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Association between PNPLA3 and TM6SF2 gene polymorphisms and non-alcoholic fatty liver disease patients in Kazakhstan

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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) is a growing burden on a global scale and considered as the most common liver disease of the 21st century, affecting both adults and children. Genome-wide association studies (GWAS) in the field of liver diseases have made a significant contribution to the understanding of genetic background for NAFLD development. Targeted genes like PNPLA3 and TM6SF2 showed some relationship with the steatosis and hepatocellular carcinoma within NAFLD patients. In this study, we tried to analyze the frequency of PNPLA3 and TM6SF2 gene polymorphisms and their relationship to changes in instrumental and laboratory markers, the composition of the gut microbiome, the development and progression of NAFLD stage in Kazakhstan.

Materials and methods: 39 individuals were involved in this study, including 18 men and 21 women: patients with a history of heavy alcohol consumption (>20 g/day) and other specific diseases such as hepatitis B and C virus infection, etc. were excluded. The diagnosis was established based on the results of clinical assessment and laboratory-instrumental results. The microbiome composition of the large intestine was studied by semiconductor sequencing of the bacterial genome using biochips. The degree of steatosis and liver fibrosis were evaluated by fibroscanning on fibroscan touch 502. Genotyping of PNPLA3 and TM6SF2 were carried out by PCR.

Results: According to PNPLA3 genotyping: 21 patients (53.85%) were C/G, 7 (17.95%) were C/C and 11 (28.20%) were G/G. Within analyzed variables, GGT showed statistically significant difference among nucleotide variability with p-value of 0.012. Other parameters within metabolic panel also showed statistically significant difference between groups, namely, total cholesterol (p=0.036) and LDL (p=0.006). Genotyping of TM6SF2 includes 24 patients (61.54%) with C/C, 15 (38.46%) with C/T and 0 with T/T. The relationship between TM6SF2 liver function test results showed no statistically significant differences between groups. All other parameters including gut microbiome analysis are not statistically significant.

Conclusions: In this study, C/G genotype possesses the highest risk and GGT along with LDL were the statistically significant parameter between them in PNPLA3 gene. TM6SF2 and gut microbiome analysis did not reveal any statistically significant differences. Additional studies with larger sample size are recommended to obtain for more detailed and sensitive results.

Keywords: non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, PNPLA3 and TM6SF2 genes, gut microbiome

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatocellular accumulation of triglycerides in the liver in the absence of excessive alcohol consumption or any other cause of secondary liver steatosis [1, 2]. NAFLD is a growing burden on global scale and considered as the most common liver disease of the 21st century, affecting both adults and children. It is predicted that NAFLD will become the main cause of hepatocellular carcinoma (HCC) and the most common indication for liver transplantation by 2030 [3, 4]. Currently, it is the most common chronic liver disease in Western countries, the prevalence of which is estimated to be on average 25.00% worldwide and 32.00% in South America [3]. NAFLD is closely related to metabolic syndrome, which is a group of conditions including obesity, insulin resistance/type 2 diabetes mellitus and dyslipidemia leading to increased risk for developing heart diseases, stroke and diabetes mainly [5]. NAFLD covers a spectrum of liver diseases, ranging from simple steatosis, which is usually benign, to NASH, which may eventually lead to cirrhosis of the liver and HCC [6]. With the increasing number of patients with NAFLD, the risk of developing advanced stage of liver fibrosis increases in parallel [7]. In recent years, genome-

wide association studies (GWASs) specifically in the field of liver disease have made a significant contribution to the understanding genetic background for NAFLD development and their impact on the disease prognosis [8, 9]. Lipid metabolism disorders with intracellular fat accumulation are believed to be the earliest events occurring in NAFLD, and genetic predisposition also has role in accelerating the development of steatosis and the transition to NASH and eventually to cirrhosis and HCC [10].

