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Role of genetic modification of the *PNPLA3* gene in predicting metabolically unhealthy obesity and metabolic associated fatty liver disease in children

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ABSTRACT

Introduction and aim. Single nucleotide variants (SNV) of the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene play an important role in hepatic lipid remodeling and lipogenesis *de novo*, which is associated with the development of metabolically unhealthy obesity (MUO) and metabolic associated fatty liver disease (MAFLD). The aim of the study was to define the contribution of SNV *PNPLA3* gene to the development of MUO, complicated by MAFLD in children.

Material and methods. 200 obese children aged 6-18 years were examined. The main group (n=118) was represented by children with MUO. The control group (n=82) consolidated of children with metabolically healthy obesity (MHO). Whole genome sequencing (CeGat) was performed in 31 children of the main and 21 children of the control group.

Results. Among obese children, 14 variants of SNV *PNPLA3* (rs139051, rs34179073, rs2294918, rs139047, rs779127153, rs2076212, rs738409, rs738408, rs4823173, rs2072906, rs2076213, rs141106484, rs138736228) were identified, including SNV *PNPLA3* g.44322818, not described in the dbSNP core database. The role of the following SNV *PNPLA3* genotypes in the development of MUO complicated by MAFLD was revealed: rs738409 C/G (Relative risk (RR)=1.71); rs738408 C/T (RR=1.71); rs4823173 G/A (RR=1.57); rs2072906 A/G (RR=1.57) with Sensitivity (Se)=0.63 and Specificity (Sp)=0.72.

Conclusion. The contribution to the development of MUO complicated by MAFLD in children is made by the linked association of genotypes: rs738409 C/G, rs738408 C/T, rs4823173 G/A and rs2072906 A/G out of 14 *PNPLA3* SNVs diagnosed by us.

Keywords. children, metabolic associated fatty liver disease, obesity, patatin-like phospholipase domain-containing protein 3, single nucleotide variants

Introduction

The basis of metabolic associated fatty liver disease (MAFLD) is the accumulation of lipid droplets (LD) in more than 5% of hepatocytes, which are detected during histological examination, or an increase in the proton density of the fat fraction of more than 5.6% according to proton magnetic resonance spectroscopy in humans, who consume little or no alcohol, and in the absence of secondary causes of liver damage.[1] Currently, MAFLD is considered not as a primary liver disease, but as one of the components of the metabolic syndrome. An excess of nutrients entering the body causes the development of metabolically unhealthy obesity (MUO), which, unlike metabolically healthy obesity (MHO), is characterized by such changes as abdominal obesity, dyslipidemia, arterial hypertension, insulin resistance and impaired carbohydrate tolerance.[2,3]

Genome-wide association studies (GWAS) have demonstrated that single nucleotide variants (SNV) of the *Patatin-like phospholipase domain-containing protein*

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3 (PNPLA3) gene, highly expressed in the liver (43 transcripts per million - TPM), skin (10 TPM), and adipose tissue (3 TPM)[4], highly associated with MUO and MAFLD phenotype.[5-12]

PNPLA3 gene, HGNC:18590, ENSG00000100344 (adiponutrin (ADPN), calcium independent phospholipase A2 epsilon (iPLA2epsilon), dJ796I17.1, C22orf20, FLJ22012) located on chromosome 22: 43,923,792-43,964,488 has 43,964,488 strand (0GR028) forward:CM 5 transcripts (splice variants), 303 orthologues, 4 paralogues. The PNPLA3 gene encodes the LD transmembrane protein, adiponutrin. The PNPLA3 protein consists of 481 amino acids, has the enzymatic activity of triacylglycerol hydrolase 5 (TG5), lysophosphatidyl acyltransferase 6 (LPAAT6) and calcium-independent phospholipase A2 (iPLA2), play an important role in hepatic lipid remodeling and lipogenesis de novo, is involved in intracellular lipolysis of triacylglycerides (TG). Protein PNPLA3[148I] has hydrolase activity towards retinol esters, which leads to the formation of retinolic acid, which, being released from hepatic stellate cells, suppresses their activation and leads to inhibition of proliferation, migration and secretion of chemokines.[13]

However, the main function of PNPLA3 is considered to be the ability to inhibit the activity of adipose triglyceride lipase (ATGL), which is a key enzyme that controls the release of fatty acids from LD hepatocytes.[14] Activation of ATGL, which maintains an optimal LD size, is possible after ubiquitylation and proteasomal degradation of the PNPLA3 protein.

