *HFE* exon 2 PCR product has to be 378 bp. The size of *HFE* exon 4 PCR product has to be 369 bp (Fig. 2.2.6.1.1).

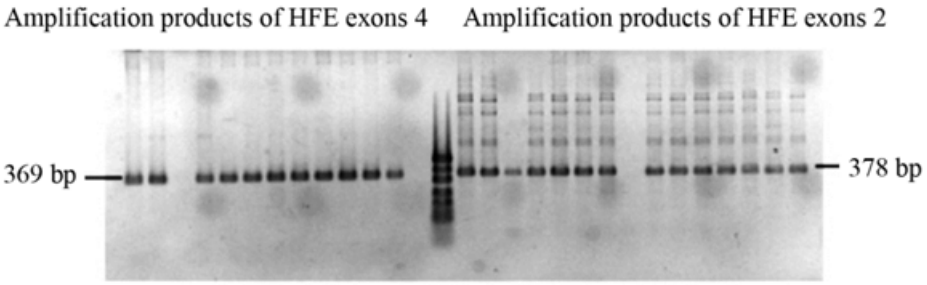

Fig. 2.2.6.1.1. *Amplification products of HFE exons 2 and 4*

2.2.7. Restriction Fragment Length Polymorphism analysis

Restriction Fragment Length Polymorphism analysis (RFLP) – by using restriction enzymes detects differences in DNA nucleotide sequence between samples.

2.2.7.1. Test of H63D mutation of the exon 2 of *HFE* gene by RFLP method

Reagents:

1. Restriction enzyme Bcl I 10 u/µl 3000 u (former "Fermentas", Lithuania).
   5'...T↓G A T C A...3'.
   3'...A C T A G↑T...5'.
2. Restriction buffer "10×Buffer G, with BSA1ml (former "Fermentas", Lithuania).

Equipment:

1. Automatic pipettes “Eppendorf Research” 0.5 ml – 10 ml and 10 ml – 100 ml. (Eppendorf AG, Germany).
2. Tubes 1.5 ml – 2 ml (Eppendorf AG, Germany).
3. Thermomixer “Eppendorf Thermomixer Comfort” (Eppendorf AG, Germany).
4. Pipette tips 0.5 µl – 10 µl; 10 µl – 100 µl.

Table 2.2.7.1.1. *H63D restriction reaction protocol*

Reagents	Final concentration	Volume 1× reactions
Water	–	3.25 µl
10× Buffer G with BSA	1×	1.5 µl
Bcl I	2.5 U	0.25 µl
PCR product	–	10 µl

Method:

1. Master mix for the restriction reaction is prepared according to the protocol presented in the Table 2.2.7.1.1.
2. 5µl of master mix and 10 µl of PCR product are combined in the test tube and incubated at 55°C for at least 1 hour.
3. The samples were stored at +4°C for short periods or at –20°C for long periods.

2.2.7.2. Test of C282Y mutation of the exon 4 of *HFE* gene by RFLP method

Reagents:

1. Restriction enzyme Rsa I 10 u/µl 3000 u (former “Fermentas”, Lithuania).

5'...GT↓AC....3'.
3'...C A↑T G...5'.

2. Restriction buffer 10× Buffer G, with BSA 1 ml (former “Fermentas”, Lithuania).

Equipment:

1. Automatic pipettes “Eppendorf Research” 0.5 ml – 10 ml and 10 ml – 100 ml. (Eppendorf AG, Germany).
2. Tubes 1.5 ml – 2 ml (Eppendorf AG, Germany).
3. Thermomixer “Eppendorf Thermomixer Comfort” (Eppendorf AG, Germany).
4. Pipette tips 0.5 µl – 10 µl; 10 µl – 100 µl.

Table 2.2.7.2.1. *C282Y restriction reaction protocol*

Reagents	Final concentration	Volume 1× reactions
Nuclease free water	–	3.25 µl
10×Buffer Tango™	1×	1.5 µl
Rsa I	2.5 U	0.25 µl
PCR product	–	10 µl

Method:

1. Master mix for the restriction reaction is prepared according to the protocol presented in the Table 2.2.7.2.1.
2. 2. 5 µl of master mix and 10 µl of PCR product are combined in the test tube and incubated at 37°C for at least 1 hour.
3. The samples were stored at +4°C for short periods or at –20°C for long periods.

2.2.8. Analysis of *HFE* gene RFLP products

After restriction of PCR product with the restriction enzymes the product is separated in agarose gel according to their size and analyzed.

Reagents:

1. Agarose “SeaKem LE Agarose” (Lonza, USA).
2. 10× TBE buffer “Rotiphorese” (Carl Roth GmbH + Co, Germany).
3. Ethidium bromide 10 mg / ml (Carl Roth GmbH + Co., Germany).
4. DNA dye “6×DNA Loading Dye” (former „Fermentas”, Lithuania)
5. DNA marker “O'GeneRuler™ 100 bp DNA Ladder, ready-to-use” (former “Fermentas”, Lithuania).
6. Pipette tips 0.5 µl – 10 µl ; 10 µl – 100 µl.

Equipment:

1. Horizontal gel electrophoresis system (Bio-Rad, USA).
2. Gel electrophoresis power supply (Bio-Rad, Singapore).
3. Microwave “Samsung G2712NR”(Samsung, Malaysia)
4. Automatic pipettes “Eppendorf research” 0.5 µl – 10 µl (Eppendorf AG, Germany).
5. Gel documentation system “Molecular Imager® Gel Doc™ XR System” (Bio-Rad, USA).
6. Glass flask.
7. Pipette tips 0.5 µl – 10 µl ; 10 µl – 100 µl.

Method:

1. 4% agarose gel is needed for analysis of *HFE* 2 H63D and *HFE* 4 C282Y mutations.
2. 6 g of agarose is mixed with 150 ml 1× TBE buffer and 50 ml of distilled water. 1ul of 10 mg/ml ethidium bromide solution is added.
3. Agarose solution is put to microwave oven and heated until the solution's volume is equal to 150 ml.
4. The melted agarose is cooled under the flow of cold water for 30 seconds and poured carefully (trying to avoid bubbles) to the gel-casting frame that has 3 gel combs of 20 wells each (60 wells).
5. When the agarose gel sets, the combs are removed carefully and gel is put into the horizontal gel electrophoresis device filled with 1x TBE buffer so that the gel was submerged in the buffer.
6. 3 µl DNA fragment marker "O'GeneRuler™ 100 bp DNA Ladder" loaded into each first well of each row of gel. 2 µl of DNA dye "6×DNA Loading Dye" is put to each tested sample with RFLP product.
7. The samples are closed and centrifuged.
8. Restriction products are loaded into the agarose gel.
9. Electrophoresis is carried out at 110 V for 120 min. The acquired results are documented using Bio-Rad gel documentation system. The tested DNA fragments are compared with the fragments of DNA fragment marker "O'GeneRuler™ 100 bp DNA Ladder".
10. The Bcl I restriction products were assessed when presence of H63D mutation was investigated:
    - Homozygotic normal individual – N/N (205 bp and 173) (Fig. 2.2.8.1).
    - Heterozygotic individual – H63D/N (378 bp, 205 bp and 173 bp). (Fig. 2.2.8.1).
    - Homozygotic individual – H63D/H63D (378 bp). (Fig. 2.2.8.1).
11. The Rsa I restriction products were assessed when presence of C282Y mutation was investigated:
    - Homozygotic normal individual – N/N (261 bp, 108 bp) (Fig. 2.2.8.2).
    - Heterozygotic individual – C282Y/N (261 bp, 108 bp, 80 bp, 29 bp) (Fig. 2.2.8.2).
    - Homozygotic individual – C282Y/C282Y (261 bp, 80 bp, 29 bp) (Fig. 2.2.8.2).

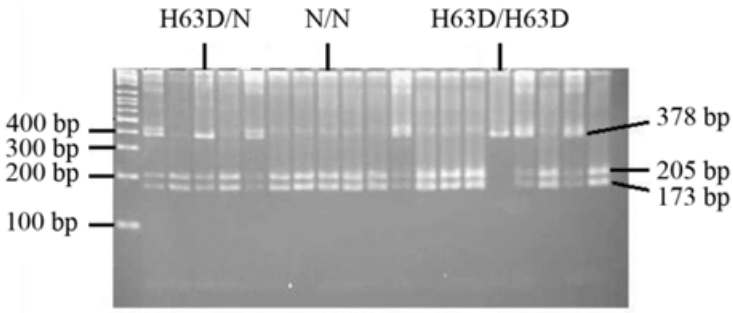

Fig. 2.2.8.1. *Bcl I restriction products*

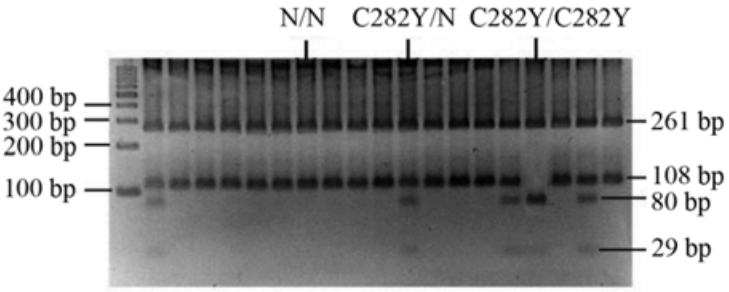

Fig. 2.2.8.2. *Rsa I restriction products*

2.2.9. TaqMan®

TaqMan® is a fluorescence-based genotyping assay (single-tube PCR assay). TaqMan® includes two allele-specific dual-labeled oligonucleotide probes and a PCR primer pair for detection of specific SNP targets. TaqMan® probes can be labelled by different 5′-end fluorophores (FAM™ - 6-carboxyfluorescein or VIC®) and by an appropriate 3′-end quencher TAMRA™ (6-carboxytetramethylrhodamine, succinimidyl ester) [86]. During PCR, due to 5′–3′exonuclease activity DNA polymerase (in our case AmpliTaqGold®) cleaves of a dual-labeled probe, which complementary to target sequence, and fluorophore is releases and detected (Fig. 2.2.9.1). For genotyping SNPs of *PNPLA3* C>G (rs738409), *RNF7* A>C (rs16851720), *MERTK* A>G (rs4374383) and *PCSK7* C>G (rs236918), TaqMan® genotyping method was chosen. The genotyping in this study was performed on 7500TM Fast real-time PCR system. All stages of the reaction were carried out in accordance with the manufacturer's protocols.

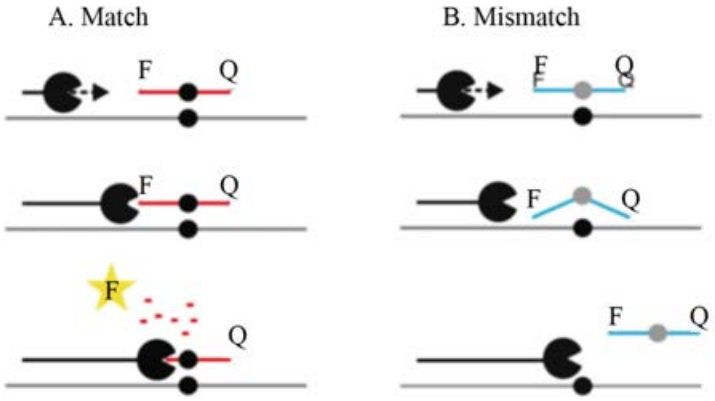

Fig. 2.2.9.1. *TaqMan® assay overview* [86] *(A)*
The polymerase (black sphere) elongatesthe primers (black arrow-line) and cleaves off the first 5' base linked with the fluorophore (F) of a matchingprobe (in red), resultingin the separation of fluorophore and quencher (Q). A fluorescence signal can bemeasured.

Reagents:

1. TaqMan® Universal PCR Master Mix (TaqMan® Universal PCR; Master Mix, No AmpErase® UNG).
2. Sigma® Water, BPC Grade 100 ml.
3. TE Buffer ("Roti stock 100× TE ready to use") 1000 ml.
4. The sets of factory-validated primers and probes.

Equipment:

1. Real-time PCR thermocycler "Applied Biosystems 7500; Fast".
2. Centrifuge "Eppendorf Centrifuge 5810 R".
3. Vortex mixer "IKA Yellow line lab dancer vario".
4. Vertical laminar Flow "Airstream Class II Biohazard Safety. Cabinet".
5. Automatic pipettes "Eppendorf Research" 10–100 µl, 0.5–10 µl.
6. PCR plate "Microamps ™ Fast Optical 96-Well", 0.1 ml.
7. PCR plate base "MicroAmp™ Splash Free 96-Well Base".
8. Transparent adhesive film "MicroAmps ™ Optical Adhesive Film".
9. Automatic pipettes tips 10 µl –100 µl, 0.1 µl –10 µl.
10. 1.5 ml tubes.

2.2.10. Genotyping procedure using Real-time PCR

Method:

1. Reaction mixture is prepared according to the protocol presented in Table 2.2.10.1 (5ul/sample) and divided in 96-well PCR plate. 1 ul/sample of test DNA is added.

Table 2.2.10.1. *Preparation of the reaction mix*

Reagents	Volume (µl) for 1 reaction
TaqManUniversal PCR Master Mix	3.25
Primer and TaqMan Probe dye mix	0.16
Nuclease free water	1.59
Total	5

2. The gene amplification is carried out according to PCR protocol (Table 2.2.10.2).

Table 2.2.10.2. *TaqMan® PCR protocol*

Step	Temperature	Time	Cycle(s)	Function
1	95ºC	10 min	1	Start
2	92ºC	15 sec	45	Denaturation
3	60 ºC	90 sec		Elongation, nucleolytic cleavage of hybridized probes
4	4 ºC	∞	1	Finish

3. After PCR amplification, genotyping fluorescence results was detected by using “7500 Software v2.0.5”. Allele selection for each plate was done automatically (Fig. 2.2.10.1).

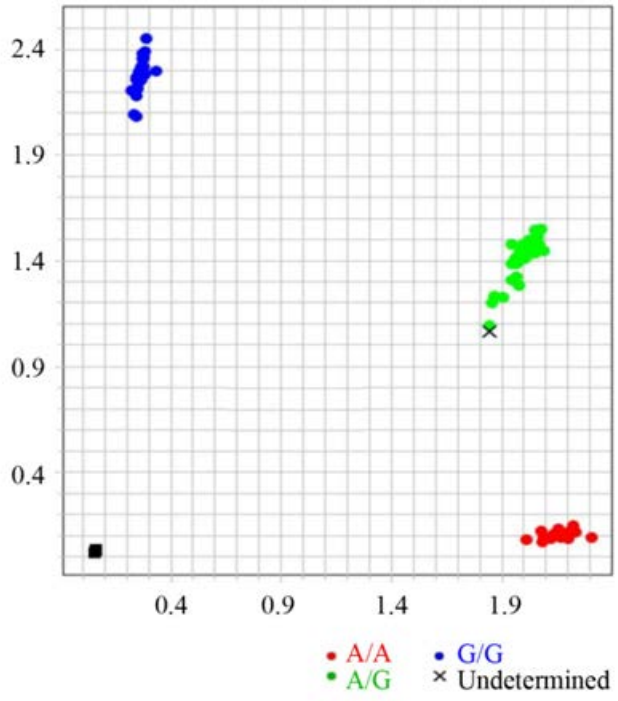

Fig.2.2.10.1. *Allelic Discrimination Plot*

2.2.11. Calculation of the study power

The statistical power is one of the basic limiting factors for the discovering the real associations in the genetic studies. The statistical power within a given sample size is influenced by several factors: the effect size (OR) of variant, sample size, and allele frequency of the risk variant in controls. Statistical power rises with increasing sample size. Statistical power of this study to detect a given allelic disease association (calculations performed for carriership of the rarer SNP allele) in screening panel (154 cases, 498 controls) is illustrated in Fig. 2.2.11.1. Screening panel had 80% power to detect a variant with an OR of 1.8 or higher at the 5% significance level, assuming a frequency of the disease-associated allele of at least 20 in controls. Calculations were performed for different allele frequencies using PS Power and Sample Size v2.130 [87]

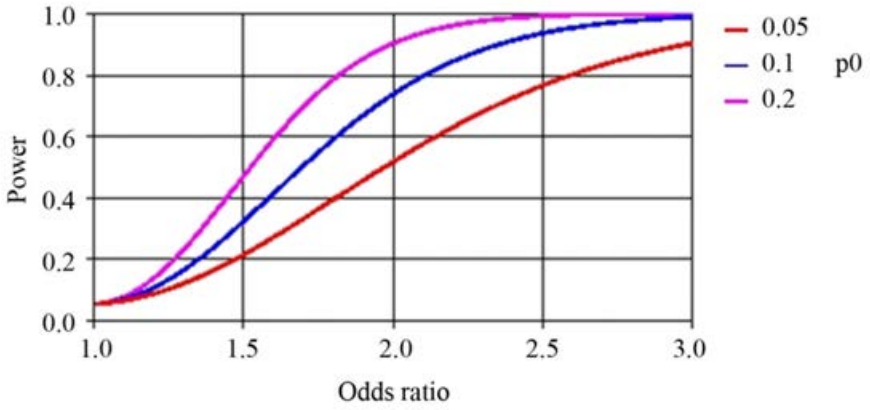

Fig. 2.2.11.1. *Power calculations.*
p0 denotes different allele frequencies.
Yellow dotted line shows the threshold of 80% power.

2.2.12. Statistical analysis

Statistical analysis of the genotyping data was performed by using PLINK software version 1.07 [88]. The distribution of *HFE*, *PNPLA3*, *RNF7*, *MERTK* and *PCSK7* genotypes in cases and controls was examined for consistency with the Hardy-Weinberg equilibrium (HWE). Allele frequencies were compared between cases and controls by Pearson's goodness-of-fit χ^2 test and Fisher exact tests. Associations between control and cases groups with *PNPLA3*, *RNF7*, *MERTK* and *PCSK7* SNP alleles and genotypes were calculated by using logistic regression analysis with adjustment for age and sex. Association analysis between control and cases groups was performed for each *HFE* SNP by using the Armitage trend test. The relative risks for mutations were estimated by using allelic, genotypic, recessive and dominant models. Recessive and dominant models led to a comparison between homozygous vs. wild type + heterozygous and heterozygous + homozygous vs. wild type carriers. Adjusted odds ratios (aOR) and 95% confidence intervals (CI) were reported. P value of 0.05 was defined to be statistically significant. Age is shown as mean with standard deviation, and was compared by using analysis of variance (ANOVA). Gender distributions were compared using χ^2 tests.

3. RESULTS

3.1. Characteristics of patients groups

1. In the first part of our study we included: 209 patients with liver cirrhosis and 1005 controls. Patient group consisted of 107 men and 102 women with a mean age of 54 years. The most common causes of liver cirrhosis in our study group were HCV infection and alcochol consumption. Distribution of patients with cirrhosis according to Child-Pugh score and disease aetiology are presented in the Tables 3.1.1 and 3.1.2. The control group consisted of 1005 individuals: 581 men and 424 women with a mean age of 37.1 years (range, 18–65 years).