To this day, the role of TM6SF2 rs5852962 and PNPLA3 rs738409 nucleotides in the NAFLD and its advanced forms development has been studied the most. In 2008, during a GWAS, Romeo and co-authors found that the polymorphism I148M (rs738409 C>G) of the PNPLA3 gene (patatin-like phospholipase domain containing 3) is associated with the NAFLD development [4]. The gene, which is normally expressed in hepatocytes, undergoes mutation leading to active deposition of lipids within liver cells, forming macrovesicular steatosis [11]. Additionally, the deposition begins the cycle of inflammation by the activation of stellate cells–end result being formation of fibrotic tissue within hepatocytes [12].

A number of studies have confirmed the association of these genotypes with NAFLD [13, 14]. Dongiovanni et al. have confirmed that patients with the TM6SF2 variant have a more severe course of NAFLD compared to other genotypes [15]. Liu et al. on their meta-analysis identified a significant association of TM6SF2 with progressive liver fibrosis/cirrhosis. This association was independent of age, BMI and genotype PNPLA3 rs738409 [16, 17].

Although, there have been tremendous breakthrough in the understanding of NAFLD development in terms gene variations, there is not a single genetic study in Kazakhstan with NAFLD patients, especially regarding the polymorphism of TM6SF2, PNPLA3. Therefore, in this paper we investigated the relationship between the PNPLA3 and TM6SF2 genotypes and NAFLD patients' clinical parameters and analyzed the genotype variations as markers of predictors for development of liver cirrhosis and HCC. Such study is being conducted in Kazakhstan for the first time, which gives it novelty and practical significance.

MATERIALS & METHODS

The patients' recruitment was carried out within the "Medical Center Hospital of President's Affairs Administration of the Republic of Kazakhstan" during outpatient visit for gastroenterologist. In total, 102 patients were selected with hepatic cytolysis syndrome with appropriate laboratory changes as well as fatty liver changes according to the abdominal/liver ultrasound examination. The exclusion criteria for patient selection included: the presence of viral hepatitis (A, B, C, D, and E), drug-induced liver injury, autoimmune hepatitis, hepatic storage diseases (glycogen-storage diseases), hereditary liver diseases (alpha-1 antitrypsin deficiency, Wilson's disease, etc.), parasitic liver diseases, liver tumors.

As a result, the main study group consisted of 39 patients. The study was approved by the local ethics committee of NJSC "Astana Medical University" and the local ethics committee of the Medical Centre Hospital of President's Affairs Administration of the Republic of Kazakhstan.

All patients signed informed consent prior to participating in the study. All patients were assessed by a gastroenterologist, a laboratory analysis including complete blood count, comprehensive metabolic panel (ALT, AST, GGTP, alkaline phosphatase, total and direct bilirubin, total protein, albumin, protein fractions, lipid profile, glucose, and HbA1c), ELISA for viral hepatitis, parasites, autoimmune markers for liver diseases, urine ceruloplasmin, ELISA for AFP. An instrumental analysis included: ultrasound of the abdominal cavity, liver fibroscanning on the fibroscan touch 502 device with the determination of the degree of steatosis. The diagnosis was established based on the results of clinical assessment and laboratory-instrumental results.

All patients underwent blood sampling, genomic DNA was extracted from the whole blood of each participant using PureLink genomic DNA mini kit in accordance with the manufacturer's instructions. Genotyping was performed using real time PCR analysis, Taqman (Life Technologies, CA, USA) in accordance with the manufacturer's instructions. Associations between the PNPLA3, TM6SF2, and NAFLD variants were evaluated. In order to study the microbiome of the large intestine, fecal samples were collected from all participants, DNA from fecal samples was isolated using the PureLink microbiome DNA purification kit, the composition of the microbiome of the large intestine was studied by semiconductor sequencing of the bacterial genome using biochips. The sequencing data were grouped into three enterotypes: 1-prevotella, 2-bacteroides, and 3-firmicutes.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2019 software, IBM SPSS statistics for Windows, version 23.0., USA.

To determine a statistically significant difference between groups of a continuous variables a parametric method was used to study one-way ANOVA (one-way analysis of variance) and Student's t-test if the variable was normally distributed otherwise equivalent non-parametric method for one-way ANOVA–Kruskal-Wallis test and Mann-Whitney U (Wilcoxon rank test) test, for post-hoc analysis Tukey method were used appropriately. p-values reported as statistically significant at <0.05 for all analyses.

