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GENETIC POLYMORPHISMS IN BULGARIAN CHILDREN WITH NONALCOHOLIC FATTY LIVER DISEASE

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a multifactorial, highly prevalent liver disease in children. There is growing evidence that some genetic variants are associated with its development and rapid progression. The aim of this study was to assess the prevalence of specific single-nucleotide polymorphisms – *PNPLA3* I148M, *GCKR* P446L, *TM6SF2* E167K in Bulgarian children with NAFLD.

We conducted a single-centre, prospective study of 32 children (22 patients with NAFLD and 10 healthy controls). Genetic testing and abdominal ultrasound were performed in all study participants. In the patients with NAFLD were additionally analyzed anthropometric parameters and standard biochemical tests, 10 of them underwent a liver biopsy for assessing disease activity and fibrosis.

The most common polymorphism was *GCKR* P446L, detected in 75% of the studied population, followed by *PNPLA3* I148M (50.0% of the studied population) and *TM6SF2* E167K (9.4% of the studied population). The *GCKR* P446L gene variant was more common in patients with NAFLD than in healthy controls (86.4% vs. 50.0%, $p = 0.03$). Furthermore, significantly more patients with NAFLD were homozygous carriers of *GCKR* P446L compared to healthy

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controls (54.5% vs. 10.0%, $p = 0.025$). Both patients with histologically proven significant liver fibrosis were carriers of *GCKR* P446L.

In the present study we found a high prevalence of *GCKR* P446L polymorphism among children with NAFLD, suggesting that this gene variant is associated with development of NAFLD in the Bulgarian pediatric population.

Key words: children, *GCKR* P446L, NAFLD, *PNPLA3* I148M, *TM6SF2* E167K

Introduction. The term non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of diseases that ranges from non-alcoholic fatty liver to non-alcoholic steatohepatitis, NAFLD with fibrosis or NAFLD with cirrhosis. Pediatric NAFLD is defined as chronic hepatic steatosis in children, which is not secondary to genetic/metabolic disorders, infections, use of steatogenic medications, ethanol consumption, or malnutrition [1]. NAFLD is a common disease in children. The prevalence rate varies between 3% and 10% depending on method of detection, which may include screening by alanine aminotransferase, imaging for steatosis, or confirmation by liver biopsy [1, 2]. In Europe NAFLD shows still an increasing trend that parallels the rising prevalence of obesity in the pediatric population [2, 3]. However, obesity is only one of the predisposing factors to NAFLD [2] Recent studies in adults and children showed that some genetic variants in the patatin-like phospholipase domain containing protein 3 (*PNPLA3)*, glucokinase regulatory protein *(GCKR)* and transmembrane 6 superfamily member 2 (*TM6SF2)* genes are associated with development of NAFLD and play a key role in the disease progression [4, 5]. The aim of this study was to assess the prevalence of specific single-nucleotide polymorphisms – *PNPLA3* I148M, *GCKR* P446L and *TM6SF2* E167K in Bulgarian pediatric patients with NAFLD.

Materials and methods. This was a single-centre prospective study conducted at the Department of Gastroenterology and Hepatology, at University Pediatric Hospital "Prof. Dr. Ivan Mitev" Sofia, between September 2016 and November 2018. The inclusion criteria were: age below 18 years and diagnosis of NAFLD, defined by ultrasound examination after exclusion of viral and autoimmune hepatitis, Wilson's disease, metabolic liver disease and drug-induced liver injury. A group of healthy volunteers was also recruited.

Standard tests. Standard anthropometric measures and laboratory tests were performed in all patients with NAFLD. In addition, in all of them was calculated a homeostatic model assessment for insulin resistance (HOMA – IR), using the following formula: HOMA-IR = fasting plasma glucose (mmol/l) × fasting serum insulin (μIU/ml)/22.5. Insulin resistance (IR) was defined as a value of HOMA-IR > 2.5. All study participants were examined by abdominal ultrasonography.