Table 3.1.1. *Characteristics of liver cirrhosis and control groups*

	Liver cirrhosis (n=209)	**Controls (n=1005)**
Gender, n (%) Male Female	 107 (51.2) 102 (48.8)	 581 (57.8) 424 (42.2)
Age, years (SD)	54.0 (8.1)	37.1 (4.3)
Child–Pugh class, n (%) A B C	 65 (31.1) 100 (47.8) 44 (21.1)	

SD, standard deviation

Table 3.1.2. *Etiology of liver cirrhosis*

Etiology of liver cirrhosis, n (%)[a]	**Liver cirrhosis (n=209)**
HCV	71 (33.9)
HBV	28 (13.6)
Autoimmune	15 (7.1)
Alcohol	54 (25.8)
Other causes	12 (5.7)
Cryptogenic	19 (9.1)

HBV, hepatitis B virus; HCV, hepatitis C virus.
[a]17 of HCV and 5 of HBV patients had a mixed (viral and alcohol) etiology.

2. In the second part of our study we included: 317 patients with liver cirrhosis, 154 patients with liver fibrosis and 498 controls: 318 men and 180 women with a mean age of 46.8 years (range,18–65 years). Characteristics of study participants are represented in Table 3.1.3.

Table 3.1.3. *Characteristics of patients with liver cirrhosis, fibrosis and controls*

	Liver cirrhosis (n=317)	**Liver fibrosis (n=154)**	**Controls (n=498)**	**ANOVA (age)[a] and χ^2 test P value**
Age (Mean±SD)	54.5±12.2	49.9±11.5	46.8 ± 6.5	<0.001
Gender (n, %) Male Female	 150 (47.3%) 167 (52.7%)	 95 (61.7%) 59 (38.3%)	 318 (63.9%) 180 (36.1%)	 <0.001
Child-Pugh class (n, %) A B C	 99 (31.2%) 152 (48.0%) 66 (20.8%)			
Liver fibrosis stage[b] (n, %) 1 2 3		 97 (63.0%) 28 (18.2%) 29 (18.8%)		

[a]Statistical analysis was performed for all groups.
[b]Histological degree of liver fibrosis was evaluated according to METAVIR.

The most common causes of liver cirrhosis were chronic HCV infection and alcohol. Distribution of ptients according aetiology and Child-Pugh score is shown in Tables 3.1.3 and 3.1.4. The most common cause of liver injury in the fibrosis group was HCV infection. The subjects differed in age and sex between the study groups (Table 3.1.3). Men were predominant in control and liver fibrosis groups and accounted for 63.9% and 61.7%, respectively. Liver cirrhosis patients were significantly (P<0.001) older than liver fibrosis and control patients (Table 3.1.3). To eliminate the potential bias of differences in age and sex distribution among the groups, these parameters were included as covariates in further logistic regression analysis.

Table 3.1.4. *Etiology of liver cirrhosis and fibrosis*

Etiology of liver disease (n, %)	**Liver cirrhosis (n=317)**	**Liver fibrosis (n=154)**
HCV	117 (36.9%)	136 (88.3%)
HBV	43 (13.5%)	6 (3.9%)
Autoimmune	23 (7.2%)	1(0.65%)
Alcohol	82 (25.7%)	4 (2.6%)
Other causes	18 (5.8%)	6 (3.9%)
Cryptogenic	28 (9.0%)	1 (0.65%)

HBV – hepatitis B virus; HCV – hepatitis C virus.

The observed genotype frequencies for two *HFE* C282Y, H63D gene mutations and for all four SNPs in: *PNPLA3, RNF7, MERTK* and *PCSK7* genes included in the study were in Hardy-Weinberg equilibrium (P > 0.05).

3.2. Association between *HFE* C282Y mutation and liver cirrhosis

The association between *HFE* C282Y mutation and liver cirrhosis is presented in Table 3.2.1. The carriage of C282Y risk allele was significantly more frequent in patients with liver cirrhosis than in controls (OR = 2.1, P = 0.005). This association was also evident in genotypic analysis where heterozygous genotype C282Y/wt and wt/wt carriers were compared (OR = 2.0, P = 0.01) and in recessive model (OR = 2.07, P = 0.008)

Table 3.2.1. *The frequencies of HFE C282Y mutations in cirrhotic patients and controls*

Genotype C282Y	**Liver cirrhosis (n=209) (%)**	**Controls (n=1005) (%)**	**OR (95% CI)**	**P**
C282Y allee	21 (5.02)	50 (2.49)	2.07(1.23–3.49)	**0.005**
wt/wt	189 (90.43)	956 (95.5)	1.00 (reference)	
C282Y/wt	19 (9.09)	48 (4.79)	2 (1.15–3.48)	**0.012**
C282Y/C282Y	1 (0.48)	1 (0.09)	5.06(0.32–81.22)	0.203
wt/wt vs. C282Y/wt + C282Y/C282Y			2.07 (1.20–3.55)	**0.008**
wt/wt + C282Y/wt vs. C282Y/C282Y			0.21 (0.01–3.33)	0.219
wt allele	397 (94.98)	1952 (97.51)		

OR, odds ratio; CI, confidence interval; significant p-values are marked in bold.

Gender-based stratification analysis revealed significant gender-related differences in the carriage of C282Y mutation between liver cirrhosis patients and controls (Tables 3.2.2 and 3.2.3). The carriage of heterozygous C282Y/wt genotype in men was associated with liver cirrhosis (OR=2.48, P=0.008 Table 3.2.2) whereas no significant associations were found in the female group (Table 3.2.3).

Table 3.2.2. *The frequencies of HFE C282Y mutations in cirrhotic men patients and controls*

Genotype C282Y	Men (n=107) (%)	Controls (n = 581) (%)	OR (95% CI)	P
C282Y allele	15 (7.01)	33 (2.84)	2.58 (1.38–4.84)	**0.002**
wt/wt	93 (86.92)	549 (94.49)	1.00 (reference)	
C282Y/wt	13 (12.15)	31 (5.33)	2.48 (1.25–4.91)	**0.008**
C282Y/C282Y	1 (0.93)	1 (0.18)	5.90 (0.37–95.20)	0.155
wt/wt vs. C282Y/wt + C282Y/C282Y			2.58 (1.33–5.02)	**0.004**
wt/wt + C282Y/wt vs. C282Y/C282Y			0.18 (0.01–2.95)	0.178
wt allele	199 (92.99)	1129 (97.16)		

OR, odds ratio; CI, confidence interval; significant p-values are marked in bold.

Table 3.2.3. *The frequencies of HFE C282Y mutations in cirrhotic women patients and controls*

Genotype C282Y	Women (n=102) (%)	Controls (n=424) (%)	OR (95% CI)	P
C282Y allele	6 (2.94)	17 (2.01)	1.48 (0.58–3.81)	0.412
wt/wt	96 (94.12)	407 (95.91)	1.00 (reference)	
C282Y/wt	6 (5.88)	17 (4.01)	1.50 (0.58–3.90)	0.406
C282Y/C282Y	0 (0)	0 (0)		
wt/wt vs. C282Y/wt + C282Y/C282Y			1.50 (0.58–3.90)	0.406
wt/wt + C282Y/wt vs. C282Y/C282Y			0.24(0.01-12.24)	1
wt allele	98 (97.06)	407 (97.99)		

OR, odds ratio; CI, confidence interval.

3.3. Association between *HFE* H63D mutation and liver cirrhosis

The carriage of H63D alleles was distributed equally in the control (15.5%) and cirrhotic groups (18.1%; P=0.244) Genotyping analysis of *HFE* gene H63D mutation did not reveal significant association with liver cirrhosis (Table 3.3.1).

Table 3.3.1. *The frequencies of HFE H63D mutations in cirrhotic patients and controls*

Genotype H63D	Liver cirrhosis (n=209) (%)	Controls (n=1005) (%)	OR (95% CI)	P
H63D allele	76 (18.1)	320 (15.53)	1.18 (0.89–1.55)	0.244
wt/wt	142 (67.9)	712 (70.84)	1.00 (reference)	
H63D/wt	58 (27.8)	267 (26.57)	1.09 (0.78–1.53)	0.618
H63D/H63D	9 (4.3)	26 (2.58)	1.74 (0.80–3.78)	0.161
wt/wt vs H63D/wt + H63D/H63D			1.15 (0.83–1.58)	0.403
wt/wt + H63D/wt vs. H63D/H63D			0.59 (0.83–1.58)	0.403
wt allele	342 (81.9)	1682 (84.47)		

OR, odds ratio; CI,confidence interval

Significant gender – related differences were revealed in carriage of H63D mutation between liver cirrhosis patients and controls (Tables 3.3.2 and 3.3.3). The carriage of H63D risk allele (OR = 1.54, P = 0.02) and homozygous H63D/H63D genotype (OR = 4.13, P = 0.005) were associated with liver cirrhosis in men but not in women.

Table 3.3.2. *The frequencies of HFE H63D mutations in cirrhotic male patients and controls*

Genotype H63D	Men (n=107) (%)	Controls (n = 581) (%)	OR (95% CI)	P
H63D allele	46 (21.49)	175 (15.06)	1.54 (1.07–2.22)	**0.019**
wt/wt	67 (62.61)	415 (71.41)	1.00 (reference)	
H63D/wt	34 (31.77)	157 (27.02)	1.34 (0.85–2.11	0.202
H63D/H63D	6 (5.62)	9 (1.57)	4.13(1.42–11.98)	**0.005**
wt/wt vs H63D/wt + H63D/H63D			1.49 (0.97–2.30)	0.067
wt/wt + H63D/wt vs. H63D/H63D			0.27 (0.09–0.76)	**0.008**
wt allele	168 (78.51)	987 (84.94)		

OR, odds ratio; CI, confidence interval; significant p-values are marked in bold.

Table 3.3.3. *The frequencies of HFE H63D mutations in cirrhotic female patients and controls.*

Genotype H63D	Women (n = 102) (%)	Controls (n=424) (%)	OR (95% CI)	P
H63D allele	30 (14.71)	144 (16.98)	0.84 (0.55–1.29)	0.432
wt/wt	75 (73.53)	297 (70.05)	1.00 (reference)	
H63D/wt	24 (23.53)	110 (25.94)	0.86 (0.52–1.44)	0.573
H63D/H63D	3 (2.94)	17 (4.01)	0.70 (0.20–2.45)	0.573
wt/wt vs H63D/wt + H63D/H63D			0.84 (0.52–1.37)	0.488
wt/wt + H63D/wt vs. H63D/H63D			1.38 (0.40–4.80)	0.613
wt allele	174 (85.29)	704 (83.02)		

OR, odds ratio; CI, confidence interval

3.4. Association between the carriage of two or more *HFE* gene alleles and liver cirrhosis

The cariership of two or more risk alleles was significantly higher in the group of patients with liver cirrhosis than in controls (7.66% vs 3.98%) and resulted in significant association (P = 0.021; OR: 2.00, 95% CI: 1.10–3.64). Gender-based analysis revealed that male patients higher carriership was increased of two or more risk alleles compared with controls (11.22% vs. 3.78%, P=0.001, OR: 3.22, 95% CI: 1.54–6.71), whereas no significant difference was observed in female. The prevalence of particular C282Y/ H63D compound heterozygous genotype in Lithuanian cirrhotic patients is presented in Table 3.4.1. Only small number of patients and controls were carriers of C282Y/H63D compound heterozygous genotype. The genotyping analysis showed higher incidence of C282Y/H63D genotype in cirrhotic patients (2.87%) than in healthy individuals (1.29%); however, the differences did not reach statistical significance (OR: 2.26; P=0.118, Table 3.4.1).

Table 3.4.1. *The frequencies of HFE C282Y/H63D genotype in cirrhotic patients and controls*

Genotype	Liver cirrhosis	Controls	OR (95% CI)	P
Total patients with liver cirrhosis (n=209) (%)		Total (n=1005) (%)		
C282Y/H63D	6 (2.87)	13 (1.29)	2.26 (0.85–6.00)	0.118
Women with liver cirrohsis (n=102) (%)		Women (n=424) (%)		
C282Y/H63D	1 (0.98)	1 (0.24)	4.78(0.30–77.10)	0.350
Men with liver cirrhosis (n=107) (%)		Men (n=581) (%)		0.163
C282Y/H63D	5 (4.67)	12 (2.06)	2.32 (0.80–6.73)	0.163

OR, odds ratio; CI, confidence interval

3.5. Association of *PNPLA3* gene single nucleotide polymorphism 3.6.with liver fibrosis and cirrhosis

Tables 3.5.1, 3.5.2 and 3.5.3 presents the frequencies of alleles and genotypes of *PNPLA3*, SNP in controls, liver fibrosis, liver cirrhosis and combined liver cirrhosis + liver fibrosis groups. The *PNPLA3* rs738409 risk allele G was less frequent in controls (17.5%) than in the combined liver fibrosis and cirrhosis group (28.3%) and was associated with liver damage in the combined group (aOD: 1.84, CI: 1.47–2.30, $P=9.27\times10^{-8}$) . The G allele of rs738409 was also more frequent in the cirrhosis group (29.3%; $P=5.57\times10^{-7}$) and in the fibrosis group (26.3%; P=0.001) as compared to controls (Tables 3.5.1 and 3.5.2). And it also was significantly associated with liver cirrhosis(aOD: 1.92, CI: 1.49–2.48, $P=5.57\times10^{-7}$) and liver fibrosis (aOD: 1.65; CI–1.22-2.23; P=0.001).The distribution of *PNPLA3* genotypes differed significantly between controls, the combined liver cirrhosis and fibrosis group (P=0.001) and patients with cirrhosis (P=0.002; Tables 3.5.2 and 3.5.3). The frequency of the *PNPLA3* rs738409 GG genotype was significantly (P=0.001) lower in controls (3.4%) than in patients with liver fibrosis (9.7%, Table 3.5.2). *PNPLA3* rs738409 genotypes were associated with liver fibrosis and cirrhosis when comparing both dominant (aOD: 1.98; CI: 1.44–2.72, $P=2.20\times10^{-5}$; aOD:1.67, CI: 1.14–2.43, P= 0.008, respectively) and recessive (aOD: 3.94, CI: 2.03–7.67, $P=5.16\times10^{-5}$; aOD: 3.02, CI: 1.45–6.28; P=0.003, respectively) inheritance models (Tables 3.5.1 and 3.5.2).

Table 3.5.1. *Distribution of PNPLA3 gene polymorphisms in liver cirrhosis and control groups*

Allele/Genotypes	Liver cirrhosis (n=317) n (%)	Controls (n=498) n (%)	aOR (95% CI)	P
PNPLA3 rs738409				
G	93 (29.3)	87 (17.5)	1.92(1.49–2.48)	**5.57×10^{-7}**
C	224 (70.7)	411(82.5)		
GC	124 (39.1)	140(28.1)	1.69 (1.21–2.36)	**0.002**
GG	31 (9.8)	17 (3.4)	4.74 (2.41–9.30)	**6.21×10^{-6}**
CC	162 (51.1)	341(68.5)	1 (Reference)	
GG+GC vs. CC			1.98 (1.44–2.72)	**2.20×10^{-5}**
GG vs. GC+CC			3.94 (2.03–7.67)	**5.16×10^{-5}**

aOR – adjusted odds ratio;
CI – confidence interval; significant *P*-values are marked in bold.

Table 3.5.2. *Distribution of PNPLA3 gene polymorphisms in liver fibrosis and control groups*

Allele/Genotypes	Liver fibrosis (n=154) n (%)	Controls (n=498) n (%)	aOR (95% CI)	P
PNPLA3 rs738409				
G	41 (26.3)	87 (17.5)	1.65(1.22–2.23)	**0.001**
C	113 (73.7)	411(82.5)		
GC	51 (33.1)	140(28.1)	1.45 (0.97–2.17)	0.071
GG	15 (9.7)	17 (3.4)	3.41 (1.62–7.20)	**0.001**
CC	88 (57.2)	341(68.5)	1 (Reference)	
GG+GC vs. CC			1.67 (1.14–2.43)	**0.008**
GG vs. GC+CC			3.02 (1.45–6.28)	**0.003**

aOR – adjusted odds ratio; CI – confidence interval;
significant *P*-values are marked in bold.

Table 3.5.3. *Distribution of PNPLA3 gene polymorphisms in both patients groups(liver cirrhosis and liver fibrosis) and control groups*

Allele/Genotypes	Liver cirrhosis+liver fibrosis (n=471) n (%)	Controls (n=498) n (%)	aOR (95%CI)	P
PNPLA3 rs738409				
G	133 (28.3)	87 (17.5)	1.84(1.47–2.30)	**9.27×10^{-8}**
C	338 (71.7)	411(82.5)		
GC	175 (37.2)	140(28.1)	1.64 (1.23–2.20)	**0.001**
GG	46 (9.8)	17 (3.4)	4.28 (2.33–7.85)	**2.67×10^{-6}**
CC	250 (53.0)	341(68.5)	1 (Reference)	
GG+GCvs. CC			1.91 (1.45–2.51)	**4.39×10^{-6}**
GGvs. GC+CC			3.61 (1.98–6.56)	**2.64×10^{-5}**

aOR – adjusted odds ratio; CI – confidence interval;
significant *P*-values are marked in bold.

3.6. Association of *RNF7* gene single nucleotide polymorphism with liver fibrosis and cirrhosis

Alleles of RNF7 rs16851720 were distributed evenly between the study groups. Meanwhile, the rs16851720 CC genotype was more frequent in controls (4.6%) when compared with the combined liver cirrhosis and fibrosis group (1.5%; aOD: 0.33; CI: 0.13–0.80, P=0.014) or cirrhosis patients (1.3%; aOD: 0.26, CI: 0.08–0.80, P=0.019, Tables 3.6.1, 3.6.2 and 3.6.3). Similarly, considering recessive inheritance, the genotype CC was less frequent in both liver cirrhosis and liver fibrosis (aOD: 0.34, CI: 0.14–0.82, P=0.016) and in patients with liver cirrhosis (aOD: 0.26; CI: 0.09–0.81, P=0.020; Tables 3.6.1and 3.6.3).

Table 3.6.1. *Distribution of RNF7 gene polymorphisms in liver cirrhosis and control groups*

Allele/Genotypes	Liver cirrhosis (n=317) n (%)	Controls (n=498) n (%)	aOR (95% CI)	P
RNF7 rs16851720				
C	57 (18.0)	105(21.1)	0.80(0.60–1.06)	0.115
A	260 (82.0)	393(78.9)		
CA	106 (33.4)	164(32.9)	0.95 (0.68–1.32)	0.755
CC	4 (1.3)	23 (4.6)	0.26 (0.08–0.80)	**0.019**
AA	207 (65.3)	311(62.5)	1(Reference)	
CC+CA vs. AA			0.86(0.63–1.19)	0.367
CC vs. CA+AA			0.26(0.09–0.81)	**0.020**

aOR – adjusted odds ratio; CI – confidence interval;
significant *P*-values are marked in bold.