95% confidence interval [CI] for nominal data was calculated using the Clopper-Pearson method. For the analysis of nominal data, Pearson Chi-square test or the Fischer exact test were used depending on the data value for each variable.

RESULTS

In total 39 patients were included in this study, male 46.20% and female 53.80%, the average age of participants was 48.05±8.75 [95% CI: 45.21-50.89], average BMI was 30.80±5.28 corresponding to the obesity 1 (stage 2) and 87.10% (34) is considered as obese. Among study population 34 patients (87.10%) with fibrosis score zero and five patients (12.90%) with fibrosis score 1. The fibroscan results revealed 11 patients (28.20%) with CAP score between 238-260 dB/m with Steatosis S1 grade, seven patients (17.90%) with Steatosis S2 grade and 21 patients (53.80%) with Steatosis S3 grade. Additionally, only four patients had diabetes mellitus type 2 and 48.70% (19) patients were on statin treatment, all descriptive data are presented within **Table 1**.

Table 1. Demographics characteristics & main variables

Variables	Mean±SD/(%)	95% CI
Age, years	48.05±8.75	[45.21-50.89]
Male	18 (46.20%)	
Female	21 (53.80%)	
Hight, cm	167.15±9.75	[163.99-170.32]
Weight, kg	86.18±17.87	[80.39-91.97]
BMI, kg/m^2	30.80±5.28	[29.09-32.51]
ALT, U/l	81.79±44.9	[67.23-96.35]
AST, U/l	58.20±25.56	[49.91-66.48]
GGT, U/l	100.98±96.83	[69.59-132.37]
ALP U/l	104.61±39.68	[91.74-117.48]
Glucose, mmol/l	5.77±0.88	[5.48-6.06]
HbA1c %	5.92±0.53	[5.75-6.10]
Fibrosis 0(0-2)	34 (87.10%)	
Fibrosis 1(3-4)	5 (12.90%)	
Steatosis S I (238-260 dB/m)	11 (28.20%)	
Steatosis S II (261-290 dB/m)	7 (17.90%)	
Steatosis S III >291dB/m	21 (53.80%)	
BMI <25	5 (12.80%)	
Overweight, 25<BMI<29.9	15 (38.50%)	
Obesity 1, BMI 30-34.9	10 (25.60%)	
Obesity 2, BMI 35-39.9	7 (17.90%)	
Obesity 3, BMI >40	2 (5.10%)	
Diabetes mellitus	4 (10.30%)	
Treatment with statins	19 (48.70%)	

Note. SD: Standard deviation & CI: Confidence interval; & n=39

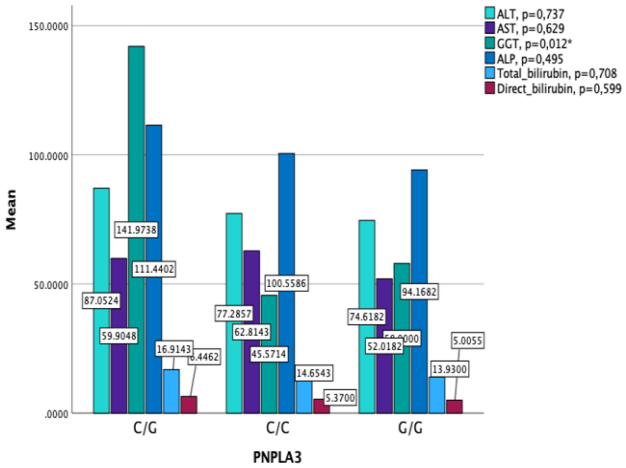

Figure 1. Comparison of liver function parameters depending on PNPLA3 genotypes (Source: Authors' own elaboration)

Table 2. Post-hoc analysis of GGT level with Tukey method within PNPLA3 genotypes

Genotype	GGT		p-value
	Mean±SD	95% CI	
C/G	141.97±45.97	89.84-194.10	p1-2=0.043* & p1-3=0.038*
C/C	45.57±29.91	17.90-73.25	p2-1=0.043* & p2-3=0.950
G/G	83.97±12.42	35.17-80.82	p3-1=0.038* & p3-2=0.950

Note. SD: Standard deviation & CI: Confidence interval

According to PNPLA3 genotyping: 21 patients (53.85%) were C/G, seven (17.95%) were C/C and 11 (28.20%) were G/G. The relationship between PNPLA3 gene polymorphism and liver function test outcomes are represented in **Figure 1**.