Replacing isoleucine with methionine at position 148 of the PNPLA3 gene (rs738409 C>G) leads to the formation of a mutated PNPLA3[I148M] protein highly resistant to proteasomal degradation, inactivation of ATGL, and accumulation of fatty acids in LD of hepatocytes.[6]

Protein PNPLA3[I148M] is the target of transcription factor NF-kB[15] and has the selective ability to activate oxidative stress through the IRE-1α/JNK/c-Jun signaling pathway in the endoplasmic reticulum. Unlike the PNPLA3 protein, the PNPLA3[I148M] protein is localized in the cytoplasm, which promotes c-Jun-dependent expression of pro-inflammatory cytokines such as TNF-α, causing the development of steatohepatitis and liver fibrosis.[16]

Patients with hepatic steatosis (obese and non-obese) have been found to have an increased incidence of the rs738409 C/G genotype compared to a population of people with obesity but without hepatic steatosis.[17] It has been proven that the formation of the mutant protein PNPLA3[I148M] causes an increase in the level of the serum biomarker of microvesicular steatosis 3-methylglutarylcarnitine.[18]

The contribution of SNV rs738409 PNPLA3 predicts the severity of MAFLD and the degree of activity of non-alcoholic steatohepatitis (NASH): $p = 3.94 \times 10^{-8}$, in both adults ($p = 9.73 \times 10^{-15}$) and children ($p = 9.92 \times 10^{-6}$) and is the most significant.[19,20] The relative risk of MAFLD in individuals with minor variants is, according to different authors, from 1.58 to 2.29.[21,22] The role of rs738409 PNPLA3 is especially significant in males and in children with elevated levels of basal insulinemia and hypertriglyceridemia.[23]

The results of GWAS meta-analyses demonstrated that SNV PNPLA3 (rs139051, rs12483959 and rs2072907) also have a significant impact on childhood obesity; SNV rs4823173 PNPLA3 determine excessive accumulation of LD in hepatocytes in adult patients and ALT increase ($p = 3.44 \times 10^{-10}$), causing a high risk of developing hepatocellular carcinoma.[5,24-26]

The contribution of other SNVs of the PNPLA3 gene, identified in our research work by whole genome sequencing, to the development of MUO in children complicated by MAFLD, as the most common liver disease worldwide, remains poorly understood.

Aim
The aim of the study was to define the contribution of SNV of the PNPLA3 gene to the development of MUO, complicated by MAFLD in children.

Material and methods
Ethical approval
Participants provided written informed consent, and research protocols and procedures were approved according to the ethical standards of the Helsinki Declaration 2013 and by the Human Research Ethics Committee of Dnipro State Medical University (ethical approval DSMU/EC/19/1107). Time of data collection: January 2020 – August 2022.

Study design
Observational, analytical, longitudinal, cohort study.[27] Inclusion criteria: children with polygenic obesity (BMI≥97th percentiles) 6-18 years old. Exclusion criteria: children with monogenic and/or syndromic obesity, pregnancy.

To test the hypothesis about the association of the studied SNVs with obesity phenotypes, an analysis of the frequency of PNPLA3 genetic variants, along with measurements of anthropometric and biochemical parameters, according to the recommendations of IDEFICS 2014, was carried out in a cohort of 200 obese children aged 6–18 years in the children's endocrinology department of the CNE Dnipro Clinical Hospital No. 9 of the Dnipro City Council (children from an urban obesity clinic).[28] For the examination of children, the consent of their parents was obtained. The main group (n=118) was represented by children with MUO. The control group (n=82) consisted of children with MHO. Each participant was identified by a code used in the database.