Table 3.6.2. *Distribution of RNF7 gene polymorphisms in liver fibrosis and control groups*

Allele/Genotypes	Liver fibrosis (n=154) n (%)	Controls (n=498) n (%)	aOR (95% CI)	P
RNF7 rs16851720				
C	25 (16.2)	105(21.1)	0.74(0.53–1.05)	0.091
A	129 (83.8)	393(78.9)		
CA	44 (28.6)	164(32.9)	0.79(0.53–1.19)	0.256
CC	3 (1.9)	23 (4.6)	0.42(0.12–1.42)	0.161
AA	107 (69.5)	311(62.5)	1(Reference)	
CC+CA vs. AA			0.75(0.50–1.11)	0.146
CC vs. CA+AA			0.45(0.13–1.52)	0.198

aOR – adjusted odds ratio; CI – confidence interval.

Table 3.6.3. *Distribution of RNF7 gene polymorphisms in both patients groups (liver fibrosis and liver cirrhosis) and control groups.*

Allele/Genotypes	Liver cirrhosis + liver fibrosis (n=471) n (%)	Controls (n=498) n (%)	aOR (95% CI)	P
RNF7 rs16851720				
C	82 (17.4)	105(21.1)	0.79(0.62–1.01)	0.055
A	389 (82.6)	393(78.9)		
CA	150 (31.8)	164(32.9)	0.90(0.68–1.20)	0.478
CC	7 (1.5)	23 (4.6)	0.33(0.13–0.80)	**0.014**
AA	314 (66.7)	311(62.5)	1(Reference)	
CC+CA vs. AA			0.83(0.63–1.10)	0.196
CC vs. CA+AA			0.34(0.14–0.82)	**0.016**

aOR – adjusted odds ratio; CI – confidence interval;
significant *P*-values are marked in bold.

3.7. Association of *MERKT* gene single nucleotide polymorphism with liver fibrosis and cirrhosis

The distributions of alleles and genotypes of *MERKT* SNP were similar between the control, liver fibrosis and cirrhosis groups. The risk allele A of *MERKT* rs4374383 was detected in 36.1% of controls, in 37.1% of the combined liver cirrhosis and fibrosis group (Table 3.7.3), in 38.3% of patients with cirrhosis (Table 3.7.1) and in 34.4% of the liver fibrosis group (P>0.05; Table 3.7.2). *MERKT* rs4374383 was not linked with the liver fibrosis (aOD: 0.92, CI: 0.71–1.19, P=0.530) or cirrhosis (aOD: 1.13, CI: 0.90–1.40, P=0.287).

Table 3.7.1. *Distribution of MERKT gene polymorphisms in liver cirrhosis and control groups*

Allele/Genotypes	Liver cirrhosis (n=317) n (%)	Controls (n=498) n (%)	aOR (95% CI)	P
MERTK rs4374383				
A	121 (38.3)	180 (36.1)	1.13 (0.90–1.40)	0.287
G	196 (61.7)	318 (63.9)		
AG	149 (47.0)	216 (43.4)	1.13 (0.81–1.58)	0.467
AA	47 (14.8)	72 (14.5)	1.27 (0.80–2.02)	0.320
GG	121 (38.2)	210 (42.1)	1 (Reference)	
AA+AG vs. GG			1.16 (0.85–1.59)	0.343
AA vs. AG+GG			1.19 (0.77–1.83)	0.436

aOR – adjusted odds ratio; CI – confidence interval.

Table 3.7.2. *Distribution of MERKT gene polymorphisms in liver fibrosis and control groups*

Allele/Genotypes	Liver fibrosis (n=154) n (%)	Controls (n=498) n (%)	aOR (95% CI)	P
MERTK rs4374383				
A	53 (34.4)	180 (36.1)	0.92 (0.71–1.19)	0.530
G	101 (65.6)	318 (63.9)		
AG	58 (37.7)	216 (43.4)	0.78 (0.52–1.16)	0.215
AA	24 (15.6)	72 (14.5)	0.94 (0.55–1.62)	0.831
GG	72 (46.7)	210 (42.1)	1 (Reference)	
AA+AG vs. GG			0.82 (0.57–1.18)	0.286
AA vs. AG+GG			1.06 (0.64–1.77)	0.813

aOR – adjusted odds ratio; CI – confidence interval.

Table 3.7.3. *Distribution of MERKT gene polymorphisms in both patients groups (liver cirrhosis and liver fibrosis) and control groups*

Allele/Genotypes	Liver cirrhosis+liver fibrosis (n=471) n (%)	Controls (n=498) n (%)	aOR (95% CI)	P
MERTK rs4374383				
A	175 (37.1)	180 (36.1)	1.04 (0.86–1.26)	0.682
G	296 (62.9)	318 (63.9)		
AG	207 (43.9)	216 (43.4)	1.01 (0.76–1.34)	0.959
AA	71 (15.1)	72 (14.5)	1.10 (0.74–1.65)	0.630
GG	193 (41.0)	210 (42.1)	1 (Reference)	
AA+AG vs. GG			1.03 (0.79–1.35)	0.822
AA vs. AG+GG			1.10 (0.76–1.60)	0.619

aOR – adjusted odds ratio; CI – confidence interval.

3.8. Association of *PCSK7* gene single nucleotide polymorphism with liver fibrosis and cirrhosis

The distributions of alleles and genotypes of PCSK7 SNP was similar between the control, liver fibrosis and cirrhosis groups. The frequencies of risk allele C of PCSK7 rs236918 were 13.9% in controls, 12.5% in the combined liver cirrhosis and fibrosis group (Table 3.8.3) 12.3% in liver cirrhosis, and 13.0% in liver fibrosis (P>0.05; Tables 3.8.1 and 3.8.2). PCSK7 SNP was not associated with liver fibrosis (aOD: 0.86; CI: 0.59–1.26; P=0.434) or cirrhosis (aOD: 0.86, CI: 0.63–1.19, P=0.362; Tables 3.8.2 and 3.8.1).

Table 3.8.1. *Distribution of PCSK7 gene polymorphisms in liver cirrhosis and control groups*

Allele/Genotypes	Liver cirrhosis (n=317) n (%)	Controls (n=498) n (%)	aOR (95% CI)	P
PCSK7 rs236918				
C	39 (12.3)	69 (13.9)	0.86 (0.63–1.19)	0.362
G	278 (87.3)	429 (86.1)		
CG	70 (22.1)	114 (22.9)	0.97 (0.67–1.40)	0.865
CC	4 (1.3)	12 (2.4)	0.39 (0.11–1.36	0.139
GG	243 (76.6)	372 (74.7)	1 (Reference)	
CC+CT vs. TT			0.91 (0.63–1.30)	0.589
CC vs. CG+GG			0.39 (0.11–1.37)	0.141

aOR – adjusted odds ratio; CI – confidence interval.

Table 3.8.2. *Distribution of PCSK7 gene polymorphisms in liver fibrosis and control groups*

Allele/Genotypes	Liver fibrosis (n=154) n (%)	Controls (n=498) n (%)	aOR (95% CI)	P
PCSK7 rs236918				
C	20 (13.0)	69 (13.9)	0.86(0.59–1.26)	0.434
G	134 (87.0)	429(86.1)		
CG	34 (22.1)	114(22.9)	0.86(0.55–1.34)	0.495
CC	3 (2.0)	12 (2.4)	0.75(0.20–2.73)	0.661
GG	117 (75.9)	372(74.7)	1(Reference)	
CC+CT vs. TT			0.85(0.55–1.30)	0.447
CC vs. CG+GG			0.78(0.21–2.82)	0.701

aOR – adjusted odds ratio; CI – confidence interval.

Table 3.8.3. *Distribution of PCSK7 gene polymorphisms in both patients groups (liver cirrhosis and liver fibrosis) and control groups*

Allele/Genotypes	Liver cirrhosis+liver fibrosis (n=471) n (%)	Controls (n=498) n (%)	aOR (95% CI)	P
PCSK7 rs236918				
C	59 (12.5)	69 (13.9)	0.84 (0.64–1.10)	0.209
G	412 (87.5)	429 (86.1)		
CG	104 (22.1)	114 (22.9)	0.89 (0.65–1.22)	0.469
CC	7 (1.5)	12 (2.4)	0.52 (0.19–1.41)	0.200
GG	360 (76.4)	372 (74.7)	1 (Reference)	
CC+CT vs. TT			0.85 (0.63–1.16)	0.313
CC vs. CG+GG			0.53 (0.20–1.44)	0.214

aOR – adjusted odds ratio; CI – confidence interval.

3.9. Association of *PNPLA3, RNF7, MERTK* and *PCSK7* genes single nucleotide polymorphisms with liver cirrhosis in hepatitis C virus – induced and non-hepatitic C virus – induced liver cirrhosis

Since large proportion of patients with in cirrhosis group were HCV patients we carried out sub-analysis for the risk of developing liver cirrhosis in HCV and non-HCV patients. Tables 3.9.1 and 3.9.2 presents the frequencies of alleles and genotypes of *PNPLA3*, SNP in controls, liver cirrhosis in HCV and non-HCV groups. *PNPLA3* SNP was associated with both HCV and non-HCV induced liver cirrhosis. The *PNPLA3* rs738409

risk allele G was less frequent in controls 17.5% than in hepatitis C virus–induced cirrhosis group 31.2% and was associated with liver cirrhosis in HCV group (aOD: 2.04, CI: 1.41–2.94, P=1.43×10^{-4} Table 3.9.1).

Table 3.9.1. *Distribution of PNPLA3 gene polymorphisms in hepatitis C virus-induced cirrhosis and control groups*

Allele/Genotypes	**Hepatitis C virus–induced cirrhosis (n=117) n (%)**	**Controls (n=498) n (%)**	**aOR (95% CI)**	**P**
PNPLA rs738409				
G	37 (31.2)	87 (17.5)	2.04 (1.41–2.94)	**1.43×10^{-4}**
C	80 (68.8)	411 (82.5)		
GC	49 (41.9)	140 (28.1)	1.99 (1.22–3.26)	**0.006**
GG	56 (47.9)	17 (3.4)	4.30 (1.75–10.59)	**0.001**
CC	12 (10.2)	341 (68.5)	1 (Reference)	
GG+GC vs. CC			2.25 (1.41–3.57)	**0.001**
GG vs. GC+CC			3.34 (1.39–8.03)	**0.007**

aOR – adjusted odds ratio; CI – confidence interval;
significant *P*-values are marked in bold.

And the *PNPLA3* rs738409 risk allele G was less frequent in controls 17.5% than in non hepatitis C virus-induced cirrhosis 28.3 % and was associated with liver cirrhosis in non-HCV group. (aOD: 1.81, CI: 1.36–2.42, P=5.64×10^{-5}; Table 3.9.2).

Table 3.9.2. *Distribution of PNPLA3 gene polymorphisms in non- hepatitis C virus–induced cirrhosis and control groups*

Allele/Genotypes	**Non hepatitisC virus–induced cirrhosis (n=200) n (%)**	**Controls (n=498) n (%)**	**aOR (95% CI)**	**P**
*PNPLA*rs738409				
G	57 (28.3)	87 (17.5)	1.81 (1.36–2.42)	**P=5.64×10^{-5}**
C	143 (71.7)	411 (82.5)		
GC	75 (37.5)	140 (28.1)	1.56 (1.06–2.28)	**0.023**
GG	106 (53)	17 (3.4)	4.37 (2.08–9.19)	**1.01×10^{-4}**
CC	19 (9.5)	341(68.5)	1 (Reference)	
GG+GC vs. CC			1.82 (1.27–2.60)	**0.001**
GG vs. GC+CC			3.76 (1.81–7.82)	**3.91×10^{-4}**

aOR – adjusted odds ratio; CI – confidence interval;
significant *P*-values are marked in bold.

RNF7 rs16851720 CC genotype was less frequent than AA genotype in non-HCV cirrhosis group if compared to the control group (aOD: 0.28, CI: 0.08–1.00, P=0.050; Table 3.9.4). Interestingly, frequencies of *RNF7* rs16851720 genotypes and alleles in HCV cirrhosis group did not differ from the controls; however, the potential association could be blunted by a relatively small number of individuals within this group.

Table 3.9.3. *Distribution of RNF7 gene polymorphisms in hepatitis C virus-induced cirrhosis and control group*

Allele/Genotypes	**Hepatitis C virus–induced cirrhosis (n=117) n (%)**	**Controls (n=498) n (%)**	**aOR (95% CI)**	**P**
RNF7 rs16851720				
C	21 (18.0)	105 (21.1)	0.86 (0.57–1.30)	0.469
A	96 (82.0)	393 (78.9)		
CA	40 (34.2)	164 (32.9)	1.04 (0.64–1.68)	0.874
CC	1 (0.9)	23 (4.6)	0.23 (0.03–1.78)	0.158
AA	76 (64.9)	311 (62.5)	1 (Reference)	
CC+CA vs. AA			0.94 (0.59–1.51)	0.811
CC vs. CA+AA			0.22 (0.03–1.74)	0.153

aOR – adjusted odds ratio; CI, confidence interval.

Table 3.9.4. *Distribution of RNF7 gene polymorphisms in non-hepatitis C virus-induced cirrhosis and control groups*

Allele/Genotypes	**Non hepatitisC virus-induced cirrhosis (n=200) n (%)**	**Controls (n=498) n (%)**	**aOR (95% CI)**	**P**
RNF7 rs16851720				
C	36 (18.0)	105 (21.1)	0.78 (0.57–1.08)	0.129
A	164 (82.0)	393 (78.9)		
CA	66 (33.0)	164 (32.9)	0.91 (0.63–1.33)	0.632
CC	3 (1.5)	23 (4.6)	0.28 (0.08–1.00)	**0.050**
AA	131 (65.5)	311 (62.5)	1 (Reference)	
CC+CA vs. AA			0.83 (0.58–1.20)	0.327
CC vs. CA+AA			0.29 (0.08–1.03)	0.055

aOR – adjusted odds ratio; CI – confidence interval;
significant *P*–values are marked in bold.

The distributions of alleles and genotypes of SNPs are presented in Tables 3.9.5 and 3.9.6. *PCSK7* rs236918 was not linked with liver cirrhosis in HCV(aOD: 0.86, CI: 0.54–1.37, P=0.517, Table 3.9.5) or non-HCV patients aOD: 0.86 CI: 0.60–1.24, P=0.416, Table 3.9.6).

***Table 3.9.5**. Distribution of gene PCSK7 polymorphisms in hepatitis C virus-induced cirrhosis and control groups*

Allele/Genotypes	HepatitisC virus-induced cirrhosis (n=117)	Controls (n=498) n (%)	aOR (95% CI)	P
PCSK7 rs236918				
C	14 (12.0)	69 (13.9)	0.86(0.54–1.37)	0.517
G	103 (88.0)	429(86.1)		
CG	26 (22.2)	114(22.9)	1.02(0.60–1.74)	0.951
CC	1 (0.9)	12 (2.4)	0.25(0.03–2.26)	0.216
GG	90 (76.9)	372(74.7)	1(Reference)	
CC+CT vs. TT			0.93(0.55–1.56)	0.773
CC vs. CG+GG			0.25(0.03–2.25)	0.214

aOR – adjusted odds ratio; CI – confidence interval .

***Table 3.9.6.** Distribution of gene PCSK7 polymorphisms in non-hepatitis C virus-induced cirrhosis and control groups*

Allele/Genotypes	Non- hepatitis Cvirus-induced cirrhosis (n=200)	Controls (n=498) n (%)	aOR (95% CI)	P
PCSK7 rs236918				
C	25 (12.5)	69 (13.9)	0.86(0.60–1.24)	0.416
G	175 (87.5)	429(86.1)		
CG	44 (22.0)	114(22.9)	0.93(0.61–1.42)	0.747
CC	3 (1.5)	12 (2.4)	0.49(0.12–1.93)	0.306
GG	153 (76.5)	372(74.7)	1(Reference)	
CC+CT vs. TT			0.89(0.59–1.34)	0.562
CC vs. CG+GG			0.49(0.12–1.96)	0.316

aOR – adjusted odds ratio; CI – confidence interval.

The distributions of alleles and genotypes of *MERKT* SNP were similar between the control, cirrhosis in HCV and non-HCV groups. The risk allele A of *MERKT* rs4374383 was detected in 36.1% of controls, 41.0% of hepatitis C virus-induced cirrhosis group and 36.8% of non-hepatitis C virus-induced cirrhosis group (P>0.05; Tables 3.9.7 and 3.9.8). rs4374383

(*MERTK*) was not linked with liver cirrhosis in HCV (aOD: 1.16, CI: 0.84–1.61; P = 0.355, Table 3.9.7) or non-HCV patients (aOD:1.09, CI: 0.84–1.40; P=0.513 Table 3.9.8).

Table 3.9.7. *Distribution of gene MERTK polymorphisms in hepatitis C virus-induced cirrhosis and control groups*

Allele/Genotypes	**HepatitisC virus–induced cirrhosis (n=117) n (%)**	**Controls (n=498) n (%)**	**aOR (95% CI)**	**P**
MERTK rs4374383				
A	48 (41.0)	180 (36.1)	1.16 (0.84–1.61)	0.355
G	69 (59.0)	318 (63.9)		
AG	54 (46.2)	216 (43.4)	1.09 (0.66–1.80)	0.726
AA	42 (35.9)	72 (14.5)	1.40 (0.72–2.75)	0.324
GG	21 (17.9)	210 (42.1)	1 (Reference)	
AA+AG vs. GG			1.17 (0.73–1.86)	0.522
AA vs. AG+GG			1.34 (0.72–2.47)	0.355

aOR – adjusted odds ratio; CI – confidence interval.

Table 3.9.8. *Distribution of gene MERTK polymorphisms in non - hepatitis C virus-induced cirrhosis and control groups*

Allele/Genotypes	**Non-hepatitis C virus-induced cirrhosis (n=200) n (%)**	**Controls (n=498) n (%)**	**OR (95% CI)**	**P**
MERTK rs4374383				
A	74 (36.8)	180 (36.1)	1.09 (0.84–1.40)	0.513
G	126 (63.2)	318 (63.9)		
AG	95 (47.5)	216 (43.4)	1.13 (0.77–1.64)	0.536
AA	79 (39.5)	72 (14.5)	1.16 (0.67–2.00)	0.491
GG	26 (13)	210 (42.1)	1 (Reference)	
AA+AG vs. GG			1.13 (0.79–1.62)	0.491
AA vs. AG+GG			1.09 (0.65–1.80)	0.749

OR, odds ratio; CI – confidence interval.