Within analyzed variables on GGT showed statistically significant difference among nucleotide variability with p-value of 0.012. Other parameters within metabolic panel also showed statistically significant difference between groups, namely, total cholesterol (p=0.036) and LDL (p=0.006).

Table 3. Analysis of total cholesterol depending on PNPLA3 genotypes

Genotype	Total chholesterol		p-value: 0.036*
	Mean±SD	95% CI	
C/G	5.76±1.00	5.30-6.21	p1-2=0.810 & p1-3=0.640
C/C	6.00±0.79	5.26-6.73	p2-1=0.810 & p2-3=0.620
G/G	4.98±0.71	4.49-5.46	p3-1=0.640 & p3-2=0.620

Note. SD: Standard deviation & CI: Confidence interval

Table 4. LDL analysis depending on PNPLA3 genotypes

Genotype	LDL		p-value: 0.006*
	Mean±SD	95% CI	
C/G	3.90±1.03	3.42-4.37	p1-2=0.980 & p1-3=0.007*
C/C	3.96±0.58	3.42-4.50	p2-1=0.980 & p2-3=0.031*
G/G	2.82±0.71	2.34-3.30	p3-1=0.007* & p3-2=0.031*

Note. SD: Standard deviation & CI: Confidence interval

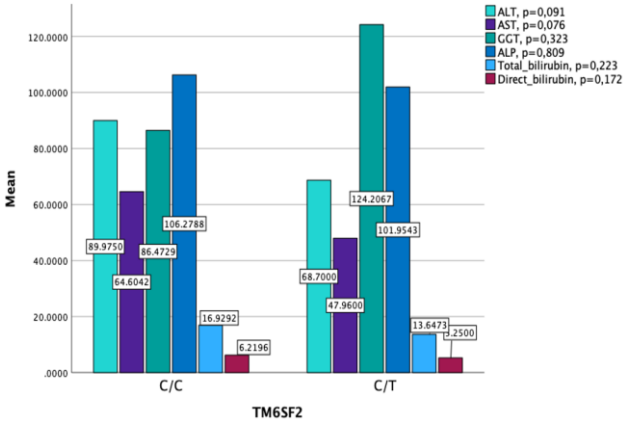

Figure 2. Comparison of liver function parameters depending on TM6SF2 genotypes (Source: Authors' own elaboration)

In **Table 2**, post-hoc analysis with Tukey method revealed a statistically significant GGT increase in genotype C/G with average being 141.90±45.97 U/l compared to the C/C genotype (p=0.043) and to G/G genotype (p=0.038). However, there were no statistically significant difference between C/C and C/G genotypes (p=0.950).

Along with GGT, other components of metabolic panel including total cholesterol and LDL also showed statistically significant differences within PNPLA3 genotypes with p values of 0.036 and 0.006, respectively.

Corresponding post-hoc analyses are represented in **Table 3** and **Table 4**, where total cholesterol level differences within PNPLA3 genotypes did not show any statistical significance, on the other hand LDL levels within G/G genotype with average value of 2.82±0.71 mmol/l has statistically significant difference from C/G (p=0.007) and C/C (p=0.031) genotypes.

Genotyping of TM6SF2 includes 24 patients (61.54%) with C/C, 15 (38.46%) with C/T and 0 with T/T.

The relationship between TM6SF2 liver function test results is illustrated in **Figure 2**, showing no statistically significant differences between groups. The same also true for differences in lipid profile parameters (total cholesterol and LDL levels), unlike in PNPLA3 genotyping.

Regarding the categorical variables, obesity and steatosis stages were evaluated for their relationship with different PNPLA3 and TM6SF2 genotypes. For obesity, there were no statistically significant differences between groups with p-value of 0.546 and 0.600 for PNPLA3 and TM6SF2, respectively.