For inclusion in the main observation group, the presence of abdominal obesity and two of the presented criteria were taken into account: 1). Fasting glycemia ≥ 5.6 mmol/L; 2). High-density lipoprotein (HDL) ≤ 1.03 mmol/L or less than 10th percentile of the age norm; 3). TG ≥1.7 mmol/L or more than the 90th percentile of the age norm; 4) Systolic blood pressure (SBP) above the 90th percentile for a given age, gender and height; 5). Diastolic blood pressure (DBP) above the 90th percentile for a given age, gender and height.[29-32]

The abdominal type of obesity was determined according to the consensus of the International Diabetes Federation (IDF), based on the excess of the waist circumference over the 90th percentile for children.[33,34]

Anthropometric measurements were made in a child in underwear and without shoes. Height (cm) was measured using Heightronic Digital Stadiometer® to the nearest 0.1 cm. Weight (kg) and body fat (BF) percentage was measured using Tefal Bodysignal body composition analyzer (France). The calculation of the percentage of fat or BF in the body was performed automatically with a discreteness of 0.1%, according to the requirements of Tefal Bodysignal, with the evaluation of results according to the unified centile scales for children of this age.[35] Waist circumference (WC), hip circumference (HC) was measured using a standardized anthropometric tape, measuring the circumference at the midpoint between the top of the iliac crest and the lower part of the lateral rib cage to the nearest 0.1 cm.[36] BMI was converted to SDS by means of the current WHO growth references.[37]

Systolic and diastolic blood pressure (DBP) were measured using a digital oscillimetric device, Dinamap ProCare (GE Healthcare).

Laboratory examination for the formation of observation groups for obesity phenotypes included general clinical methods. Blood samples were obtained after an overnight fast by venipuncture in vacutainer gel tubes, and serum was separated from cells by centrifugation in a certified laboratory "Synevo" (Ukraine) using an analyzer and a Cobas 6000 test system; Roche Diagnostics (Switzerland). The analysis of serum glucose was carried out by the hexokinase method; the determination of triglycerides and high-density lipoproteins of blood plasma was carried out by the enzymatic – colorimetric method. The study of the levels of alanine aminotransaminase (ALT) and aspartate transaminase (AST) was performed by the kinetic method and assessed according to NASPGHAN guidelines.[38] The determination of the level of basal insulin was performed using the immunochemical testing method with electrochemiluminescent detection (ECLIA). The level of basal insulin in the venous blood was considered normal 2.6–24.9 mcU/ml.

Additionally, in the comparison groups, we assessed biochemical markers (AST/ALT ratio index, where an indicator of more than 1 was considered pathological; As-partate aminotransferase/platelet ratio index (APRI), where an indicator of more than 0,76 (Metavir F0-F1) was considered pathological; visceral adiposity index (VAI), according to Amato.[39-41] The threshold values determined for VAI in predicting MUO were 1.58, 1.30 and 1.78 for the general population, boys and girls, respectively.[42]

To study the role of pro-inflammatory markers in the development of meta-inflammation in children with obesity, the serum levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), C-reactive protein (CRP). IL-1β was detected by the immunochemical method with chemi-luminescence immunoassay (CLIA). Analyzer and test – system: Immulite (Siemens AG), Germany. The reference value of IL-1β level was 0–5 pg/ml. IL-6 was determined by an enzyme-linked immunosorbent assay (ELISA) using a Cobas 6000/Cobas 8000 kit provided by Roche Diagnostics (Switzerland). The reference value of IL-6 level was 1.5–7.0 pg/ml. The level of CRP was measured using the turbidimetric immunoassay method. Analyzer and test – system: Cobas 6000 (with 501 modules), Roche Diagnostics (Switzerland). The CRP level of 0–5 mg/dl was considered the reference value. Leptin was determined using ELISA. Analyzer and test system: Tecan Sunrise, LDN (Germany). The reference value of leptin level for boys was 2–5.6 ng/ml, for girls – 3.7-11.1 ng/ml. Adiponectin was tested using ELI-SA. Analyzer and test system: Mediagnost GmbH (Germany). Interpretation of the results was carried out as follows: low cardiovascular risk - more than 10 µg/ml; moderate cardiovascular risk – 7-10 µg/ml; high cardiovascular risk – 4-7 µg/ml; very high cardiovascular risk – less than 4 µg/ml.