3.10. Association analysis of combined *PNPLA3* and *RNF7* gene single nucleotide polymorphisms effects on hepatitis C virus – induced liver fibrosis and liver cirrhosis

In order to evaluate the combined effect of *PNPLA3* and *RNF7* SNPs genotypes on association with HCV fibrosis or cirrhosis, we performed an additional analysis. Table 3.10.1 and Table 3.10.2 represents the frequencies of combined *PNPLA3* and *RNF7* SNPs genotypes in HCV induced cirrhosis, and HCV induced fibrosis and controls. Chi-squared test and Fisher Exact probability tests were used to compare combined SNP genotypes frequencies between groups. Each combination of *PNLPA3* and *RNF7* genotypes was compared with the rest of *PNPLA3* and *RNF7* genotypes combinations. CC_{PNPLA3} CC_{RNF7} and CC_{PNPLA3} AA_{RNF7} genotype combined effect showed reduced risk in developing HCV induced cirrhosis (OD:0.63, CI:0.41–0.96, P=0.033; P=0.032 respectively; Table 3.10.1). CG_{PNPLA3} AA_{RNF7} and GG_{PNPLA3} AA_{RNF7} genotype combinations were associated with HCV cirrhosis (OD: 1.69, CI: 1.04–2.76, P=0.035; OD: 3.11, CI: 1.30–7.46, P=0.011, respectively; Table 3.10.1). Combined effect of GG_{PNPLA3} CA_{RNF7} genotype revealed a significantly asocciation in HCV induced fibrosis (OD: 5.98, CI: 1.66–21.50, P=0.006; Table 3.10.2).

Table 3.10.1. *Combined effects of PNPLA3 and RNF7 gene polymorphisms on hepatitis C virus-induced liver cirrhosis*

Combined genotypes	Hepatitis C virus–induced cirrhosis (n=117) n (%)	Controls (n=498)	OR (95% CI)	P
CC_{PNPLA3} CC_{RNF7}	0 (0.0)	19 (3.8)		**0.032**
CC_{PNPLA3} CA_{RNF7}	17(14.5)	102 (20.5)	0.66(0.38–1.15)	0.145
CC_{PNPLA3} AA_{RNF7}	39(33.3)	220 (44.2)	0.63(0.41–0.96)	**0.033**
CG_{PNPLA3} CC_{RNF7}	1 (0.9)	4 (0.8)	1.0 (0.18–9.61)	1.000
CG_{PNPLA3} CA_{RNF7}	20(17.1)	58 (11.6)	1.56(0.90–2.72)	0.113
CG_{PNPLA3} AA_{RNF7}	28(23.9)	78 (15.7)	1.69(1.04–2.76)	**0.035**
GG_{PNPLA3} CC_{RNF7}	0 (0.0)	0 (0.0)	–	
GG_{PNPLA3} CA_{RNF7}	3 (2.6)	4 (0.8)	1.69(1.04–2.76)	0.126
GG_{PNPLA3} AA_{RNF7}	9 (7.7)	13 (2.6)	3.11(1.30–7.46)	**0.011**

OR – odds ratio; CI – confidence interval; significant p-values are marked in bold.

Table 3.10.2. *Combined effects of PNPLA3 and RNF7 gene polymorphisms on hepatitis C virus-induced liver fibrosis*

Combined genotypes	**Hepatitis C virus-induced fibrosis (n=130) n (%)**	**Controls (n=498)**	**OR (95% CI)**	**P**
CC_{PNPLA3} CC_{RNF7}	8 (1.5)	19 (3.8)	1.65(0.71–3.87)	0.246
CC_{PNPLA3} CA_{RNF7}	20 (15.4)	102 (20.5)	0.71(0.42–1.19)	0.193
CC_{PNPLA3} AA_{RNF7}	57 (43.8)	220 (44.2)	0.99(0.67–1.46)	0.946
CG_{PNPLA3} CC_{RNF7}	1 (0.8)	4 (0.8)	0.96(0.11–8.64)	0.969
CG_{PNPLA3} CA_{RNF7}	11 (8.5)	58 (11.6)	0.70(0.36–1.38)	0.303
CG_{PNPLA3} AA_{RNF7}	25 (19.2)	78 (15.7)	1.28(0.78–2.11)	0.329
GG_{PNPLA3} CC_{RNF7}	0 (0.0)	0 (0.0)	–	
GG_{PNPLA3} CA_{RNF7}	6 (4.6)	4 (0.8)	5.98(1.66–21.50)	**0.006**
GG_{PNPLA3} AA_{RNF7}	8 (6.2)	13 (2.6)	2.45(0.99–6.03)	0.052

OR – odds ratio; CI – confidence interval; significant p-values are marked in bold.

4. DISCUSSION

4.1. *HFE* gene C282Y and H63D mutations in liver cirrhosis

The major finding in our first study part is significant gender related association of C282Y and H63D mutations in the *HFE* gene with liver cirrhosis in the Lithuanian population. The impact of *HFE* mutations was found to be significant in male, but not in female subjects. These findings suggest that *HFE* mutations may contribute to hepatic fibrogenesis process during the natural history of chronic liver diseases. Individuals with chronic liver diseases may have mild to moderate iron overload, but the mechanism is not fully understood [89]. An increased hepatic iron content is known to have the potency to exacerbate liver injury [12, 90]. Different groups have provided evidences that level of iron near the upper limit of normal is associated with different pathological processes including cardiovascular diseases and even cancer [56, 91, 92]. As mentioned before C282Y and H63D are the most common mutations causing HH in Caucasians, but have lower penetration and cannot be responsible alone for the development of HH [39, 50]. Different studies showed that elevated liver enzymes were observed only in 30% of males, while elevated transferrin saturation in combination with an elevated ferritin was present in 43.4% of males and 23.3% of females homozygous for C282Y [39] and cirrhosis was diagnosed only in 6% of males and in 2% of females in a population-based screening setting among C282Y homozygotes [93]. Studies in different populations examining the relationship between *HFE* mutations and chronic liver diseases have produced varying outcomes. A study including 587 patients from Italy with NAFLD and 184 control subjects did not find a link between *HFE* mutations and hepatocellular iron accumulation [12]. A Canadian study has demonstrated that Caucasian C282Y heterozygotes were more likely to have bridging fibrosis or cirrhosis in non-alcoholic steatohepatitis (NASH)[45]. A Polish study showed a trend toward a more common occurrence of ALD in homozygous individuals for the H63D mutation [94], while another study found no differences in the prevalence of *HFE* mutations between Polish cirrhotic patients and healthy individuals [95]. A Germany study in patients with chronic HCV infection showed that C282Y or H63D heterozygosity is an independent risk factor for liver fibrosis and cirrhosis [96]. Whereas Scottish study has shown that carriage of *HFE* mutations does not have any role in the accumulation of iron or the progression of liver disease in HCV infection [57]. A Czech study also has demonstrated that *HFE* mutations do not play an important role in the pathogenesis of chronic

hepatitis B, chronic hepatitis C or alcoholic liver disease.[97] Varying results between previous studies have urged us to examine the above-discussed associations in a cohort of Lithuanian cirrhotic patients. The results of our study support those studies that have revealed a significant role of C282Y and H63D mutations in liver cirrhosis in Caucasian populations. Overall analysis has revealed that C282Y mutation is associated with liver cirrhosis in our study population, but this observation has not been found for H63D carriers. Gender based stratification analysis of our data revealed that carriage of *HFE* risk allele C282Y was associated with liver cirrhosis in males, but not in females. Association of liver cirrhosis in men was also evident among the carriers of heterozygous C282Y genotype. Furthermore, in males the carriage of homozygous H63D genotypes were also associated with liver cirrhosis, while this relationship was not present in females. There was a trend for increased risk of liver cirrhosis among female carriers of C282Y mutation, but due to a relatively small sample size the difference did not reach statistical significance. It is well known that the penetrance of *HFE* C282Y homozygous subjects is higher in males than in females, which due to physiological mechanisms, are less likely to develop iron overload [89]. The strength of our study is a large, well-selected control group, which offers a good representation of the overall Lithuanian population, and which has been used for determination of *HFE* gene mutation frequencies in Lithuania [83]. The major limitation of our research is the retrospective design of the study. For this reason, full-scale information on iron parameters including ferritin levels, transferrin saturation and hepatic iron content was available only for a small proportion of cirrhotic patients. Due to a relatively small sample size, subgroup analysis in different etiological entities of liver cirrhosis (hepatitis C and B, alcoholic liver disease, etc.) was not performed. We admit that the relatively small and heterogeneous sample size of cirrhotic patients in our study does not carry high statistical power; however, significant associations between *HFE* mutations and liver cirrhosis determined by our group suggest a possible role of these genetic alterations in chronic liver diseases.

4.2. PNPLA3, RNF7, MERTK, PCSK7 gene variants association with liver fibrosis and cirrhosis

In the second part our study we aimed to replicate the associations between key *PNPLA3, RNF7, MERTK* and *PCSK7* gene polymorphisms and liver fibrosis and cirrhosis that have been reported in recent GWAS. We show that *PNPLA3 rs738409* is associated both with liver fibrosis and cirrhosis and that *RNF7 rs16851720* is associated with liver cirrhosis. Also

revealed that *PNPLA3 rs738409* is associated with liver cirrhosis both in HCV and non-HCV patients. The major finding in our second part of the study is that *PNPLA3* rs738409 is linked to liver fibrosis and cirrhosis, confirming an association that has been reported in distinct ethnic groups [14, 16, 98, 99]. We confirmed this association both in HCV and non-HCV cirrhosis groups. Highly significant differences and odds ratios observed for liver fibrosis and cirrhosis point the importance of this genetic variant for liver damage.

A large GWAS reported that in the combined cohort of 2,342 HCV-infected patients *RNF7*rs16851720 were associated with fibrosis progression. [17] The results of our study support these findings, because the rs16851720 AA genotype conferred asocciation with liver cirrhosis. We did not observe differences in genotype frequencies between controls and patients with liver fibrosis for *RNF7* rs16851720. In order to evaluate the combined effect of *PNPLA3* and *RNF7* SNPs genotypes on association with HCVfibrosis or cirrhosis, we performed an additional analysis. Interestingly, combined GG_{PNPLA3} CA_{RNF7} genotype revealed a very high odds ratio of 5.98 (P=0.006) for HCV related fibrosis;. However, the subgroups of patients within this analysis were not very large and these findings must be validated in larger groups of patients.

A recent meta-analysis of GWAS results revealed a novel association between variation in *PCSK7* gene and iron overload [18]. *Stickel* et al. Reported that the *PCSK7* variant rs236918 was a risk factor for the development of cirrhosis in HH patients homozygous for the *HFE* p.C282Y mutation [35]. Hypothesizing that this SNP might contribute to the development of liver damage in other chronic liver diseases, we genotyped PCSK7 rs236918 in our cohort of patients with liver fibrosis and cirrhosis. Based on our findings, alleles and genotypes of this SNP were distributed equally between the study groups. A recent Italian study has confirmed that the *PCSK7* rs236918 C allele represents a risk factor for cirrhosis development in Italian patients with HH [36]. Replication studies on *PCSK7* rs236918 in patients with liver diseases are scarce. Further large scale studies are needed to evaluate whether the *PCSK7* SNP affects the development of liver cirrhosis in other patients than HH.

In our study *MERTK* rs4374383 SNP alleles and genotypes were distributed equally between the control, liver fibrosis and cirrhosis groups. GWAS reported association between *MERTK* rs4374383 and development of fibrosis in chronic HCV disease. A Swiss study showed that *MERTK* SNP significantly added risk of accelerated fibrosis progression rate in the chronic HCV disease [15]. One of the potential explanations for differences between previously published data and our results could be linked to the

sample size of our patient groups. Our study has certain limitations that need to be acknowledged. Firstly, the cohort of our cirrhotic patients is heterogonous with respect to etiology and specific genetic variants might be more important for different types of cirrhosis. In particular, we couldnot assess the association between SNPs and risk of NAFLD related cirrhosis, because we had only very few patients with this diagnosis in our study group. We also did not have complete information on alcohol consumption, metabolic syndrome and diabetes status, that might be modify the effects on analyzed genetic variants. Our liver fibrosis and fibrosis cohorts were also not very large and might be underpowered to detected subtle genetic associations. The groups of patients with different liver function impairment levels were also not very large and this aspect needs to be reassessed in further larger scale studies.

5. CONCLUSION

1. Heterozygous C282Y mutation of the *HFE* gene is associated with liver cirrhosis in Lithuanian populiation.
2. In gender-related analysis heterozygous C282Y and homozygous H63D mutations are linked with liver cirrhosis in men, but not in women.
3. *PNPLA3* rs738409 and *RNF7* rs16851720 are associated with liver fibrosis, whereas *MERTK* rs4374383 and *PCSK7* rs236918 SNPs are not linked with liver fibrosis in Lithuanian population.
4. *PNPLA3* rs738409 and *RNF7* rs16851720 are associated with liver cirrhosis, however *MERTK* rs4374383 and *PCSK7* rs236918 SNPs are not linked with liver cirrhosis in Lithuanian populiation.
5. Sub-analysis of single nucleotide polymorhisms based on etiology of liver cirrhosis in both hepatitis C virus-induced and non-hepatitis C virus-induced liver cirrhosis patients groups revealed significant association with *PNPLA3* rs738409 and *RNF7* rs16851720. Analysis of combined effect of *PNPLA3* and *RNF7* SNPs genotypes revealed significant associations between: CG_{PNPLA3} AA_{RNF7} and $GG_{PNPLA3}AA_{RNF7}$ genotype and HCV induced cirrhosis, GG_{PNPLA3} CA_{RNF7} genotype and HCV induced fibrosis.

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LIST OF PUBLICATIONS

1. Publication related to the results of dissertation

1.1. Juzėnas S, Kupčinskas J, Valantienė I, Šumskienė J, Petrenkienė V, Kondrackienė J, et al. Association of HFE gene C282Y and H63D mutations with liver cirrhosis in the Lithuanian population. Medicina (Kaunas) 2016. doi: 10.1016/j.medici.2016.09.004. (Impact factor: 0.609).

1.2. Kupčinskas J, Valantienė I,Varkalaitė G, Steponaitienė R, Skiecevičienė J, Šumskienė, Petrenkienė V, Kondrackienė J, Kiudelis G, Lammert F, Kupčinskas L. *PNPLA3* and *RNF7* gene variants are associated with the risk of developing liver fibrosis and cirrhosis in Eastern European population. J Gastrointestin Liver Dis. 2017, March; 26,(1): 37-43. (Impact factor:1.891) doi:http://dx.doi.org/10.15403/jgld.2014.1121.261.pnp.

LIST OF PRESENTATIONS AT SCIENTIFIC CONFERENCES

The results of dissertation were presented in the scientific conferences:

1. 22nd united European Gastroenterology Week, Vienna, Austria, October 18–22, 2014 Poster presentation: *HFE* gene C282Y and H63D mutations are associated with overall risks of developing liver cirrhosis. Abstract Book. 2014, October; 2,(1): 452, doi. 10.1177/2050640614 548980.

2. 23nd united European Gastroenterology Week, Barselona, Spain, October 24–28, 2015 Poster presentation: Association of Genetic Variants in *PNPLA3*, *MERTK*, *PCSK7* and *RNF7* with Liver Cirrhosis. Abstract Book. 2015,October; 3,(5): 333-334, doi: 10,1177/205064061 623.

3. 24nd united European Gastroenterology Week, Vienna, Austria, October 15–19, 2016 Poster presentation: The role of *PNPLA3*, *RNF7*, *MERTK* and *PCSK7* gene polymorphisms in the development of liver fibrosis and cirrhosis. Abstract Book. 2016, October;4, (5): 348-348, doi 10.1177/2050640616663689.

4. 75th conference of the University of Latvia, Riga, February 24, 2017 Oral presentation: The role of *PNPLA3*, *RNF7*, *MERTK* and *PCSK7* gene polymorphisms in the development of liver fibrosis and cirrhosis. Abstract Book. 2017, February; 3: 75-75.

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Original Research Article

Association of *HFE* gene C282Y and H63D mutations with liver cirrhosis in the Lithuanian population

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Liver cirrhosis

ABSTRACT

Background and objective: Liver cirrhosis is the end-stage disease of chronic liver injury. Due to differences in the natural course of chronic liver diseases, identification of genetic factors that influence individual outcomes is warranted. *HFE*-linked hereditary hemochromatosis (HH) predisposes disease progression to cirrhosis; however, the role of heterozygous C282Y or H63D mutations in the development of cirrhosis in the presence of other etiological factors is still debated. The aim of this study was to determine the association between heterozygous C282Y and H63D mutations and non-HH liver cirrhosis in Lithuanian population.

Materials and methods: The patient cohort consisted of 209 individuals. Diagnosis of cirrhosis was confirmed by clinical, laboratory parameters, liver biopsy, and radiological imaging. Control samples were obtained from 1005 randomly selected unrelated healthy individuals. *HFE* gene mutations were determined using the PCR-RFLP method.

Results: The most common causes of cirrhosis were hepatitis C (33.9%), hepatitis B (13.6%), and alcohol (25.8%). C282Y allele was associated with the presence of cirrhosis (OR = 2.07; P = 0.005); this was also observed under recessive model for C282Y (OR = 2.06, P = 0.008). The prevalence of C282Y allele was higher in cirrhotic men than in controls (7.0% vs. 2.8%, P = 0.002). The carriage of H63D risk allele (OR = 1.54; P = 0.02), heterozygous C282Y/wt and homozygous H63D/H63D genotypes were associated with liver cirrhosis in males (OR = 2.48, P = 0.008, and OR = 4.13, P = 0.005, respectively).

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Conclusions: Heterozygous C282Y mutation of the *HFE* gene was associated with liver cirrhosis in the Lithuanian population. In gender-related analysis, heterozygous C282Y and homozygous H63D mutations were linked to liver cirrhosis in men, not in women.

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1. Introduction

Liver cirrhosis is the end-stage disease of chronic liver injury. Cirrhosis is caused by different etiological factors; however, progression of liver injury varies considerably among individuals independently of the cause [1]. Different research groups over the last decade have attempted to identify crucial cofactors that contribute to the development of liver damage [2,3]. A growing number of studies show that, apart from the main underlying causative agent in liver cirrhosis, the process may be reinforced by confounding factors such as diet, alcohol consumption, etc. [4–6]. Interindividual variation of time span from normal liver to fibrotic and cirrhotic stages suggested potential influence of congenital variations. Advances in genotyping techniques allowed to identify coexisting genetic alterations in relation to liver fibrosis [6] and cirrhosis of different etiologies [4,7].

C282Y and H63D mutations of the *HFE* gene are now recognized as the most common genetic disorders in populations of European ancestry. Carriage of heterozygous hemochromatosis (HH) gene mutations has been attributed as the risk factor of iron overload and liver damage, but equivocal conclusion on the role of these mutations has not been achieved [8,9]. The rationale that suggested iron as a susceptible hepatotoxic factor is based on the ability of this metal to induce oxidative stress by stimulating free radical formation in liver tissue [10,11]. Furthermore, increased contents of iron have been attributed to progression to liver cirrhosis of chronic viral hepatitis C (HCV) infection [12], nonalcoholic fatty liver disease (NAFLD) [11] or alcoholic liver disease (ALD) [13].

As noted above, development of liver cirrhosis regardless of etiology in separate individuals may have enormous variation in terms of time frame and severity. Carriage of *HFE* gene mutations has been linked with increased risk of liver fibrosis or liver cirrhosis; however, published studies report conflicting results [8]. The presence of the C282Y mutation was associated with more advanced degrees of fibrosis or cirrhosis [12,14], but these findings were not confirmed in other studies [11,15]. The prevalence of *HFE* C282Y mutations varies significantly across Europe, with highest estimated in Ireland (>10%), intermediate frequencies (2.7%–7%) in neighboring countries Latvia [16] and Poland [17], and very low rates of (0%–2%) in Mediterranean areas [18]. *HFE* H63D mutation also occurs at different frequencies in separate regions [18]. Therefore, the discordance among the findings in previous studies on association of *HFE* mutations with non-HH liver cirrhosis/fibrosis might be related to variations in study design and differences in *HFE* mutation prevalence in individual populations.