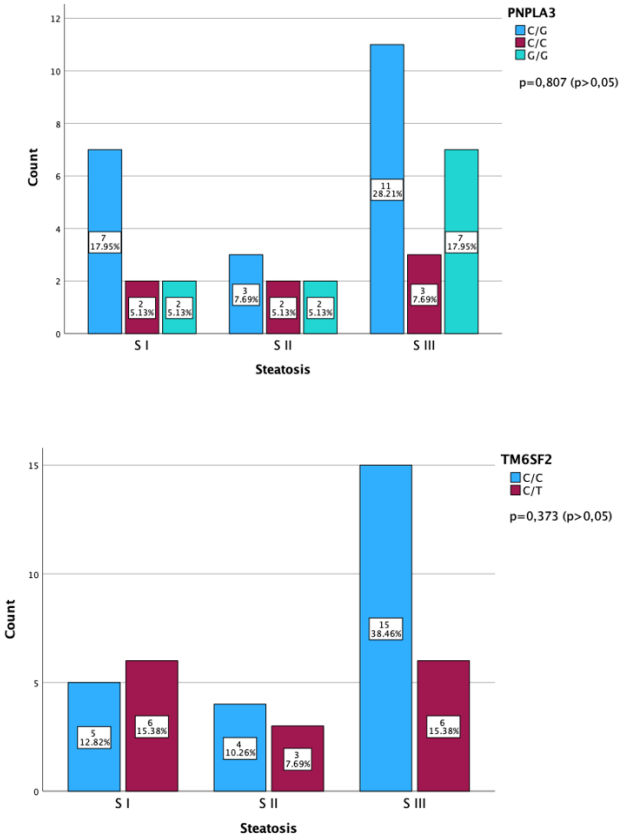

Figure 3. PNPLA3 & TM6SF2 genotypes & steatosis stages (Source: Authors' own elaboration)

Table 5. Analysis of fibrosis on results of liver fibroscan (fibrosis score)

Nucleotide	Percentage (%)		Exact Fischer test (p-value)
	Fibrosis 0: n=34	Fibrosis 1: n=5	
TM6SF2 C/C	21 (61.70%)	3 (60.00%)	1.00 (p>0.05)
TM6SF2 C/T	13 (38.30%)	2 (40.00%)	
PNPLA3 C/G	19 (55.80%)	2(40.00%)	0.767 (p>0.05)
PNPLA3 C/C	6 (17.70%)	1(20.00%)	
PNPLA3 G/G	9 (26.50%)	2(40.00%)	
TM6SF2 C/C	21 (61.70%)	3 (60.00%)	

In **Figure 3**, the distribution of PNPLA3 and TM6SF2 genotypes depending on steatosis stages are illustrated. For all PNPLA3 and TM6SF2 genotypes, Fisher exact test showed no apparent statistically significant differences between groups regarding the steatosis stage (p>0.050).

Table 5 illustrates PNPLA3 and TM6SF2 genotypes and their corresponding fibrosis score obtained from liver fibroscan. In general, for both genes with nucleotide variations the fibrosis score mostly zero (87.17%), with no apparent statistically significant differences (p>0.050).

Additionally, we also investigated the relationship between liver function test results relationship to the large intestine microbiome differences, specifically identifying three subgroups: enterotype 1 (prevotella, n=26), enterotype 2 (bacteriodes, n=12) and enterotype 3 (firmicutes, n=1), the last one excluded from further statistical analysis due to small sample size.

Analyzing liver parameters between remaining enterotypes (**Table 6**) revealed that only AST level was significantly higher in the group with Bacteriodes [M=73.7±9.31 95% CI 53.27-94.28 p=0.042] compared to enterotype 1 [M=51.40±3.75 95%CI 43.67-59.13, p=0.042], all other parameters without any statistically significant difference.