The sample population examined by whole genome sequencing (NGS, Illumina CSPro®, CeGat, Germany) consisted of 31 children of the main and 21 children of the control group and was qualitatively homogeneous in relation to the general population). Average amount of DNA (µg) in samples – 0.875. Library Preparation: Quantity used 50 ng. Library Preparation Kit: Twist Human Core Exome plus Kit (Twist Bioscience). Sequencing parameters: NovaSeq 6000; 2 x 100 bp.

Bioinformatic analysis – demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (version 2.20). Adapters were trimmed with Skewer, version 0.2.2.[43] DNA-Seq: Trimmed raw reads were aligned to the human reference genome (hg19-cegat) using the Burrows-Wheeler Aligner, BWA – mem version 0.7.17-cegat.[44] ABRA, version 2.18 and Genotype-Harmonizer v.1.4.20 were used for local restructuring of readings in target regions to improve more accurate detection of indels in the genome during mutagenesis.[45,46]

All children underwent an ultrasound examination of the liver using the Simens Sonoline G 40 device (Ja-pan) using of MAFLD ultrasound criteria according to Saverymuttu.[47]

To recognize the functional effects of SNV *PNPLA3* in the development of MUO complicated by MAFLD, statistical methods were used: analysis of variance, Odds Ratio (OR) with calculation of 95% Confidence Interval (CI), Spearman correlation analysis, sequential Wald analysis with calculation of Relative Risk (RR), Predictive Coefficient (PC), Sensitivity (Se), Specificity (Sp) and p-value for each variable.[48] Statistical processing of the results was performed using Microsoft Excel (Office Home Business 2KB4Y-6H9DB-BM47K-749PV-PG3KT) and STATISTICA 6.1 software (StatSoftInc, no. AGAR909E415822FA).

Results

The proportion of boys in the main group was 40% (47/118), in the control group – 50% (41/82). In this connection, RR MUO in girls was 1.2 times higher than in boys, p<0.05.

In the comparison groups, there was a statistically significant difference in anthropometric data, indicators of carbohydrate and fat metabolism, adipocytokine status of patients in the form of hyperleptinemia, adiponectinemia, increased levels of IL-6 and CRP among children with MUO. The results of clinical (Table 1) and paraclinical examinations (Table 2) among children with various obesity phenotypes allowed us to identify the most frequent clinical associations of markers of a complicated course of obesity (dyslipidemia, arterial hypertension, hyperglycemia).

Table 1. Average values of anthropometric and manometric examination of children with different phenotypes of obesity

Indicator	Children with MUO (M±m)	Children with MHO (M±m)	p
BMI in percentiles, %	99.54±0.31	98.74±0.39	0.12
Physical development in percentiles	74.9±4.85	55±6.86	0.02
BF in girls, %	38.2±2.3	28.9±0.8	0.0004
BF in boys, %	35.5±2.5	25.0±2.1	0.02
WC in girls, cm	97,6±4.2	79.9±3.1	0.001
WC in boys, cm	101.5±3.2	86.8±4.8	0.014
Correlation WC/H in girls	0.61±0.02	0.57±0.02	0.16
Correlation WC/H in boys	0.6±0.02	0.54±0.02	0.03
Correlation WC/HC in girls	0.9±0.04	0.88±0.1	0.85
Correlation WC/HC in boys	0.94±0.04	0.87±0.02	0.12
A body shape index (ABSI), %	0.072±0.001	0.071±0.02	0.96
SBP in percentiles	83.4±3.13	72.5±4	0.04
DBP in percentiles	87.1±2.81	67.15±4.22	0.0004

In the main group, the most frequent clinical association was a combination of abdominal obesity, dyslipidemia and arterial hypertension (33.3%), the presence of four markers of a complicated course of obesity was noted in every fifth patient with MUO (20%). Hyperglycemia was diagnosed only in the main group in the association of abdominal obesity with dyslipidemia (6.7%) or in combination with abdominal obesity and arterial hypertension (6.7%). In the control group, every fourth patient (25%) was diagnosed with isolated abdominal obesity, in 85% of patients a combination of two markers of a complicated course of obesity was found, namely obesity with initial manifestations of arterial hypertension (SBP/DBP=85–90th percentiles) – in 35% of patients or associations of obesity with initial manifestations of dyslipidemia (HDL=11–25th percentile or TG=85–90th percentile) – in 20% of the examined.