In this study we performed analysis of *HFE* gene C282Y and H63D mutations in consecutive 209 cirrhotic patients and 1005 voluntary, unrelated blood donors of the Caucasian ethnicity. The aim of this study was to determine the association between *HFE* gene C282Y and H63D mutations and liver cirrhosis in the Lithuanian population. This was the first study assessing the prevalence of *HFE* gene mutations in Lithuanian cirrhotic patients and adds additional insights on the impact of *HFE* mutations in development of non-HH cirrhosis.

2. Materials and methods

2.1. Patients and control subjects

A cohort of liver cirrhosis patients consisted of 209 consecutive patients referred to the Department of Gastroenterology, Hospital of the Lithuanian University of Health Sciences. The diagnosis and etiology of liver cirrhosis was confirmed by laboratory tests, clinical features, liver biopsy and radiological imaging tests. ALD was confirmed when daily consumption of alcohol was >30/20 g/day for males/females, respectively, as confirmed by at least 1 family member of affected individuals [19]. Control samples came from our previous genotyping study on the prevalence of *HFE* mutations in the Lithuanian population [20] and included 1005 voluntary, unrelated Lithuanian blood donors. The study design met ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Lithuanian Bioethics Committee (Protocol No. 2/2008) and Kaunas Regional Biomedical Research Ethics Committee (Protocol No. BE-2-10). Informed consent was obtained from all participants.

2.2. DNA extraction and genotyping

Genomic DNA was isolated from whole blood containing EDTA by using salting-out procedure. *HFE* mutations C282Y c.845 G>A (p.Cys282Tyr) and H63D c.187 C>G (p.His63Asp) were detected after DNA amplification by polymerase chain reaction and restriction with RsaI (for C282Y) and BclI (for H63D). For identification of the C282Y mutation, the fragment was amplified using primer forward 5′-TCCAGTCTTCCTGGCAA-3′ and primer reverse 5′-TTCTAGCTCCTGGCTCTCA-3′. The exon 2 containing S65C and H63D mutations were amplified with primer forward 5′-TGTGGAGCCTCAACATCCT-3′ and primer reverse 5′-TGAAAAGCTCTGACAACCTCA-3′. PCR amplification was performed in a total volume of 25 μl, which contained 100 ng of genomic DNA, 200 μM of each dNTP, 200 nM of each primer, 1.0 mM $MgCl_2$, 10× PCR buffer solution, and 2.5 U Taq polymerase (Thermo Scientific, Vilnius, Lithuania). PCR

consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s with final extension at 72 °C for 10 min. The restrictions reactions were performed according manufacturer's protocol. The RFLP fragments were analyzed electrophoretically in 3% of agarose gel.

2.3. Statistical analysis

The distribution of *HFE* genotypes in both cases and controls was examined for deviation from Hardy Weinberg equilibrium (HWE) using the chi-square (χ^2) test in each single nucleotide polymorphism (SNP). Comparisons of carriage frequencies for alleles between cases and controls were analyzed by Pearson chi-square and Fisher exact tests. Association analysis based on the case-control design was performed for each SNP by using the Armitage trend test. To estimate relative risks for mutations, odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated using recessive and dominant models. A P value of 0.05 was defined to be statistically significant. Statistical analysis was performed using statistical software for genetic association studies PLINK v2.050 [21].

3. Results

3.1. Characteristics of the study group

The characteristics of the study groups are presented in Table 1. The group of cirrhotic patients consisted of 209 individuals: 107 men and 102 women with a mean age of 54.0 years (range, 25–84 years). The most common cause of liver cirrhosis was HCV infection and alcohol consumption. Distribution of Child–Pugh classes A, B, and C in the cirrhotic group was 31.1%, 47.8%, and 21.1%, respectively. The control group consisted of 1005 individuals: 581 men and 424 women with a mean age of 37.1 years (range, 18–65 years). In more detail, the control group was described in our previous study [20].

Table 1 – Characteristics of the subject groups.

	Liver cirrhosis (*n* = 209)	Controls (*n* = 1005)
Gender, *n* (%)		
Male	107 (51.2)	581 (57.8)
Female	102 (48.8)	424 (42.2)
Age, years (SD)	54.0 (8.1)	37.1 (4.3)
Etiology of liver cirrhosis, *n* (%)[a]		
HCV	71 (33.9)	
HBV	28 (13.6)	
Autoimmune	15 (7.1)	
Alcohol	54 (25.8)	
Other causes	12 (5.7)	
Cryptogenic	19 (9.1)	
Child–Pugh class, *n* (%)		
A	65 (31.1)	
B	100 (47.8)	
C	44 (21.1)	

HBV, hepatitis B virus; HCV, hepatitis C virus.
[a] 17 of HCV and 5 of HBV patients had a mixed (viral and alcohol) etiology.

3.2. Association between HFE C282Y mutation and liver cirrhosis

The association between *HFE* C282Y mutation and liver cirrhosis is presented in Table 2. The carriage of C282Y risk allele was significantly more frequent in patients with liver cirrhosis than in controls (OR = 2.1, P = 0.005). This association was also evident in genotypic analysis where heterozygous genotype C282Y/wt and wt/wt carriers were compared (OR = 2.0, P = 0.01) and in recessive model (OR = 2.1, P = 0.007).

Table 2 – The frequencies of *HFE* C282Y and H63D mutations in cirrhotic patients and controls.

Genotype	Liver cirrhosis (*n* = 209) *n* (%)	Controls (*n* = 1005) *n* (%)	OR (95% CI)	P
H63D				
wt/wt	142 (67.9)	712 (70.84)	1.00 (reference)	
H63D/wt	58 (27.8)	267 (26.57)	1.09 (0.78–1.53)	0.618
H63D/H63D	9 (4.3)	26 (2.58)	1.74 (0.80–3.78)	0.161
wt/wt vs H63D/wt + H63D/H63D			1.15 (0.83–1.58)	0.403
wt/wt + H63D/wt vs. H63D/H63D			0.59 (0.83–1.58)	0.403
wt allele	342 (81.9)	1682 (84.47)		
H63D allele	76 (18.1)	320 (15.53)	1.18 (0.89–1.55)	0.244
C282Y				
wt/wt	189 (90.43)	956 (95.5)	1.00 (reference)	
C282Y/wt	19 (9.09)	48 (4.79)	2 (1.15–3.48)	**0.012**
C282Y/C282Y	1 (0.48)	1 (0.09)	5.06 (0.32–81.22)	0.203
wt/wt vs. C282Y/wt + C282Y/C282Y			2.07 (1.20–3.55)	**0.008**
wt/wt + C282Y/wt vs. C282Y/C282Y			0.21 (0.01–3.33)	0.219
wt allele	397 (94.98)	1952 (97.51)		
C282Y allele	21 (5.02)	50 (2.49)	2.07 (1.23–3.49)	**0.005**
C282Y/H63D	6 (2.87)	13 (1.29)	2.26 (0.85–6.00)	0.118

OR, odds ratio; CI, confidence interval; In bold, significant p-values.

Table 3 – The frequencies of *HFE* C282Y and H63D mutations in male cirrhotic patients and controls.

Genotype	Men with liver cirrhosis (*n* = 107) *n* (%)	Controls (*n* = 581) *n* (%)	OR (95% CI)	P
H63D				
wt/wt	67 (62.61)	415 (71.41)	1.00 (reference)	
H63D/wt	34 (31.77)	157 (27.02)	1.34 (0.85–2.11)	0.202
H63D/H63D	6 (5.62)	9 (1.57)	4.13 (1.42–11.98)	**0.005**
wt/wt vs H63D/wt + H63D/H63D			1.49 (0.97–2.30)	0.067
wt/wt + H63D/wt vs. H63D/H63D			0.27 (0.09–0.76)	**0.008**
wt allele	168 (78.51)	987 (84.94)		
H63D allele	46 (21.49)	175 (15.06)	1.54 (1.07–2.22)	**0.019**
C282Y				
wt/wt	93 (86.92)	549 (94.49)	1.00 (reference)	
C282Y/wt	13 (12.15)	31 (5.33)	2.48 (1.25–4.91)	**0.008**
C282Y/C282Y	1 (0.93)	1 (0.18)	5.90 (0.37–95.20)	0.155
wt/wt vs. C282Y/wt + C282Y/C282Y			2.58 (1.33–5.02)	**0.004**
wt/wt + C282Y/wt vs. C282Y/C282Y			0.18 (0.01–2.95)	0.178
wt allele	199 (92.99)	1129 (97.16)		
C282Y allele	15 (7.01)	33 (2.84)	2.58 (1.38–4.84)	**0.002**
C282Y/H63D	5 (4.67)	12 (2.06)	2.32 (0.80–6.73)	0.163

OR, odds ratio; CI, confidence interval; In bold, significant *p*-values.

Gender-based stratification analysis revealed significant gender-related differences in the carriage of C282Y mutation between liver cirrhosis patients and controls (Tables 3 and 4). The carriage of heterozygous C282Y/wt genotype in men was associated with liver cirrhosis (OR = 2.48, P = 0.008), whereas no significant associations were found in the female group (Table 4).

3.3. *Association between HFE H63D mutation and liver cirrhosis*

Genotyping analysis of *HFE* gene H63D mutation did not reveal significant association with liver cirrhosis (Table 2). The carriage of H63D alleles was distributed equally in the control (15.5%) and cirrhotic groups (18.1%, P = 0.244). However, significant gender-related differences were revealed in carriage of H63D mutation between liver cirrhosis patients and controls (Tables 3 and 4). The carriage of H63D risk allele (OR = 1.54, P = 0.02) and homozygous H63D/H63D genotypes (OR = 4.13, P = 0.005) were associated with liver cirrhosis in men, but not women.

3.4. *Association between the carriage of two or more HFE gene alleles and liver cirrhosis*

Carriage of two or more risk alleles was significantly higher in the group of patients with liver cirrhosis than in controls and resulted in significant association (7.66% vs. 3.98%, P = 0.021;

Table 4 – The frequencies of *HFE* C282Y and H63D mutations in female cirrhotic patients and controls.

Genotype	Women with liver cirrhosis (*n* = 102) *n* (%)	Controls (*n* = 424) *n* (%)	OR (95% CI)	P
H63D				
wt/wt	75 (73.53)	297 (70.05)	1.00 (reference)	
H63D/wt	24 (23.53)	110 (25.94)	0.86 (0.52–1.44)	0.573
H63D/H63D	3 (2.94)	17 (4.01)	0.70 (0.20–2.45)	0.573
wt/wt vs H63D/wt + H63D/H63D			0.84 (0.52–1.37)	0.488
wt/wt + H63D/wt vs. H63D/H63D			1.38 (0.40–4.80)	0.613
wt allele	174 (85.29)	704 (83.02)		
H63D allele	30 (14.71)	144 (16.98)	0.84 (0.55–1.29)	0.432
C282Y				
wt/wt	96 (94.12)	407 (95.91)	1.00 (reference)	
C282Y/wt	6 (5.88)	17 (4.01)	1.50 (0.58–3.90)	0.406
C282Y/C282Y	0 (0)	0 (0)	4.22 (0.08–214.11)	1
wt/wt vs. C282Y/wt + C282Y/C282Y			1.50 (0.58–3.90)	0.406
wt/wt + C282Y/wt vs. C282Y/C282Y			0.24 (0.01–12.24)	1
wt allele	98 (97.06)	407 (97.99)		
C282Y allele	6 (2.94)	17 (2.01)	1.48 (0.58–3.81)	0.412
C282Y/H63D	1 (0.98)	1 (0.24)	4.78 (0.30–77.10)	0.350

OR, odds ratio; CI, confidence interval.

OR = 2.00, 95% CI = 1.10–3.64). Gender-based analysis revealed that in male patients relative risk was increased when bearing two or more risk alleles compared with controls (11.22% vs. 3.78%, P = 0.001; OR = 3.22, 95% CI = 1.54–6.71), whereas no significant difference was observed in women. The prevalence of particular C282Y/H63D compound heterozygous genotype in Lithuanian cirrhotic patients is presented in Table 2. Only a small number of individuals were carriers of the C282Y/H63D genotype, and the difference between cirrhotic patients and controls was not significant.

4. Discussion

The major finding of our present study is a significant gender-related association of C282Y and H63D mutations in the *HFE* gene with liver cirrhosis in the Lithuanian population. The impact of *HFE* mutations was found to be significant in male, but not in female subjects. These data suggest that *HFE* mutations may contribute to hepatic fibrogenesis process during the natural history of chronic liver diseases. This is the first study to assess the prevalence of *HFE* gene C282Y and H63D mutations in Lithuanian cirrhotic patients.

Individuals with chronic liver diseases may have mild to moderate iron overload, but the mechanism behind this phenomenon in not fully understood [22]. Increased hepatic iron content is known to have the potential to exacerbate liver injury [10,11]. Furthermore, different groups have provided evidence that levels of iron near the upper limit of normal are associated with different pathological processes including cardiovascular diseases and even cancer [23–25]. C282Y and H63D are the most common mutations causing HH in Caucasians, but studies over the last years have revealed that these mutations have lower penetration than previously estimated, and cannot be advocated alone for the development of HH [26]. Elevated liver enzymes were observed only in 30% of males, while elevated transferrin saturation in combination with an elevated ferritin was present in 43.4% of males and 23.3% of females homozygous for C282Y [26]. Further studies showed that cirrhosis was diagnosed only in 6% of males and in 2% of females in a population-based screening setting among C282Y homozygotes [27]. Nevertheless, even carriage of heterozygous C282Y and H63D variants has been suggested to increase iron overload and exacerbate chronic non-HH related liver injury [12,14].

Studies in different populations examining the relationship between *HFE* mutations and chronic liver diseases have produced varying outcomes. A study including 587 patients from Italy with NAFLD and 184 control subjects did not find a link between *HFE* mutations and hepatocellular iron accumulation [11]. A Canadian study has demonstrated that Caucasian C282Y heterozygotes were more likely to have bridging fibrosis or cirrhosis in non-alcoholic steatohepatitis (NASH) [14], meanwhile this link was not present in a study by Chitturi et al. [15]. A Polish study conducted by Raszeja-Wyszomirska et al. showed a trend toward a more common occurrence of ALD in individuals homozygous for the H63D mutation [28], while another study found no differences in the prevalence of *HFE* mutations between Polish cirrhotic patients and healthy individuals [29]. C282Y or H63D heterozygosity was found as an independent risk factor for liver fibrosis and cirrhosis in a German study including 401 patients with chronic HCV infection and 295 healthy controls [30]. The presence of *HFE* mutations was independently associated with iron loading and advanced fibrosis in patients with HCV, especially after controlling for duration of disease [12]. Whereas, a Scottish study has shown that carriage of *HFE* mutations does not have any role in the accumulation of iron or the progression of liver disease in HCV infection [31]. Similar results were observed in the Czech study which has demonstrated that *HFE* mutations do not play an important role in the pathogenesis of chronic hepatitis B, chronic hepatitis C or alcoholic liver disease [32]. Interestingly, an Indian study has observed significant associations of common *HFE* mutations (C282Y and H63D) with HCV and ALD related liver cirrhosis, even though the mutations are relatively rare in this population [33]. Another study in non-European population has suggested that iron overload and *HFE* gene mutations do not play a primary role in cryptogenic cirrhosis in the south Iranian population [34].

Varying results between previous studies have urged us to examine the above-discussed associations in a cohort of Lithuanian cirrhotic patients. The results of our study support those studies that have revealed a significant role of C282Y and H63D mutations in non HH-related liver cirrhosis in Caucasian populations. Overall analysis has revealed that C282Y mutation is associated with liver cirrhosis in our study population, but this observation has not been found for H63D carriers. Gender based stratification analysis of our data revealed that carriage of *HFE* risk allele C282Y was associated with liver cirrhosis in males, but not in females. Association of liver cirrhosis in men was also evident among the carriers of heterozygous C282Y genotype. Furthermore, in males the carriage of homozygous H63D genotypes were also associated with liver cirrhosis, while this relationship was not present in females. There was a trend for increased risk of liver cirrhosis among female carriers of C282Y mutation, but due to a relatively small sample size the difference did not reach statistical significance. It is well known that the penetrance of *HFE* C282Y homozygous subjects is higher in males than in females, who, due to physiological mechanisms, are less likely to develop iron overload [22,27]. As pointed out by Fargion et al., discrepant results that have been reported on the association between *HFE* mutations and different liver diseases might be influenced by ethnic differences and small sample sizes of the individual studies, as well as by variable penetrance of *HFE* gene mutations [22]. The strength of our study is a large, well-selected control group, which offers a good representation of the overall Lithuanian population, and which has been used for determination of *HFE* gene mutation frequencies in Lithuania [20]. Overall, the ultimate role of *HFE* mutations for chronic liver injury has to be determined in further large-scale, well-designed prospective studies.

The major limitation of our research is the retrospective design of the study. For this reason, full-scale information on iron parameters including ferritin levels, transferrin saturation and hepatic iron content was available only for a small proportion of cirrhotic patients. Due to a relatively small sample size, subgroup analysis in different etiological entities of liver cirrhosis (hepatitis C and B, alcoholic liver disease, etc.) was not performed. In addition, the spectrum of etiology of

cirrhosis in a tertiary-level hospital (one having a liver transplantation unit) patients' cohort could be influenced by referral and selection bias. We admit that the relatively small and heterogeneous sample size of cirrhotic patients in our study does not carry high statistical power; however, significant associations between *HFE* mutations and liver cirrhosis determined by our group suggest a possible role of these genetic alterations in chronic liver diseases.

5. Conclusions

Heterozygous C282Y mutation of the *HFE* gene was associated with liver cirrhosis. In gender-related analysis, heterozygous C282Y and homozygous H63D mutations were linked with liver cirrhosis in men, but not in women.

Conflict of interest

All the authors declare to have no conflict of interest.

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Authors' contribution

S.J. and J.K.: performed genotyping experiments, analyzed data, and wrote the manuscript; I.V.: analyzed and collected data; J.Š., V.P., and J.K.: collected data; L.K.: performed genotyping experiments; J.S.: study design, performed genotyping experiments, and analyzed data; G.K. and L.K.: study design and project supervision.

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PNPLA3 and *RNF7* Gene Variants are Associated with the Risk of Developing Liver Fibrosis and Cirrhosis in an Eastern European Population

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ABSTRACT

Background & Aims: Genome-wide association studies have revealed an association between the risk of developing liver fibrosis or cirrhosis and the single nucleotide polymorphisms (SNPs) of the *PNPLA3*, *RNF7*, *MERTK* and *PCSK7* genes. We aimed to validate these results in an Eastern European population.