Table 6. Analysis of liver parameters between enterotypes

P	Enterotypes				p-value
	Prevotella: n=26		Bacteriodes: n=12		
	Mean±SD	95% CI	Mean±SD	95% CI	
AST	51.40±3.75	43.67-59.13	73.7±9.31	53.27-94.28	0.042*
ALT	74.69±4.25	65.93-83.44	97.99±21.38	50.92-145.05	0.144
GGT	90.34±15.69	58.02-122.65	98.48±27.40	38.16-158.80	0.785
ALP	102.37±8.84	84.16-120.58	111.83±7.51	95.29-128.38	0.505
TB	14.38±1.74	10.78-17.99	17.708±3.59	9.87-25.69	0.343
DB	5.16±0.60	3.92-6.41	7.19±1.58	3.71-10.67	0.152

Note. P: Parameter; TB: Total bilirubin; DB: Direct bilirubin; SD: Standard deviation; & CI: Confidence interval

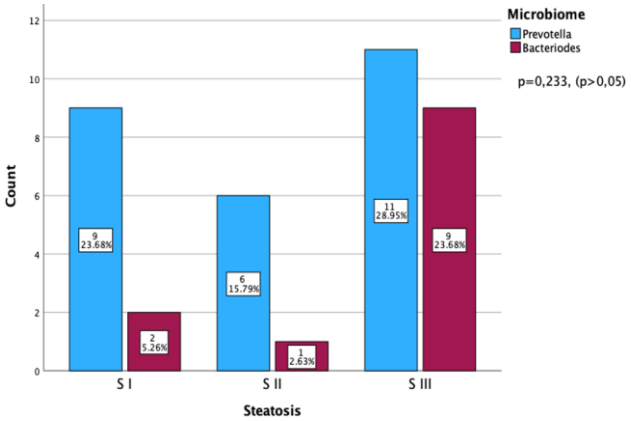

Figure 4. Gut microbiome & steatosis stages (Source: Authors' own elaboration)

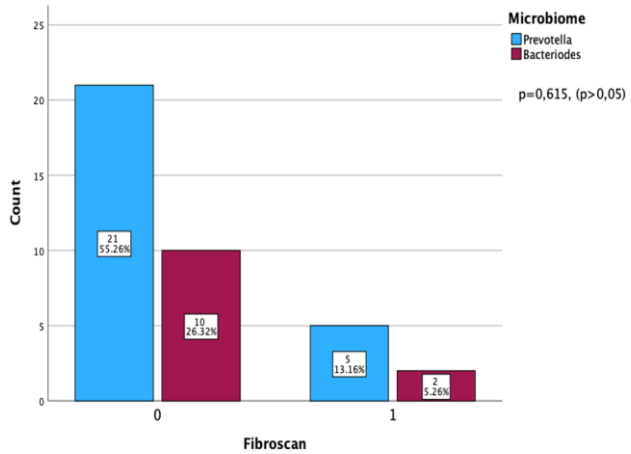

Figure 5. Gut microbiome & fibrosis score (Source: Authors' own elaboration)

Analysis of the gut microbiome alteration on the liver steatosis and fibrosis showed no statistically significant differences with p-value of 0.233 and 0.615, respectively (**Figure 4** and **Figure 5**).

DISCUSSION

In recent decades, NAFLD has become the most common liver disease worldwide, and the frequency of its complications, such as cirrhosis and HCC, has increased rapidly. Obesity and diabetes are considered not only the main

triggers of the disease development, but also two independent risk factors for the development of cirrhosis and HCC [8].

The genetics underlying inflammation and fibrosis in NAFLD are not clear yet, mainly due to the lack of sufficient clinical and research data. Although susceptible genes may increase the development of the disease, but they cannot be the primary cause for disease manifestation alone. This paradigm is most prominent in PNPLA3, where I148M carriers are much more likely to develop advanced NAFLD stages, but with some other additional triggering factors (obesity, insulin resistance). Therefore, correct understanding of the genetic basis of NAFLD provides an excellent opportunity to improve in the management of a patient with NAFLD [8, 18].