Table 2. Features of carbohydrate and fat metabolism in children with different obesity phenotypes

Indicator	Children with MUO (M±m)	Children with MHO (M±m)	p
Fasting blood glucose, mmol/L	5.23±0.13	4.85±0.11	0.03
HbA1c,%	5.36±0.11	5.22±0.22	0.57
Basal insulin, mcU/ml	29.58±2.13	12.9±1.44	0.0001
HOMA-IR	6.76±0.5	2.61±0.22	0.0001
Leptin in girls, ng/ml	51.97±2.92	26.04±2.92	0.0001
Leptin in boys, ng/ml	43.1±2.92	12.51±2.92	0.0001
Adiponectin, mcg/ml	4.99±0.57	11.13±1.7	0.001
IL-6, pg/ml	4.36±0.82	1.97±0.22	0.007
IL-1β, pg/ml	3.89±0.63	3.3±0.92	0.59
CRP, mg/ml	5.67±0.96	2.57±0.57	0.007
HDL, mmol/L	1.34±0.1	1.29±0.04	0.64
HDL in percentiles	30.83±4.04	33.2±2.9	0.63
TAG, mmol/L	1.45±0.01	1.25±0.1	0.05
TAG in percentiles	88±1.27	84±1.5	0.04
VAI in girls	2.6±0.31	1.5±0.14	0.002
VAI in boys	1.48±0.31	0.81±0.14	0.05
ALT, UI/L	27.54±1.4	23.75±0.8	0.02
AST, UI/L	27.57±1.65	21.55±1.32	0.006
AST/ALT ratio index	1.12±0.06	0.8±0.03	0.0002
APRI	0.93±0.01	0.74±0.01	0.0002

Impaired fasting glycemia and/or carbohydrate tolerance during an oral glucose tolerance test was detected in 33.3% and 16.7% of patients in the main group and was not observed in patients in the control group.

The greatest contribution to the development of MVR was noted at: the value of the HOMA index exceeding the 95th percentile (RR=9.33); ultrasound signs of steatohepatosis (RR=6.33); extreme obesity (RR=6); AST/ALT ratio index>1 (RR=3.56); ultrasound signs of hepatomegaly according to Saverimutt (RR=3.33); DBP above 90th percentile (RR=3.07); SBP above 90th percentile (RR=2.27); HDL less than 25th percentile (RR=1.87) (Table 3).

MAFLD was diagnosed among children of the main group in 66.6%, and in the control group - in 10% of patients in the following association with other markers of a complicated course of obesity (Table 4).

Table 3. Relative risk of MUO calculation with 95% confidence interval*

Indicator	MUO, %	MHO, %	EER$_{MUO}$	CER$_{MHO}$	RR	S	95% CI	Se	Sp
HOMA-IR exceeding the 95th percentile	93.3	10	0.933	0.1	9.33	0.67	2.49-34.87	0.93	0.9
Ultrasound findings regarding steatohepatosis according to Saverymuttu	63	10	0.63	0.1	6.33	0.68	1.65-24.25	0.91	0.62
Extreme obesity (with body weight more than 120% from 95 percentile)	16.6	5	0.35	0.05	6	1.01	1-43.76	0.9	0.48
AST/ALT ratio index>1	53.3	15	0.53	0.15	3.56	0.56	1.19-10.64	0.84	0.55
Ultrasound findings regarding hepatomegaly	66.7	20	0.67	0.2	3.33	0.47	1.34-8.3	0.83	0.62
DBP exceeding the 90th percentile	76.7	25	0.77	0.25	3.07	0.4	1.4-6.71	0.82	0.68
SBP exceeding the 90th percentile	56	25	0.57	0.25	2.27	0.42	1-5.15	0.77	0.53
HDL less than 25 percentile	46	25	0.47	0.25	1.87	0.43	0.8-4.37	0.74	0.48