Methods: We evaluated the associations between the *PNPLA3* (rs738409), *RNF7* (rs16851720), *MERTK* (rs4374383) and *PCSK7* (rs236918) variants and liver fibrosis and cirrhosis in a series of consecutive patients recruited at the Department of Gastroenterology, Lithuanian University of Health Sciences Hospital, during the period 2012-2015. The study included 317 individuals with liver cirrhosis, 154 individuals with liver fibrosis, and 498 controls. The studied SNPs were determined using RT-PCR TaqMan assays.

Results: *MERTK* and *PCSK7* SNPs were not associated with liver fibrosis or cirrhosis. The *PNPLA3* SNP rs738409 was associated with a higher risk of developing liver fibrosis (aOR: 1.65, P=0.001) and cirrhosis (aOR: 1.92, P=5.57*10^{-7}). *PNPLA3* genotypes were also associated with higher risk of developing liver fibrosis and cirrhosis in dominant (aOR: 1.98, P=2.20*10^{-5}; aOR: 1.67, P=0.008, respectively) and recessive (aOR: 3.94, P=5.16*10^{-5}; aOR: 3.02, P=0.003, respectively) models. *RNF7* rs16851720 was associated with liver cirrhosis comparing CC vs. AA + CA genotypes (aOR: 0.26, P=0.020).

Conclusion: Our study showed that *PNPLA3* rs738409 and *RNF7* rs16851720 confer an increased risk of developing liver fibrosis and cirrhosis in this Eastern European population, while the *MERTK* and *PCSK7* SNPs are not associated with these conditions.

Key words: *PNPLA3* – *RNF7* – *MERTK* – *PCSK7* – liver cirrhosis – liver fibrosis – SNP – gene polymorphism.

Abbreviations: GWAS: Genome-wide association studies; HBV: hepatitis B virus; HCV: hepatitis C virus; HH: hereditary hemochromatosis; MERTK: proto-oncogene tyrosine-protein kinase MER; NAFLD: non-alcoholic fatty liver disease; PCSK7: proprotein convertase 7; PNPLA3: patatin-like phospholipase domain containing 3; RNF7: SAG sensitive to apoptosis gene; SNP: single nucleotide polymorphism.

INTRODUCTION

Liver cirrhosis is the common end stage of chronic liver diseases and significantly reduces the life quality and expectancy [1, 2]. The most common causes of chronic liver injury include hepatitis C virus (HCV) and hepatitis B virus (HBV) infections, alcohol, non-alcoholic fatty liver disease (NAFLD), autoimmune hepatitis, as well as other rare conditions [3]. The natural course of liver diseases varies considerably between individual patients [3]. This inter-individual variability might be related to different confounding factors, including clinical, environmental, and host factors [4]. The known risk factors for progression of liver injury such as age, gender, alcohol consumption, and obesity cannot explain all the clinically evident differences observed in patients [5].

The advent of new genomic technologies and the decreased costs of genotyping have been followed by multiple studies that point to the importance of genetic predisposition in different liver diseases [6]. To date, several gene polymorphisms have been linked with the progression of liver fibrosis and the development of liver cirrhosis [6]. Initially, Huang et al. (2007) developed a cirrhosis risk score signature containing seven predictive single nucleotide polymorphisms (SNPs) that were associated with the risk for HCV cirrhosis [7]. A later, large scale genome-wide associated study (GWAS) demonstrated

that the patatin-like phospholipase domain containing 3 (*PNPLA3*) gene polymorphism rs738409 was associated with NAFLD [8] and affects the activities of liver enzymes in plasma [9]. The *PNPLA3* gene polymorphism rs738409 was also shown to accelerate liver fibrosis progression rate in HCV-infected patients [10] and contribute to the development of alcoholic liver cirrhosis [11]. Further genetic association studies discovered two other loci harboring *RNF7*, which is also known as *SAG* (sensitive to apoptosis gene), and proto-oncogene tyrosine-protein kinase MER (*MERTK*), which may influence the development of fibrosis in chronic hepatitis C [12]. A meta-analysis of GWAS results on the soluble transferrin receptor (sTfR) and ferritin reported a novel association between sTfR and the variation in the proprotein convertase 7 (*PCSK7*) gene and the iron overload [13]. These polymorphisms were extensively studied by Stickel et al. [14], who showed that the *PCSK7* variant rs236918 was a risk factor for the development of cirrhosis in hereditary hemochromatosis (HH).

The aforementioned genetic variants have been shown to affect the development of liver fibrosis or cirrhosis. However, the relevance of these results for different populations remains to be established. Due to a large number of analyzed genetic variants, the validation in independent cohorts of patients from different populations of the GWAS results is necessary. In this study, we aimed to evaluate the association between four SNPs reported in GWAS (*MERTK* rs4374383, *PCSK7* rs236918, *PNPLA3* rs738409, *RNF7* rs16851720) and the risk of liver fibrosis and cirrhosis.

METHODS

Patients

A number of 969 individuals were included in the study: 498 controls, 317 patients with liver cirrhosis, and 154 patients with liver fibrosis. The consecutive patients with liver cirrhosis and liver fibrosis were recruited at the Department of Gastroenterology, Lithuanian University of Health Sciences Hospital, during the period 2012-2015. The diagnosis and etiology of liver cirrhosis were confirmed by laboratory tests, clinical features, liver biopsy, and radiological imaging. The patients in liver fibrosis group underwent percutaneous liver biopsy and were included in the study if stage 1 to 3 fibrosis was documented by histological evaluation using METAVIR score [15]. Control samples came from our previous genotyping study on the prevalence of HFE mutations in the Lithuanian population and included 498 voluntary, unrelated Lithuanian blood donors [16]. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008). The study was approved by the Ethics Committee of the Lithuanian University of Health Sciences (Protocol Nr. BE-2-10). All patients and controls gave their informed consent to take part in this study.

Genotyping

Genomic DNA from samples was isolated from whole blood mononuclear cells using a salting-out method and stored at –20°C until analysis. *PNPLA3* C>G (rs738409), *RNF7* A>C (rs16851720), *MERTK* A>G (rs4374383) and *PCSK7* C>G (rs236918) SNPs were genotyped by real-time PCR (RT-PCR), using TaqMan® allelic discrimination assays with a 7500TM Fast real-time PCR system (Life Technologies, Carlsbad, California, USA).

Statistical analysis

The distribution of the *PNPLA3*, *RNF7*, *MERTK* and *PCSK7* genotypes in cases and controls was examined for consistency with the Hardy-Weinberg equilibrium (HWE). Allele frequencies were compared between cases and controls by Pearson's goodness-of-fit χ^2 test. Associations between control and cases groups with SNP alleles and genotypes were calculated using logistic regression analysis with adjustment for age and sex. The relative risks for mutations were estimated using allelic, genotypic, recessive and dominant models. Recessive and dominant models led to a comparison between homozygous vs. wild type + heterozygous and heterozygous + homozygous vs. wild type carriers. Adjusted odds ratios (aORs) and 95% confidence intervals (CI) are reported. A p value of 0.05 was defined to be statistically significant. Age is shown as a mean with a standard deviation, and was compared using analysis of variance (ANOVA). Gender distributions were compared using the χ^2 tests. Statistical analysis of the genotyping data was performed using PLINK software version 1.07 [17].

RESULTS

Characteristics of study participants

Table I summarizes the characteristics of study participants. The most common causes of liver cirrhosis were chronic HCV infection and alcohol. The distribution of Child-Pugh classes A, B and C in the cirrhotic group was 31.2%, 48.0% and 20.8%, respectively. The most common cause of liver injury in the fibrosis group was HCV infection. The subjects differed in age and sex between the study groups. Men were predominant in the control and liver fibrosis groups and accounted for 63.9% and 61.7%, respectively. Patients with liver cirrhosis were significantly ($P<0.001$) older than those with liver fibrosis and control groups. To eliminate the potential bias of differences in age and sex distribution among the groups, these parameters were included as covariates in further logistic regression analysis.

Hardy-Weinberg equilibrium

The observed genotype frequencies for all four SNPs included in the study were concordant with the Hardy-Weinberg equilibrium (rs738409, P=0.5369; rs16851720, P=0.7885; rs4374383, P=0.1752; rs236918; P=0.3483).

Association of *PNPLA3, RNF7, MERTK* and *PCSK7* SNPs with liver fibrosis and cirrhosis

Table II presents the frequencies of the alleles and genotypes of *PNPLA3*, *RNF7*, *MERTK* and *PCSK7* SNPs in controls, in patients with liver fibrosis, liver cirrhosis and combined liver cirrhosis + liver fibrosis. The *PNPLA3* rs738409 allele G was associated with liver damage in the combined liver cirrhosis and fibrosis group (aOR: 1.84, CI: 1.47-2.30, $P=9.27*10^{-8}$), and it also conferred increased risks for liver cirrhosis (aOR: 1.92, CI: 1.49-2.48, $P=5.57*10^{-7}$) and liver fibrosis (aOR:

Table I. Characteristics of the patients with liver cirrhosis, liver fibrosis and controls

	Liver cirrhosis (n=317)	Liver fibrosis (n=154)	Controls (n=498)	ANOVA (age)[a] and χ^2 test P value
Age (mean ± SD), years	54.5 ± 12.2	49.9 ± 11.5	46.8 ± 6.5	<0.001
Gender, n (%)				
Male	150 (47.3%)	95 (61.7%)	318 (63.9%)	< 0.001
Female	167 (52.7%)	59 (38.3%)	180 (36.1%)	
Etiology of liver disease, n (%)				
HCV	117 (36.9%)	136 (88.3%)		
HBV	43 (13.5%)	6 (3.9%)		
Autoimmune	23 (7.2%)	1(0.65%)		
Alcohol	82 (25.7%)	4 (2.6%)		
Other causes	18 (5.8%)	6 (3.9%)		
Cryptogenic	28 (9.0%)	1 (0.65%)		
Child-Pugh class, n (%)				
A	99 (31.2%)			
B	152 (48.0%)			
C	66 (20.8%)			
Liver fibrosis stage[b], n (%)				
1		97 (63.0%)		
2		28 (18.2%)		
3		29 (18.8%)		

HBV: hepatitis B virus; HCV: hepatitis C virus; [a]: Statistical analysis performed globally for all four groups; [b]: Histological degree of liver fibrosis evaluated according to METAVIR.

1.65; CI-1.22-2.23; P=0.001; Table II). *PNPLA3* rs738409 genotype was associated with a higher risk of developing liver fibrosis and cirrhosis when comparing both dominant (aOR: 1.98; CI: 1.44-2.72, P=2.20*10^{-5}; aOR: 1.67, CI: 1.14-2.43, P=0.008, respectively) and recessive (aOR: 3.94, CI: 2.03-7.67, P=5.16*10^{-5}; aOR: 3.02, CI: 1.45-6.28; P=0.003, respectively) inheritance models (Table II).

The *RNF7* rs16851720 CC genotype was protective against liver damage in the combined liver cirrhosis and fibrosis group (aOR: 0.33; CI: 0.13-0.80, P=0.014) and the liver cirrhosis group (aOR: 0.26, CI: 0.08-0.80, P=0.019). Similarly, considering recessive inheritance, the genotype CC was less frequent in both liver cirrhosis and liver fibrosis (aOR: 0.34, CI: 0.14-0.82, P=0.016) and in patients with liver cirrhosis (aOR: 0.26; CI: 0.09-0.81, P=0.020). The distributions of alleles and genotypes of *MERKT* and *PCSK7* SNPs were similar between the control, liver fibrosis and cirrhosis groups. *MERKT* rs4374383 was neither associated with the risk of developing liver fibrosis (aOR: 0.92, CI: 0.71-1.19, P=0.530) nor liver cirrhosis (aOR: 1.13, CI: 0.90-1.40, P=0.109). Similarly, the *PCSK7* SNP was not linked with the risk of developing liver fibrosis (aOR: 0.86; CI: 0.59-1.26; P=0.434) or cirrhosis (aOR: 0.86, CI: 0.63-1.19, P=0.362 (Table II).

Association of *PNPLA3, RNF7, MERTK* and *PCSK7* SNPs with HCV and non-HCV liver cirrhosis

Since a large proportion of patients within the cirrhosis group were HCV patients, we carried out a sub-analysis for the risk of developing liver cirrhosis in HCV and non-HCV patients (Table III). *PNPLA3* SNP was associated with higher risk of both HCV and non-HCV induced liver cirrhosis. The *PNPLA3* rs738409 G allele was associated with an increased risk of liver cirrhosis both in HCV (aOR: 2.04, CI: 1.41-2.94, P=1.43*10^{-4}) and non-HCV groups (aOR: 1.81, CI: 1.36-2.42, P=5.64 * 10^{-5}; Table III). The *RNF7* rs16851720 CC genotype was less frequent than AA genotype in non-HCV cirrhosis group if compared to the control group (aOR: 0.28, CI: 0.08-1.00, P=0.050). Interestingly, frequencies of *RNF7* rs16851720 genotypes and alleles in HCV cirrhosis group did not differ from the controls. However, the potential association could be blunted by the relatively small number of individuals within this group. The rs236918 (*PCSK7*) and rs4374383 (*MERTK*) were not linked with the risk of developing liver cirrhosis in HCV or non-HCV patients (Table III).

Association analysis of the effect of the combined *PNPLA3* and *RNF7* SNPs for development of HCV liver fibrosis and liver cirrhosis

Table IV shows the frequencies of the combined *PNPLA3* and *RNF7* SNPs genotypes in HCV induced cirrhosis, HCV induced fibrosis and controls. Chi-squared test and Fisher exact probability tests were used to compare combined SNP genotypes frequencies between groups. Each combination of *PNPLA3* and *RNF7* genotypes was compared with the other *PNPLA3* and *RNF7* genotypes combinations. CC_{PNPLA3} CC_{RNF7} and CC_{PNPLA3} AA_{RNF7} genotype combined effect showed a reduced risk in developing HCV induced cirrhosis (OR: 0.63, CI: 0.41-0.96, P=0.033; P=0.032, respectively). CG_{PNPLA3} AA_{RNF7} and GG_{PNPLA3} AA_{RNF7} genotype combinations were associated with a higher risk in developing HCV cirrhosis (OR: 1.69, CI: 1.04-2.76, P=0.035; OD: 3.11, CI: 1.30-7.46, P=0.011, respectively). The combined effect of GG_{PNPLA3} CA_{RNF7} genotype

Table II. Distribution of the *MERTK*, *PCSK7*, *RNF7* and *PNPLA3* gene polymorphisms in liver cirrhosis, liver fibrosis and control groups.

Allele/Genotypes	Controls (n=498)	Liver cirrhosis + liver fibrosis (n=471)			Liver cirrhosis (n=317)			Liver fibrosis (n=154)		
	n (%)	n (%)	aOR (95% CI)	P	n (%)	aOR (95% CI)	P	n (%)	aOR1 (95% CI)	P
rs738409 (*PNPLA3*)										
G	87 (17.5)	133 (28.3)	1.84 (1.47-2.30)	**$9.27*10^{-8}$**	93 (29.3)	1.92 (1.49-2.48)	**$5.57*10^{-7}$**	41 (26.3)	1.65 (1.22-2.23)	**0.001**
C	411 (82.5)	338 (71.7)			224 (70.7)			113(73.7)		
GC	140 (28.1)	175 (37.2)	1.64 (1.23-2.20)	**0.001**	124 (39.1)	1.69 (1.21-2.36)	**0.002**	51 (33.1)	1.45 (0.97-2.17)	0.071
GG	17 (3.4)	46 (9.8)	4.28 (2.33-7.85)	**$2.67*10^{-6}$**	31 (9.8)	4.74 (2.41-9.30)	**$6.21*10^{-6}$**	15 (9.7)	3.41 (1.62-7.20)	**0.001**
CC	341 (68.5)	250 (53.0)	1 (Reference)		162 (51.1)	1 (Reference)		88 (57.2)	1 (Reference)	
GG+GC vs. CC			1.91 (1.45-2.51)	**$4.39*10^{-6}$**		1.98 (1.44-2.72)	**$2.20*10^{-5}$**		1.67 (1.14-2.43)	**0.008**
GG vs. GC+CC			3.61 (1.98-6.56)	**$2.64*10^{-5}$**		3.94 (2.03-7.67)	**$5.16*10^{-5}$**		3.02 (1.45-6.28)	**0.003**
rs16851720 (*RNF7*)										
C	105 (21.1)	82 (17.4)	0.79 (0.62-1.01)	0.055	57 (18.0)	0.80 (0.60-1.06)	0.115	25 (16.2)	0.74 (0.53-1.05)	0.091
A	393 (78.9)	389 (82.6)			260 (82.0)			129 (83.8)		
CA	164 (32.9)	150 (31.8)	0.90 (0.68-1.20)	0.478	106 (33.4)	0.95 (0.68-1.32)	0.755	44 (28.6)	0.79 (0.53-1.19)	0.256
CC	23 (4.6)	7 (1.5)	0.33 (0.13-0.80)	**0.014**	4 (1.3)	0.26 (0.08-0.80)	**0.019**	3 (1.9)	0.42 (0.12-1.42)	0.161
AA	311 (62.5)	314 (66.7)	1 (Reference)		207 (65.3)	1 (Reference)		107(69.5)	1 (Reference)	
CC+CA vs. AA			0.83 (0.63-1.10)	0.196		0.86 (0.63-1.19)	0.367		0.75 (0.50-1.11)	0.146
CC vs. CA+AA			0.34 (0.14-0.82)	**0.016**		0.26 (0.09-0.81)	**0.020**		0.45 (0.13-1.52)	0.198
rs236918 (*PCSK7*)										
C	69 (13.9)	59 (12.5)	0.84 (0.64-1.10)	0.209	39 (12.3)	0.86 (0.63-1.19)	0.362	20 (13.0)	0.86 (0.59-1.26)	0.434
G	429 (86.1)	412 (87.5)			278 (87.3)			134 (87.0)		
CG	114 (22.9)	104 (22.1)	0.89 (0.65-1.22)	0.469	70 (22.1)	0.97 (0.67-1.40)	0.865	34 (22.1)	0.86 (0.55-1.34)	0.495
CC	12 (2.4)	7 (1.5)	0.52 (0.19-1.41)	0.200	4 (1.3)	0.39 (0.11-1.36)	0.139	3 (2.0)	0.75 (0.20-2.73)	0.661
GG	372 (74.7)	360 (76.4)	1 (Reference)		243 (76.6)	1 (Reference)		117 (75.9)	1 (Reference)	
CC+CT vs. TT	-	-	0.85 (0.63-1.16)	0.313	-	0.91 (0.63-1.30)	0.589	-	0.85 (0.55-1.30)	0.447
CC vs. CG+GG	-	-	0.53 (0.20-1.44)	0.214	-	0.39 (0.11-1.37)	0.141	-	0.78 (0.21-2.82)	0.701
rs4374383 (*MERTK*)										
A	180 (36.1)	175 (37.1)	1.04 (0.86-1.26)	0.682	121 (38.3)	1.13 (0.90-1.40)	0.287	53 (34.4)	0.92 (0.71-1.19)	0.530
G	318 (63.9)	296 (62.9)			196 (61.7)			101 (65.6)		
AG	216 (43.4)	207 (43.9)	1.01 (0.76-1.34)	0.959	149 (47.0)	1.13 (0.81-1.58)	0.467	58 (37.7)	0.78 (0.52-1.16)	0.215
AA	72 (14.5)	71 (15.1)	1.10 (0.74-1.65)	0.630	47 (14.8)	1.27 (0.80-2.02)	0.320	24 (15.6)	0.94 (0.55-1.62)	0.831
GG	210 (42.1)	193 (41.0)	1 (Reference)		121 (38.2)	1 (Reference)		72 (46.7)	1 (Reference)	
AA+AG vs. GG	-	-	1.03 (0.79-1.35)	0.822	-	1.16 (0.85-1.59)	0.343	-	0.82 (0.57-1.18)	0.286
AA vs. AG+GG	-	-	1.10 (0.76-1.60)	0.619	-	1.19 (0.77-1.83)	0.436	-	1.06 (0.64-1.77)	0.813

aOR - adjusted odds ratio; CI - confidence interval; significant p-values are marked in bold

showed a significantly higher risk in developing HCV induced fibrosis (OR: 5.98, CI: 1.66-21.50, P=0.006) (Table IV).