PNPLA3 and TM6SF2 are the two most studied genes that highly associated to NAFLD development nowadays. PNPLA3 normally encoding for protein adiponutrin seems to play important role in adipocytes and hepatocytes with lipolytic and lipogenic properties [19]. In general, C/C genotype of PNPLA3 seems to have protective effect and homozygous G/G genotype has the highest odds ratio for development of NAFLD compared to other. In other words, there is some dose dependent relationship of G allele especially in PNPLA3 polymorphism for the NAFLD development [20]. Similarly, it was reported that patients with PNPLA3 homozygous G/G genotype had an increased risk (3.29 times) of developing NAFLD, a higher level of AST with the presence of liver fibrosis, compared with C/C subjects [21]. It was shown that PNPLA3 genotype C/G and G/G were associated with the presence of higher steatosis (S2-S3) and fibrosis (F2-F4) stages, whereas the TM6SF2 genotype was not associated with fibrosis, but only with liver steatosis [13]. Furthermore, it was noted that PNPLA3 nucleotide G allele increased the likelihood of NAFLD and NASH (OR=3.50, 95% CI: 1.84-6.64, p<0.001). The probability of NASH was even higher with G/G homozygosity (OR=5.53, 95% CI: 2.04-14.92, p=0.001). No connection was found between the G allele and the features of the metabolic syndrome. The presence of T allele within TM6SF2 was not associated with NAFLD or NASH and not associated with typical histological features characteristic for NASH/NAFLD [22].

Despite all the previously published data, according to our results, the highest values in liver function tests were observed in patients with C/G genotype but not with homozygous G/G genotype. Interestingly, all parameters seem to be lower in G/G genotype compared to others except maybe GGT, second high mean value after C/G. The mean GGT was the highest in C/G genotype and was the one of two statistically significant (p=0.012) parameters between groups second being LDL (p=0.006). Although, Xu M. et.al. reported that TM6SF2 polymorphism associated with more advanced steatosis stages there were no association between steatosis stage, fibrosis score and different genotypes of PNPLA3 and TM6SF2 in our studies.

Along with PNPLA3 and TM6SF2 genotyping, in this study we additionally analyzed gut microbiome differences depending on the subsequent gene polymorphisms. In general, two subgroups of gut microbiome (Prevotella and Bacteroides) were chosen for further analysis, which resulted in statistically significant difference only in AST levels. Despite insignificant data regarding gut microbiome differences (p=0.233 and p=0.615 for steatosis and fibrosis, respectively), we also need to mention that Bacteriodes relatively common with steatosis stage 3 group but without significant fibrosis.

There are several studies, which compared the association of gut microbiome to the severity and progress of disease in biopsy proven NAFLD patients. It was studied combined effects of gut microbiota and genetic alteration in PNPLA3 gene and identified that prevotella and faecalibacterium species are relatively low in NAFLD patients and gemmiger species are associated with more severe disease progression [23]. Similar, results were also obtained although study was done on pediatric population [24]. The study of microbiome of the gut and its association to NAFLD development is relatively new and considering multifactorial origin of NAFLD, further studies regarding the effect of microbiome difference needs to be investigated as well in details in the future.

Limitations

The small sample size is the primary limitation of the study. Relatively limited data on gut microbiome also contributes to the limitation. Further investigation with larger sample size as well as comprising most gut microbiome is recommended for more detailed analysis of PNPLA3 and TM6SF2 gene polymorphism effect on NAFLD development and its progression.

CONCLUSIONS

According to the results of our study, polymorphisms in PNPLA3 and TM6SF2 genes were diagnosed in patients with NAFLD, namely the NASH stage, which shows their important role in the development of advanced NAFLD stages. Despite previous study results suggesting homozygous G/G genotype being the highest risk group for NAFLD development, in our study G/G genotype was the lowest risk, instead C/G genotype possesses the highest risk and GGT being the statistically significant difference between them in PNPLA3 gene. TM6SF2 and gut microbiome analysis did not reveal any statistically significant differences. Considering the relatively small sample size of our study, further studies are required with a larger sample to obtain more sensitive results.

Author contributions: All authors have sufficiently contributed to the study and agreed with the results and conclusions.

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Ethical statement: The authors stated that the study was approved by Medical Center Hospital of President's Affairs Administration of the Republic of Kazakhstan on 24 January 2020. Informed consents were obtained from the participants.

Declaration of interest: No conflict of interest is declared by authors.

Data sharing statement: Data supporting the findings and conclusions are available upon request from the corresponding author.

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