* Absolute risk in the main group: EER – experimental group event rate; absolute risk in the control group: CER – control group event rate; RR – relative risk; S – relative risk standard error; Se – sensitivity; Sp –specificity

Table 4. Types of association of metabolic markers and triglycerides in obesity phenotypes in children aged 6-18 years

Association types of metabolic markers	MUO, %	MHO, %
Isolated abdominal obesity + MAFLD	0	5
Abdominal obesity + Dyslipidemia + MAFLD	13.3	5
Abdominal obesity + Arterial hypertension + MAFLD	13.3	0
Abdominal obesity + Arterial hypertension + Dyslipidemia + MAFLD	13.3	0
Abdominal obesity + Hyperglycemia + Dyslipidemia + MAFLD	6.7	0
Abdominal obesity + Arterial hypertension + Hyperglycemia + Dyslipidemia + MAFLD	20	0

MAFLD was registered in 50% of girls and 33% of boys with various obesity phenotypes, p<0.05. We have established a correlation between an early indicator of nonspecific hepatocellular damage (ALT) and the following clinical/biochemical parameters: APRI (r=0.77); AST (r=0.62); steatohepatosis on ultrasound examination (r=0.6); body weight (r=0.59); BMI (r=0.59); the presence of extreme obesity (r=0.47); SBP (r=0.47); hepatomegaly on ultrasound examination (r=0.45); impaired fasting glycemia (r=0.44); waist circumference (r=0.43); hip circumference (r=0.43); impaired tolerance to carbohydrates (r=0.35); AST/ALT ratio index<1 (r= -0.37); growth (r=0.3); hyperleptinemia (r=0.29); ABSI (r= -0.28); arterial hypertension (r=0.28); hyperuricemia (r=0.28) and age (r=0.28).

Among obese children, 14 variants of SNV *PNPLA3* (rs139051, rs34179073, rs2294918, rs139047, rs779127153, rs2076212, rs738409, rs738408, rs4823173, rs2072906, rs2076213, rs141106484, rs138736228) were identified, including SNV *PNPLA3* at position 44322818

Table 5. Characterization of the SNV *PNPLA3* in obesity phenotypes in children*

Position	GnomAD_maxPOP	dbSNP	Ref/Alt	Zygosity (Proportion HOM^p/HET/HOM^n, %) MUO	Zygosity (Proportion HOM^p/HET/HOM^n, %) MHO	Consequence	BaseChange	CADD	RawScore	Clinical Significanse (gnomAD browser)
44324676	NFE	rs139051[a]	A/G	23.3/46.7/30	30/55/15	intronic	c.421-28A>G	4.31	0.103	Not Reported in ClinVar
44328832	NFE	rs34179073	C/T	73.3/26.7/0	75/25/0	synonymous	c.561C>T	10.46	0.62	Benign
44342116	AFR	rs2294918	A/G	10/50/40	10/55/35	missense	c.1300A>G	0.02	-0.67	Benign
44323074	EAS	rs139047	G/A	36.7/43.3/20	40/40/20	intronic	c.420+27G>A	2.91	0.02	Not Reported in ClinVar
44323036	SAS	rs779127153	G/A	100/0/0	95/5/0	missense	c.409G>A	29.8	4.23	Not Reported in ClinVar
44322970	AFR	rs2076212	G/T	86.7/13.3/0	100/0/0	missense	c.343G>T	0.19	0.19	Benign
44324727	AMR	rs738409[a]	C/G	50/43.3/0.67	55/35/10	missense	c.444C>G	15.73	1.4	Benign/ risk factor
44324730	AMR	rs738408[a]	C/T	50/43.3/0.67	55/35/10	synonymous	c.447C>T	1.13	-0.13	Benign
44328730	AMR	rs4823173	G/A	53.3/40/25	55/35/10	intronic	c.487-28G>A	0.22	-0.37	Not Reported in ClinVar
44333172	AMR	rs2072906	A/G	53.3/40/25	55/35/10	intronic	c.979+20A>G	0.69	-0.21	Not Reported in ClinVar
44322922	AMR	rs2076213	T/G	90/10/0	85/15/0	missense	c.295T>G	1.28	-0.11	Benign/VUS
44324767	AMR	rs141106484[a]	G/A	93.3/0.67/0	95/5/0	splice_region	c.484G>A	25.9	3.73	VUS
44336019	SAS	rs138736228	G/A	100/0/0	95/5/0	intronic	c.1112+14G>A	1.01	-0.15	Likely benign
44322818	–	–	A/G	96.7/0.33/0	100/0/0	missense	c.191A>G	0.01	-1.12	Variant is not available in the dbSNP core database