Liver biopsy and histology. Ten of the children with NAFLD underwent a liver biopsy. It was performed on the same day with the ultrasound examination. All of the specimens contained at least 5 portal spaces and were stained with

hematoxylin-eosin and with Masson trichrome for the evaluation of fibrous tissues. Liver fibrosis and necroinflammatory activity were evaluated using the METAVIR scoring system.

Genetic tests. Blood samples of the patients were taken after written informed consent from their parents was obtained. Genomic DNA was extracted from peripheral white blood cells according to standard salting-out procedure. PCR amplification with specific primer pairs for variants in the genes *PNPLA3* (c.444C>G, p.I148M), *GCKR* (c.1337C>T, p.P446L) and *TM6SF2* (c.499C>T, p.E167K) was performed (Primer sequences available upon request) followed by direct sequencing using BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Foster city, CA). The sequencing profiles are interpreted by the software Sequencing Analysis v.5.1.1.

Statistical analysis. Statistical analysis was performed by using software (SPSS version 13.0). The difference between the NAFLD and the control group was analyzed using a Fisher's exact test or Chi-squared test, as appropriate. *P*-values < 0.05 were defined as statistically significant level.

Ethics. This study was approved by the Ethics Committee of the Medical University of Sofia (Permit Number 10/16.03.2017) and all research activities were conducted in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individuals participating in the study.

Results. Twenty-two patients with NAFLD (fifteen boys and seven girls) and ten healthy controls (five boys and five girls) took part in the study. The mean age of the study participants was 10.53±3.78 years. Twenty-one of the patients were obese (BMI-for-age > 95th percentile) and one was overweight (BMI-for-age > 85th percentile). Liver synthetic function in all NAFLD patients was normal. Twelve (54.54%) of the NAFLD patients had elevated ALT and six (27.27%) elevated AST. The anthropometric and laboratory characteristics of the children with NAFLD are shown in Table 1.

The *GCKR* P446L gene polymorphism was found in 75.0% (24/32) of the study participants (homo-or heterozygous carriers). It was more common in patients with NAFLD than in healthy controls (86.4% vs. 50.0%, $p = 0.03$). Significantly more patients with NAFLD were homozygous carriers of *GCKR* P446L compared to healthy controls (54.5% vs. 10.0%, $p = 0.025$). One patient who was homozygous carrier of *GCKR* P446L had also low glucose levels 2 mmol/l. *PNPLA3* I148M was detected in 50% (16/32) of the study participants. It was less common in the patients with NAFLD than in healthy controls (40.9% vs. 70.0%, $p = 0.1331$). *TM6SF2* E167K was the rarest genetic variant in the study population, only 3 children (2 patients and 1 healthy child) were carriers. The frequencies of the genetic polymorphisms are presented in Table 2.

T a b l e 1

Characteristics of 22 patients with NAFLD

	N	Mean	Median	SD	Min	Max
Age, years	22	10.36	10.00	3.06	4.00	17.00
Weight, kg	22	63.62	61.65	17.99	26.00	102.40
Height, cm	22	147.97	147.95	17.09	116.00	177.50
BMI	22	28.47	28.15	3.65	19.30	35.90
BMI, Pe	22	98.3	99.3	2.98	91.00	99.80
ASAT, IU/l	22	30.86	26.5	15.27	14.00	65.00
ALAT, IU/l	22	42.22	30.50	29.65	11.00	105.00
γ-glytamil-transferase, IU/l	22	24.95	20.00	20.04	5.00	82.00
Alkaline phosphatase, IU/l	22	237.50	239.50	82.04	80.00	443.00
Cholesterol, mmol/l	22	4.16	4.09	0.88	2.38	5.83
HDL-Cholesterol, mmol/l	22	1.24	1.15	0.49	0.74	3.10
LDL-Cholesterol, mmol/l	22	2.28	2.43	0.74	0.99	3.62
Triglycerides	22	1.39	1.25	0.82	0.5	3.8
Glucose, mmol/l	22	4.94	5.08	0.92	2	6.3
HOMA-IR	22	5.32	4.09	3.28	0.69	15.34