DISCUSSION

In the present study, we aimed to replicate in our population the associations between key *PNPLA3*, *RNF7*, *MERTK* and *PCSK7* gene polymorphisms and the risk of liver fibrosis and cirrhosis that have been reported in recent GWAS. We have shown that *PNPLA3* rs738409 is associated with the risk of developing both liver fibrosis and cirrhosis and that *RNF7* rs16851720 is associated with liver cirrhosis. Our study also revealed that *PNPLA3* rs738409 is a risk factor for developing liver cirrhosis in both HCV and non-HCV patients. To date, this is the first study that has demonstrated that *PNPLA3* rs738409 confers an increased risk of liver fibrosis and cirrhosis in an Eastern European population. To our knowledge, replication analysis of the association between *RNF7* rs16851720 and liver cirrhosis has also not been reported previously. Furthermore, replication studies on *MERTK* rs4374383 and *PCSK7* rs236918 in patients with liver diseases are scarce.

The major finding in our cohort of patients is that *PNPLA3* rs738409 is linked to liver fibrosis and cirrhosis, confirming an association that has been reported in distinct ethnic groups [8, 11, 18, 19]. We confirmed this association both in HCV and non-HCV cirrhosis groups. This frequent *PNPLA3* variant is also associated with serum ALT activities already at a young age [20] and is a prominent risk factor for HCC [21]. The highly significant differences and odds ratios observed for liver fibrosis and cirrhosis point to the importance of

Table III. Distribution of the *MERTK, PCSK7, RNF7* and *PNPLA3* gene polymorphisms in hepatitis C virus–induced cirrhosis, non-hepatitis C virus–induced cirrhosis and control groups.

Allele/Genotypes	Controls (n=498)	Hepatitis C virus–induced cirrhosis (n=117)			Non-hepatitis C virus–induced cirrhosis (n=200)		
	n (%)	n (%)	aOR (95% CI)	P	n (%)	aOR (95% CI)	P
rs738409 (*PNPLA3*)							
G	87 (17.5)	37 (31.2)	2.04 (1.41-2.94)	**$1.43*10^{-4}$**	57 (28.3)	1.81 (1.36-2.42)	**$5.64*10^{-5}$**
C	411 (82.5)	80 (68.8)			143 (71.7)		
GC	140 (28.1)	49 (41.9)	1.99 (1.22-3.26)	**0.006**	75 (37.5)	1.56 (1.06-2.28)	**0.023**
GG	17 (3.4)	56 (47.9)	4.30 (1.75-10.59)	**0.001**	106 (53)	4.37 (2.08-9.19)	**$1.01*10^{-4}$**
CC	341 (68.5)	12 (10.2)	1 (Reference)		19 (9.5)	1 (Reference)	
GG+GC vs. CC			2.25 (1.41-3.57)	**0.001**		1.82 (1.27-2.60)	**0.001**
GG vs. GC+CC			3.34 (1.39-8.03)	**0.007**		3.76 (1.81-7.82)	**$3.91*10^{-4}$**
rs16851720 (*RNF7*)							
C	105 (21.1)	21 (18.0)	0.86 (0.57-1.30)	0.469	36 (18.0)	0.78 (0.57-1.08)	0.129
A	393 (78.9)	96 (82.0)			164 (82.0)		
CA	164 (32.9)	40 (34.2)	1.04 (0.64-1.68)	0.874	66 (33.0)	0.91 (0.63-1.33)	0.632
CC	23 (4.6)	1 (0.9)	0.23 (0.03-1.78)	0.158	3 (1.5)	0.28 (0.08-1.00)	**0.050**
AA	311 (62.5)	76 (64.9)	1 (Reference)		131 (65.5)	1 (Reference)	
CC+CA vs. AA			0.94 (0.59-1.51)	0.811		0.83 (0.58-1.20)	0.327
CC vs. CA+AA			0.22 (0.03-1.74)	0.153		0.29 (0.08-1.03)	0.055
rs236918 (*PCSK7*)							
C	69 (13.9)	14 (12.0)	0.86 (0.54-1.37)	0.517	25 (12.5)	0.86 (0.60-1.24)	0.416
G	429 (86.1)	103 (88.0)			175 (87.5)		
CG	114 (22.9)	26 (22.2)	1.02 (0.60-1.74)	0.951	44 (22.0)	0.93 (0.61-1.42)	0.747
CC	12 (2.4)	1 (0.9)	0.25 (0.03-2.26)	0.216	3 (1.5)	0.49 (0.12-1.93)	0.306
GG	372 (74.7)	90 (76.9)	1 (Reference)		153 (76.5)	1 (Reference)	
CC+CT vs. TT			0.93 (0.55-1.56)	0.773		0.89 (0.59-1.34)	0.562
CC vs. CG+GG			0.25 (0.03-2.25)	0.214		0.49 (0.12-1.96)	0.316
rs4374383 (*MERTK*)							
A	180 (36.1)	48 (41.0)	1.16 (0.84-1.61)	0.355	74 (36.8)	1.09 (0.84-1.40)	0.513
G	318 (63.9)	69 (59.0)			126 (63.2)		
AG	216 (43.4)	54 (46.2)	1.09 (0.66-1.80)	0.726	95 (47.5)	1.13 (0.77-1.64)	0.536
AA	72 (14.5)	42 (35.9)	1.40 (0.72-2.75)	0.324	79 (39.5)	1.16 (0.67-2.00)	0.600
GG	210 (42.1)	21 (17.9)	1 (Reference)		26 (13)	1 (Reference)	
AA+AG vs. GG			1.17 (0.73-1.86)	0.522		1.13 (0.79-1.62)	0.491
AA vs. AG+GG			1.34 (0.72-2.47)	0.355		1.09 (0.65-1.80)	0.749

aOR - adjusted odds ratio; CI - confidence interval; significant p-values are marked in bold

this genetic variant for liver damage. The exact mechanisms on how PNPLA3 contributes to fibrogenesis are still under investigation [22, 23]. It is known that PNPLA3 as a lipase is responsible for retinyl-palmitate hydrolysis in the hepatic stellate cells in humans [24]. Furthermore, the same group has recently shown that PNPLA3-mediated retinol release may protect against liver fibrosis [25]. Other studies have shown that the *PNPLA3* I148M variant is relevant for the retinyl-palmitate content in human liver [26] and influences circulating retinol levels in adults [27]. A recent elegant study in mice has shown that *PNPLA3* I148M knock-in mice accumulate PNPLA3 on lipid droplets and develop hepatic steatosis [28]. All of these studies start to unveil the role of PNPLA3 in liver damage at a molecular level and might suggest important targets for future therapies.

A large GWAS has shown that a locus in the *RNF7* gene is associated with the development of liver fibrosis in chronic hepatitis C [12]. The study reported that in the combined cohort of 2,342 HCV-infected patients rs16851720 was associated with fibrosis progression. *RNF7* encodes an antioxidant that protects against apoptosis [29]. The results of our study support these findings, because the rs16851720 AA genotype conferred an increased risk of liver cirrhosis. We did not observe differences in the genotype frequencies between controls and patients with liver fibrosis for the *RNF7* rs16851720. To our knowledge, no other studies have addressed the role of this gene polymorphism

Table IV. Combined effects of the PNPLA3 and RNF7 gene polymorphisms on the risk of hepatitis C virus–induced liver cirrhosis and hepatitis C virus–induced liver fibrosis.

Combined genotypes	Controls (n=498)	Hepatitis C virus–induced cirrhosis (n=117)			Hepatitis C virus–induced fibrosis (n=130)		
	n (%)	n (%)	OR (95% CI)	P	n (%)	OR (95% CI)	P
CC_{PNPLA3} CCR_{NF7}	19 (3.8)	0 (0.0)	-	**0.032**	8 (1.5)	1.65 (0.71 - 3.87)	0.246
CC_{PNPLA3} CA_{RNF7}	102 (20.5)	17 (14.5)	0.66 (0.38 - 1.15)	0.145	20 (15.4)	0.71 (0.42 - 1.19)	0.193
CC_{PNPLA3} AA_{RNF7}	220 (44.2)	39 (33.3)	0.63 (0.41 - 0.96)	**0.033**	57 (43.8)	0.99 (0.67 - 1.46)	0.946
CG_{PNPLA3} CC_{RNF7}	4 (0.8)	1 (0.9)	1.0 (0.18 - 9.61)	1.000	1 (0.8)	0.96 (0.11 - 8.64)	0.969
CG_{PNPLA3} CA_{RNF7}	58 (11.6)	20 (17.1)	1.56 (0.90 - 2.72)	0.113	11 (8.5)	0.70 (0.36 - 1.38)	0.303
CG_{PNPLA3} AA_{RNF7}	78 (15.7)	28 (23.9)	1.69 (1.04 - 2.76)	**0.035**	25 (19.2)	1.28 (0.78 - 2.11)	0.329
GG_{PNPLA3} CC_{RNF7}	0 (0.0)	0 (0.0)	-		0 (0.0)	-	
GG_{PNPLA3} CA_{RNF7}	4 (0.8)	3 (2.6)	3.25 (0.72 - 14.72)	0.126	6 (4.6)	5.98 (1.66 - 21.50)	**0.006**
GG_{PNPLA3} AA_{RNF7}	13 (2.6)	9 (7.7)	3.11 (1.30 - 7.46)	**0.011**	8 (6.2)	2.45 (0.99 - 6.03)	0.052

OR: odds ratio; CI: confidence interval; significant p-values are marked in bold

beyond the published GWAS [12]. In order to evaluate the combined effect of *PNPLA3* and *RNF7* SNPs genotypes for the risk of HCV fibrosis or cirrhosis, we performed an additional analysis. Interestingly, the combined GG_{PNPLA3} CA_{RNF7} genotype revealed a very high OR of 5.98 (p=0.006) for HCV related fibrosis. However, the subgroups of patients within this analysis were not very large and these findings need to be validated in larger groups of patients.

A recent meta-analysis of GWAS results revealed a novel association between the variation in *PCSK7* gene and iron overload [13]. Stickel et al. [14] reported that the *PCSK7* variant rs236918 was a risk factor for the development of cirrhosis in HH patients homozygous for the *HFE* p.C282Y mutation. Hypothesizing that this SNP might contribute to the development of liver damage in other chronic liver diseases, we genotyped *PCSK7* rs236918 in our cohort of patients with liver fibrosis and cirrhosis. Based on our findings, alleles and genotypes of this SNP were distributed equally between the study groups. A recent Italian study has confirmed that the *PCSK7* rs236918 C allele represents a risk factor for cirrhosis development in Italian patients with HH [30]. Interestingly, during the 6-month weight-loss study, the *PCSK7* rs236918 G allele was significantly associated with the decrease of fasting insulin levels in the high-dietary carbohydrate group [31]. Further, large scale studies are required to evaluate whether the *PCSK7* SNP affects the development of liver cirrhosis in other patients than HH.

In our study, the *MERTK* rs4374383 SNP alleles and genotypes were distributed equally between the control, liver fibrosis and cirrhosis groups. The initial association between *MERTK* rs4374383 and development of fibrosis in chronic HCV disease was observed in a GWAS; in this study, the association was observed only in a sub-group of HCV patients who received blood transfusions [12]. Later, the *MERTK* SNP significantly added risk to accelerated fibrosis progression rate in the Swiss Hepatitis C Cohort Study [10]. Interestingly, a recent study also showed that the rs4374383 AA genotype was protective against F2-F4 fibrosis in patients with NAFLD [32]. One of the potential explanations for differences between the previously published data and our results could be linked to the sample size of our patient groups.

Our study has certain limitations that need to be acknowledged. Firstly, the cohort of our cirrhotic patients is heterogeneous with respect to etiology and specific genetic variants might be more important for different types of cirrhosis. In particular, we could not assess the association between SNPs and risk of NAFLD related cirrhosis, because we had only a very few patients with this diagnosis in our study group. We also did not have complete information on alcohol consumption, metabolic syndrome and diabetes status, that might have modified the effects of the analyzed genetic variants. Our cohorts with liver cirrhosis and fibrosis were also not very large and might have been underpowered to detect subtle genetic associations. The groups of patients with different liver function impairment levels were also not very large and this aspect needs to be reassessed in further larger scale studies. Nevertheless, we believe that the results of our study are useful for further meta-analyses in the field. Due to the design of the study we could not include certain other polymorphisms such as the *TM6SF2* or *MBOAT7* SNPs, which have been recently associated with liver cirrhosis [11, 33], and this remains a task for further studies.

CONCLUSION

The *PNPLA3 rs738409* and *RNF7* rs16851720 SNPs were associated with the risk of developing liver fibrosis and liver cirrhosis. These genetic alterations might facilitate progression of liver fibrosis. In our study population, the *MERTK* rs4374383 and *PCSK7* rs236918 SNPs were not linked with the risk of liver fibrosis or liver cirrhosis.

Conflicts of interest: No conflicts of interest.

Authors'contribution: J.K, I.V.: study design, writing, data analysis and interpretation; G.V.: data collection, analysis, statistical analysis; R.S, J.Sk, J.S,V.P, J.K, and G.K. contributed to data collection and analysis; F.L. and L.K. contributed to the study design and gave critical review during the writing of the paper.

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SUMMARY IN LITHUANIAN

1. ĮVADAS

Paskutiniais metais sparčiai daugėja sergančiųjų kepenų ligomis, ir tai tampa viena dažniausių mirties priežasčių visame pasaulyje [1]. Dažniausios priežastys, sukeliančios lėtinį kepenų pažeidimą, yra hepatito B (HBV) ir hepatito C (HCV) virusai, piktnaudžiavimas alkoholiu, nealkoholinė suriebėjusių kepenų liga (NSKL), autoimuninės ligos ir kitos retesnės priežastys [2]. Keičiantis žmonių gyvenimo būdui visame pasaulyje sparčiai daugėja sergančiųjų alkoholine kepenų liga ir NSKL. Pasaulio gastroenterologų organizacijos (PGO) duomenimis, sergančiųjų NSKL pastaruosiais dešimtmečiai padvigubėjo ir tai tampa nauja ir didele sveikatos sutrikdymo problema visame pasaulyje [3]. Nepriklausomai nuo priežasties, lėtinis hepatitas ilgainiui sukelia kepenų parenchimos fibrozę, kuri negydant gali progresuoti iki kepenų cirozės bei kepenų funkcijos nepakankamumo, galimų komplikacijų išsivystymo ir mirties. Kepenų cirozė yra paskutinė lėtinių kepenų ligų stadija, reikšmingai bloginanti pacientų gyvenimo kokybę, daranti įtaką jų išgyvenamumą [4, 5]. Ji tampa beveik 2 procentų visų mirčių pasaulyje priežastimi [6]. Lėtinė kepenų ligos gali būti skirtinga [2]. Tai gali būti sąlygota įvairių veiksnių, tarp jų – aplinkos ar asmeninių individo savybių [7]. Klasikiniai veiksniai, įtakojantys lėtinės kepenų ligos progresavimą, tokie kaip amžius, lytis, alkoholio vartojimas, nutukimas negali paaiškinti randamų klinikinės eigos skirtumų tarp sergančiųjų [8]. Naujos genominės technologijos bei sumažėję genotipavimo kaštai leido atskleisti neabejotiną genetinės predispozicijos reikšmę sirgti tam tikromis lėtinėmis kepenų ligomis [9]. 1996m. nustatytos pirmosios mutacijos, sukeliančios *HFE*-hemochromatozę. Europiečių populiacijoje dažniausiai nustatomos dvi jų: c.845G>A (Cys282Y) ir c.187C>G (H63). *HFE* geno mutacijų nešiojimas yra susijęs su didesne kepenų fibrozės ir cirozės rizika, tačiau publikuojami įvairių mokslinių tyrimų duomenys labai prieštaringi [10]. Plataus masto viso genomo tyrimai (PMGT) nustatė keletą vieno nukleotido polimorfizmų (VNP) susijusių su lėtinėmis kepenų ligomis. Vienas jų – *PNPLA3* geno rs738409 VNP susijęs su NSKL liga [14], kepenų fibrozės progresavimu, sergantiesiems lėtiniu C virusinės kilmės hepatitu [15] ar kepenų cirozės progresavimu, sergantiesiems alkoholine kepenų ciroze [16]. Genas *RNF7*, žinomas kaip jautriai reaguojantis į apoptozę genas *SAG* ir genas *MERTK* arba protoonkogeno tirozino-proteino kinazė MER, gali daryti įtaką fibrozės vystimuisi pacientams, sergantiems lėtiniu C virusinės kilmės hepatitu. Atlikta PMGT rezultatų metaanalizė atskleidė ryšį

tarp proptroteino konvertazės 7 (*PCSK7*) geno ir geležies pertekliaus, kurį rodo feritino serumo lygiai ir transferino tirpus receptorius (sTfR**)**)[18].

2. TIKSLAS IR UŽDAVINIAI

2.1. Tikslas

Ištirti *HFE* geno mutacijų ir genų *PNPLA3, RNF7, MERTK* ir *PCSK7* vieno nukleotido polimorfizmų sąsają su kepenų fibroze ir ciroze.

2.2. Uždaviniai

1. Nustatyti ryšį tarp *HFE* geno C282Y, H63D mutacijų ir kepenų cirozės.
2. Nustatyti *HFE* geno C282Y, H63D mutacijų ir lyties sąsajas sergantiesiems kepenų ciroze.
3. Nustatyti ryšį tarp genų *PNPLA3, RNF7, MERTK, PCSK7* vieno nukleotido polimorfizmų ir kepenų fibrozės.
4. Nustatyti ryšį tarp genų *PNPLA3, RNF7, MERTK, PCSK7* vieno nukleotido polimorfizmų ir kepenų cirozės.
5. Nustatyti ryšį tarp genų *PNPLA3, RNF7, MERTK, PCSK7* vieno nukleotido polimorfizmų ir hepatito C viruso sukeltos bei kitos etiologijos kepenų fibrozės ir cirozės.

2.3. Darbo naujumas

Pastaruosius du dešimtmečius gerokai išsiplėtė mūsų žinių ratas apie genetinių veiksnių įtaką kepenų ligų vystymuisi [19]. Kaip minėta anksčiau, priežastys,sukeliančios lėtinį kepenų uždegimą, yra įvairios. Nepriklausomai nuo jų, lėtinis hepatitas gali sąlygoti kepenų audinio fibrozę, o vėliau ir cirozę, tačiau kepenų pažeidimo progresavimo laikas tarp skirtingų individų labai skiriasi [20]. Pastarąjį dešimtmetį įvairios mokslininkų grupės siekė nustatyti esminius gretutinius veiksnius, įtakojančius kepenų pažeidimo vystymąsi [21, 22]. Išaugęs tyrimų skaičius rodo, kad be pagrindinių esminių priežastinių kepenų cirozės veiksnių, šį procesą gali įtakoti tokie veiksniai kaip alkoholio vartojimas, nutukimas ir pan. [23–25]. Skirtingas laiko tarpas iki kepenų fibrozės, o vėliau ir cirozės išsivystymo tarp atskirų pacientų leidžia numatyti galimą įgimtų faktorių įtaką. Šiandien su kepenų fibrozės progresavimu ir kepenų cirozės išsivystymu jau galima susieti keletą genų polimorfizmų [9]. Pirmoji mūsų tyrimo dalis buvo skirta nustatyti *HFE* geno mutacijų C282Y ir H63D reikšmę įvairios etiologijos kepenų cirozės išsivystymui. Šis tyrimas pirmasis, įvertinantis *HFE* geno

mutacijų paplitimą tarp Lietuvos pacientų, sergančių įvairios etiologijos kepenų ciroze ir suteikiantis papildomos informacijos apie *HFE* geno mutacijų C282Y ir H63D sąsają su kepenų ciroze. Antroji mūsų tyrimo dalis buvo skirta nustatyti ryšį tarp genų *MERTK* (rs4374383), *PCSK7* (rs236918), *PNPLA3* (rs738409) ir *RNF7* (rs16851720) VNPų ir kepenų fibrozės bei cirozės. Tai pirmasis tyrimas, parodantis, kad *PNPLA3* geno rs738409 VNP yra susijęs su kepenų fibroze ir ciroze tarp Lietuvos gyventojų. Mūsų žiniomis iki šiol nėra paskelbta tyrimų apie *RNF7* geno rs16851720 VNP sąsają su kepenų ciroze, o duomenys apie *MERTK* geno rs4374383 ir *PCSK7* geno rs236918 VNPų ryšį su kepenų ligomis yra menki.

3. METODAI

Tyrimas atliktas gavus Lietuvos Bioetikos komiteto leidimą (Protokolo Nr. 2/2008) ir Kauno regioninio biomedicininių tyrimų etikos komiteto leidimą (Protokolo Nr. BE-2-10). Visi pacientai ir kontrolinės grupės asmenys pasirašė sutikimo dalyvauti tyrime formas.

3.1. Tiriamieji

Tyrime dalyvavo pacientai, tirti ir gydyti dėl bet kokios kilmės lėtinės parenchiminės kepenų ligos, LSMU ligoninės Kauno klinikų Gasroenterologijos skyriuje 2012–2015metais. Į tyrimą įtraukti pacientai, sergantys kepenų fibroze, kepenų ciroze ir sveiki kontrolinės grupės asmenys. Tyrimas sudarytas iš dviejų dalių. Pirmoji tyrimo dalis, į kurią buvo įtraukti 209 pacientai, sergantys įvairios etiologijos kepenų ciroze ir 1005 sveiki kontrolinės grupės asmenys buvo skirta nustatyti ryšį tarp *HFE* geno mutacijų C282Y ir H63D ir įvairios etiologijos kepenų cirozės. Į antrą tyrimo dalį įtraukėme 317 pacientų, sergančių kepenų ciroze, 154 pacientus, kuriems nustatyta kepenų fibrozė, ir 498 kontrolinės grupės asmenis siekiant nustatyti su fibrozės vystymusi susijusių genų *PNPLA3, RNF7, MERTK PCSK7* VNPų ryšį su kepenų fibroze ir ciroze. Abiejose tyrimo dalyse, kontrolinę grupę sudarė sveiki, savanoriai kraujo donorai, kurių bandiniai buvo renkami Nacionaliniame kraujo centre ir Kraujo donorystės centruose 2008–2009 metais. Kontrolinės grupės asmenų bandinių genetiniai duomenys pirmojoje tyrimo dalyje buvo gauti iš ankstesnio tyrimo apie *HFE* geno mutacijų paplitimą Lietuvių populiacijoje [83].

3.2. Tyrimo eiga ir metodai

Lėtinės kepenų ligos etiologija ir diagnozė buvo patvirtintos remiantis anamneze, laboratorinių, radiologinių ir histologinių tyrimų duomenimis. Alkoholinė kepenų liga buvo nustatyta, kai bent vieno paciento artimojo patvirtinimu, kasdienis alkoholio vartojimas buvo > 30 g per dieną vyrui ir > 20 g per dieną moteriai. Pacientams kepenų fibrozė buvo nustatyta histologiškai, atlikus perkutaninę kepenų parenchimos biopsiją. Į tyrimą buvo įtraukti pacientai, kuriems nustatyta 1–3 kepenų fibrozės stadija pagal METAVIR fibrozės klasifikaciją [84]. Pacientams, įtariant kepenų cirozę, buvo atlikta perkutaninė arba transjugulinė kepenų parenchimos biopsija. Pacientams su ženklia hipoakoaguliacija kepenų cirozė buvo nustatyta, remiantis klinikiniais duomenimis, laboratorinias tyrimais, endoskopiniais ir radiologiniais metodais.

Genominės DNR iš periferinio kraujo leukocitų išskyrimui naudotas išdruskinimo (angl. *salting-out*) metodas.

HFE geno mutacijos C282Y ir H63D buvo aptiktos naudojant polimerazės grandininę reakciją ir restrikcijos fragmentų ilgio polimorfizmo (PGR-RFIP) metodus.

Tiriamų genų *PNPLA3* C>G (rs738409), *RNF7* A>C (rs16851720), *MERTK* A>G (rs4374383) ir *PCSK7* C>G (rs236918) vieno nukleotido polimorfizmų nustatymui taikytas TaqMan® genotipavimo metodas, naudojant TaqMan® technologiją.

Statistinė duomenų analizė atlikta naudojant PLINK programinę įrangą, versija 1,07 [88].

4. REZULTATAI

Pirmojoje tyrimo dalyje dalyvavo 209 pacientai, sergantys įvairios etiologijos kepenų ciroze ir 1005 kontrolinės grupės asmenys. Pacientų demografiniai, klinikiniai duomenys pateikti 4.1 lentelėje.

Ištyrus 209 pacientus, sergančius įvairios etiologijos kepenų ciroze ir 1005 kontrolinės grupės asmenis rastas vienas homozigotas (0,48 proc.), turėjęs C282Y mutaciją tarp sergančiųjų kepenų ciroze. Kontrolinėje grupėje buvo rastas vienas homozigotas turėjęs C282Y (0,09 proc.) mutaciją. Statistiškai patikimo skirtumo tarp šio genotipo dažnių sergančiųjų ir kontrolės grupėse nebuvo (ŠS = 5,06, P = 0,2)

4.1 lentelė. *Demografiniai, klinikiniai pacientų duomenys*

	Kepenų cirozė (n=209)	**Kontrolinė grupė (n=1005)**
Lytis, n (proc.)		
Vyrai	107 (51,2)	581 (57,8)
Moterys	102 (48,8)	424 (42,2)
Amžius, metai, vidurkis (SD)	54,0 (8,1)	37,1 (4,3)
Child–Pugh laipsnis,n(proc.)		
A	65 (31,1)	
B	100 (47,8)	
C	44 (21,1)	

SD – standartinis nuokrypis.

C282Y alelis ir heterozigotinis genotipas (C282Y/lt) statistiškai dažnesnis pacientų, sergančiųjų kepenų ciroze nei kontrolinėje asmenų grupėje (ŠS = 2,1, P = 0,005, ŠS = 2,0, P = 0,01). Lyginant sergančiųjų ir kontrolės vyrų grupes, heterozigotinio (C282Y/lt) genotipo dažnis buvo didesnis sergančiųjų tarpe 13 (12,15 proc.) nei kontrolinės grupėje 31 (5,33 proc.) ir jis susijęs su kepenų ciroze (OR = 2,48, P = 0,008) vyrams. Tačiau tarp abiejų grupių moterų alelio C282 ir heterozigotinio (C282Y/lt) genotipo dažniai nesiskyrė.

H63D alelis ir genotipai pasisikirstę vienodai tarp sergančiųjų kepenų ciroze ir kontrolinės grupės asmenų. Rasti 9 (4,3 proc.) homozigotai, turėję H63D mutaciją tarp sergančiųjų kepenų ciroze ir 26 (2,58 proc.), homozigotai turėję H63D mutaciją kontrolinėje grupėje. Statistiškai patikimo skirtumo tarp šio genotipo dažnių sergančiųjų ir kontrolės grupėse nebuvo. Statistiškai patikimas skirtumas rastas lyginant sergančiųjų ir kontrolės vyrų grupes pagal homozigotinio (H63D/H63D) genotipo paplitimą. H63D alelis (ŠS = 1,54, P = 0,02) ir homozigotinis genotipas (H63D/H63D) (ŠS = 4,13, P = 0,005) susijęs su kepenų ciroze vyrams, bet ne moterims.

Atlikus sudėtinių heterozigotų paplitimo sergančių kepenų ciroze tyrimą, tiriamojoje grupėje buvo rasti tik 6 (2,87 proc.), o kontrolinėje 13 (1,29 proc.) individai turintys šį genotipą. Nors C282Y/H63D genotipo dažnis buvo didesnis tarp sergančiųjų , tačiau statistiškai patikimų skirtumų nebuvo (ŠS: 2,26; p=0,118).

Į antrąją tyrimo dalį įtraukėme 317 pacientų, sergančių taip pat įvairios etiologijos kepenų ciroze, 154 individus, kuriems nustatyta kepenų fibrozė ir 498 kontrolinės grupės asmenis. Dažniausia kepenų fibrozės priežastis buvo HCV infekcija, o kepenų cirozės – HCV infekcija ir alkoholis. Asmenų, kuriems nustatyta kepenų fibrozė ir kontrolinėje tiriamųjų grupėje vyravo vyrai. O sergantieji kepenų ciroze buvo statistiškai reikšmingai (P < 0,001)

vyresni, nei sergantieji kepenų fibroze ir kontrolinės grupės asmenys. Demografiniai, klinikinai pacientų duomenys pateikti 4.2 lentelėje.

4.2 lentelė. *Demografiniai, klinikiniai pacientų duomenys*

	Kepenų cirozė (n=317)	**Kepenų fibrozė (n=154)**	**Kontrolinė grupė (n=498)**	**ANOVA (amžius)[a] ir χ^2 testas P reikšmė**
Amžius,metai,vidurkis (± SD)	54,5 ± 12,2	49,9 ± 11,5	46,8 ± 6,5	<0,001
Lytis, n (proc.) Vyrai Moterys	 150 (47,3) 167 (52,7)	 95 (61,7) 59 (38,3)	 318(63,9) 180(36,1)	 <0,001
Child-Pugh laipsnis, n (proc.) A B C	 99 (31,2 152 (48,0) 66 (20,8)			
Kepenų fibrozės laipsnis, n (proc.) F1 F2 F3		 97 (63,0) 28 (18,2) 29 (18,8)		

[a] Statistinė analizė buvo atliekama visoms grupėms.
SD, standartinis nuokrypis; F- fibrozės laipsnis.

Ištyrus individus, kuriems nustatyta įvairios etiologijos kepenų fibrozė, sergančiuosius kepenų ciroze bei mišrią individų grupę (sergantieji kepenų ciroze ir turintys kepenų fibrozę) ir kontrolinės grupės asmenis nustatyta, kad *PNPLA3* geno rs738409 VNP alelis G statistiškai patikimai dažnesnis tarp sergančiųjų kepenų ciroze (29,3 proc; $P=5,57\times10^{-7}$) ir turint kepenų fibrozę (26,3 proc; P=0,001;) ir yra statistiškai reikšmingai susijęs ir su kepenų pažeidimu, sergant kepenų ciroze (ŠS: 1,92, PI: 1,49–2,48, $P = 5,57 \times 10^{-7}$) ir turint kepenų fibrozę (ŠS: 1,65; PI: 1,22–2,23; P=0,001). Mišrioje grupėje šis alelis taip pat buvo dažnesnis (28,3 proc.) nei kontrolinėje grupėje (17,5 proc.) ir statistiškai reikšmingai susijęs su kepenų pažaida (ŠS: 1,84, PI: 1,47–2,30, $P=9,27\times10^{-8}$). *PNPLA3* geno rs738409 VNP genotipų analizė parodė statistiškai reikšmingą jų sąsają su kepenų fibroze ir kepenų ciroze tiek lyginant dominantinį (ŠS: 1,98; PI: 1,44–2,72, $P = 2,20 \times 10^{-5}$; ŠS: 1,67, PI: 1,14–2,43, P = 0,008), tiek recesyvinį paveldėjimo modelius (ŠS: 3,94, PI: 2,03–7,67, $P = 5,16 \times 10^{-5}$; ŠS: 3,02, PI: 1,45–6,28; P = 0,003).

RNF7 geno rs16851720 VNP aleliai pasiskirstė tolygiai tarp tiriamųjų grupių. CC genotipas buvo dažnesnis kontrolinėje grupėje (4,6 proc.) nei tarp sergančiųjų kepenų ciroze (1,3 proc.; ŠS: 0,26, CI: 0,08–0,80,

P = 0,019) ar mišrioje grupėje (1,5%; ŠS: 0,33; CI: 0,13–0,80, P = 0,014). Taip pat šis genotipas buvo retesnis pagal recesyvinį paveldėjimo modelį mišrioje grupėje (ŠS: 0,34, PI: 0,14–0,82, P = 0,016) ir sergančiųjų kepenų ciroze grupėje (ŠS: 0,26; PI: 0,09–0,81, P = 0,020), nei tarp kontrolinės grupės individų.

MERTK geno rs437438 ir *PCSK7* geno rs236918 VNPų alelių ir genotipų pasiskirstymas tiriamosiose grupėse buvo panašus. Sąsajos *MERTK* geno rs4374383 VNP su kepenų fibroze (ŠS: 0,92, PI: 0,71–1,19, P = 0,530) ar kepenų ciroze (ŠS: 1,13, PI: 0,90–1,40, P = 0,287)nenustatyta. O ir *PCSK7* geno rs236918 VNP nesusijęs su kepenų fibroze (ŠS: 0,86; PI: 0,59–1,26; P = 0,434) ar kepenų ciroze (ŠS: 0,86, PI: 0,63–1,19, P=0,362)

Didžiausią dalį, kepenų ciroze sergančiųjų pacientų grupėje sudarė pacientai, sergantys hepatito C viruso sukelta kepenų ciroze, todėl papildomai ištirtas ryšys tarp genų *PNPLA3, RNF7, MERTK* ir *PCSK7* VNPų ir HCV sukeltos ir kitos etiologijos kepenų cirozės. Geno *PNPLA3* rs738409 VNP alelis G buvo dažnesnis sergančiųjų HCV sukelta kepenų ciroze (31,2 proc.) nei kontrolinėje grupėje (17,5 proc.) ir susijęs su HCV sąlygota kepenų ciroze (ŠS: 2,04, PI: 1,41–2,94, P = $1,43 \times 10^{-4}$). Taip pat geno *PNPLA3* rs738409 VNP alelis G buvo dažnesnis ir kitos etiologijos kepenų cirozės tiriamųjų grupėje (28,3 proc.) nei kontrolėje (17,5 proc.) bei taip pat susijęs ir su ne HCV sąlygota kepenų ciroze (ŠS: 1,81, PI: 1,36–2,42, P = $5,64 \times 10^{-5}$).

RNF7 rs16851720 CC genotipas tarp sergančiųjų ne HCV sukelta kepenų ciroze buvo retesnis nei kontrolinėje grupėje (ŠS: 0,28, PI: 0,08–1,00, P = 0,050;) Įdomu jog *RNF7* geno rs16851720 VNP alelių ir genotipų dažnis sergančiųjų HCV sąlygota kepenų ciroze ir kontrolinėje grupėje nesiskyrė, tačiau tai gali būti sąlygota santykinai mažo pacientų skaičiaus šioje grupėje.

PCSK7 geno rs236918 ir *MERTK* geno rs4374383 VNPai nesusiję su HCV sukelta ir su kitos etiologijos kepenų ciroze.

Papildomai ištyrus kombinuotą genų *PNPLA3* ir *RNF7* VNPų genotipų sąsają su HCV sukelta kepenų fibroze ir ciroze nustatyta, kad CG_{PNPLA3} AA_{RNF7} ir GG_{PNPLA3} AA_{RNF7} genotipų derinys susijęs su HCV sąlygota kepenų ciroze (ŠS: 1,69, PI: 1,04–2,76, P = 0,035; ŠS: 3,11, PI: 1,30–7,46, P = 0,011), o GG_{PNPLA3} CA_{RNF7} genotipų derinys su HCV sąlygota kepenų fibroze. (ŠS: 5,98, PI: 1,66–21,50, P = 0,006).

5. IŠVADOS

1. *HFE* geno heterozigotinė C282Y mutacija yra susijusi su kepenų ciroze Lietuvos populiacijoje.
2. *HFE* geno heterozigotinė C282Y ir homozigotinė H63D mutacijos yra susijusios su kepenų ciroze vyrams, o moterims ši sąsaja nenustatyta.
3. Lietuvos populiacijoje *PNPLA3* geno rs738409 ir *RNF7* geno rs16851720 vieno nukleotido polimorfizmai yra susiję su kepenų fibroze. *MERTK* geno rs4374383 ir *PCSK7* geno rs236918 vieno nukleotido polimorfizmų sąsajų su kepenų fibroze nenustatyta.
4. *PNPLA3* geno rs738409 ir *RNF7* geno rs16851720 vieno nukleotido polimorfizmai yra susiję su kepenų ciroze. *MERTK* geno rs4374383 ir *PCSK7* geno rs236918 vieno nukleotido polimorfizmų sąsajų su kepenų ciroze nenustatyta.
5. Išanalizavus *PNPLA3* geno rs738409 ir *RNF7* geno rs16851720 vieno nukleotido polimorfizmus priklausomai nuo kepenų cirozės etiologijos statistiškai reikšmingas ryšys nustatytas tiek su hepatito C viruso sukelta, tiek kitos kilmės kepenų ciroze. Analizuojant individų turinčių dviejų *PNPLA3* ir *RNF7* genų vieno nukleotido polimorfizmus nustatytas statistiškai reikšmingas ryšys tarp: CG_{PNPLA3} AA_{RNF7} ir GG_{PNPLA3} AA_{RNF7} genotipų ir hepatito C sąlygotos kepenų cirozės; GG_{PNPLA3} CA_{RNF7} genotipų ir hepatito C sąlygotos kepenų fibrozės.

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