T a b l e 2

Frequencies of genetic polymorphisms *PNPLA3* I148M, *GCKR* P446L, *TM6SF2* E167K in patients with NAFLD and healthy controls

Genes	NAFLD Patients		Healthy controls	
	Number	Percentage	Number	Percentage
PNPLA3				
I148M homozygous	7	31.8%	6	60.0%
I148M heterozygous	2	9.1%	1	10.0%
Wild type	13	59.1%	3	30.0%
GCKR				
P446L homozygous	12	54.55%	1	10.0%
P446L heterozygous	7	31.82%	4	40.0%
Wild type	3	13.63%	5	50.0%
TM6SF2				
E167K homozygous	1	4.55%	1	10.0%
E167K heterozygous	1	4.55%	0	0.0%
Wild type	20	90.9%	9	90.0%

Most of the patients who underwent a liver biopsy had mild fibrosis or no fibrosis. Only two of them had significant fibrosis. Patient N1, a 10-year-old boy, was homozygous carrier of *PNPLA3* I148M and heterozygous carrier of *GCKR* P446L. Patient N2, a 9-year-old girl, was a heterozygous carrier of *GCKR* P446L.

We could not find any significant associations between the prevalence of specific gene variants and the laboratory findings.

Discussion. In addition to environmental factors, recent studies suggested that genetic factors are also involved in the development and progression of NAFLD [4,5]. In this study we assessed the prevalence of three genetic polymorphisms associated with NAFLD in Bulgarian pediatric patients with NAFLD and in healthy controls. To the best of our knowledge, this study is the first investigation of the prevalence of *PNPLA3* I148M, *GCKR* P446L and *TM6SF2* E167K among children in our country.

We found that *GCKR* P446L was the most common polymorphism, detected in 75% of the studied population, followed by *PNPLA3* I148M (50.0% of the studied population) and *TM6SF2* E167K (9.4% of the studied population). *GCKR* P446L was also the most frequent polymorphism in patients with NAFLD, identified in 86.4% of them, followed by the *PNPLA3* I148M and *TM6SF2* E167K, identified in 40.9% and 9.1%, respectively. Similar to other authors we found that the prevalence of *GCKR* P446L (homo-or heterozygous state) was significantly higher in patients with NAFLD compared to healthy controls [6,7]. Although *GCKR* P446L gene polymorphism has been reported to be associated with hypoglycemia [8], low glucose levels were detected in only one patient from our cohort, who was homozygous carrier of *GCKR* P446L.

In 2019 Karamfilova et al. [9] investigated the prevalence of *PNPLA3* I148M variant in 208 adult Bulgarian patients with NAFLD. Being in agreement with our results they demonstrated that 37% of the participants were homozygous carrier and 4.8% were heterozygous carrier of the polymorphism [9].

TM6SF2 E167K was the rarest genetic variant in our cohort, identified in two patients with NAFLD and in one healthy child. These findings are in line with previous observations among adult Bulgarian patients with NAFLD [10]. Karamfilova et al. found that only 11% of the investigated patients were heterozygous carriers of *TM6SF2* E167K, no homozygous carriers were detected. Based on these data we may speculate that *TM6SF2* E167K is generally less common in the Bulgarian population in comparison to *PNPLA3* I148M and *GCKR* P446L polymorphisms.

Our study has several limitations. One of the limitations is the small sample size. Our preliminary findings should be confirmed in larger cohorts. Another limitation of the study is that we did not perform a liver biopsy in all children with NAFLD.

Conclusions. In conclusion, this study showed a high prevalence of *GCKR* P446L polymorphism among children with NAFLD, suggesting that this gene

variant is associated with development of NAFLD in the Bulgarian pediatric population.

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