* GnomAD_maxPOP – the frequency distribution of *PNPLA3* mutations. AFR, AMR, EAS, FIN, NFE, SAS and OTH represent African, American, East Asian, Finnish, Non-Finnish European, South Asian and other populations; Ref – reference allele; Alt – alternative allele; Consequence –functional consequence of the variation in relation to the transcript. The nucleotide change and position relative to the coding sequence of the affected transcript in HGVS nomenclature: c. CDS Position Reference Base > Alternative Base. Example: c.223A> T. This column is empty if the variant is intergenic; CADD – combined annotation dependent depletion; VUS – variant of uncertain significance; [a] – SNV *PNPLA3* associated with MUO

(NC_000002.11:g.44322818C>A), not described in the dbSNP core database, Table 5.

The role of the following SNV *PNPLA3* genotypes in the development of MUO has been revealed: rs139051 G/G (RR=2; PC=3); rs141106484 A/G (RR=1.3; PC=1.2); rs738409 C/G (RR=1.2; PC=1); rs738408 C/T (RR=1.2; PC=1), p<0.05.

The CADD indicators calculated by us for SNV *PNPLA3* were characterized as follows: rs139051 GG – 4.31 (mutation in the intron region); rs141106484 AG – 25.9 (mutation in the intron region); rs738409 CG – 15.73 (missense); rs738408 CG – 1.13 (synonymous variant).

We have established a correlation between an early indicator of nonspecific hepatocellular damage (ALT) and the following genotypes (SNV *PNPLA3*): rs738409 CG/GG (r=0.43); rs738408 CT/TT (r=0.43); rs4823173 GA/AA (r=0.43); rs2072906 AG/GG (r=0.43); rs141106484 AG (r=0.38); rs139051AG/GG (r=0.25); rs34179073 CT (r= -0.24); rs2294918AG/GG (r=0.20); rs2076213 TG (r= -0.19); pos. 44322818 AG (r= -0.15); rs2076212 GT (r= -0.13), p<0.05. The risk of developing MAFLD among the examined cohort of children with MUO was observed with a combination of four SNV *PNPLA3*: rs738409 C/G (RR=1.71); rs738408 C/T (RR=1.71); rs4823173 A/G (RR=1.57); rs2072906 A/G (RR=1.57) with Sensitivity (Se)=0.63 and Specificity (Sp)=0.72 (Table 6).

Table 6. Relative risk of calculating MAFLD in children with SNV *PNPLA3* with 95% confidence interval

SNV PNPLA3	EER$_{MUO}$	CER$_{MHO}$	RR	S	95% CI	Se	Sp
rs139051	0.61	0.71	0.85	0.29	0.48–1.51	0.74	0.18
rs34179073	0.5	0.68	0.73	0.38	0.35–1.55	0.21	0.63
rs2294918	0.66	0.33	2	0.82	0.4–10.13	0.94	0.18
rs139047	0.53	0.81	0.64	0.26	0.39–1.07	0.52	0.18
rs779127153	–	0.63	–	–	–	–	1
rs2076212	0.25	0.0.69	0.36	0.87	0.06–2.01	0.05	0.73
rs738409	0.8	0.46	1.71	0.3	1–3.11	0.63	0.72
rs738408	0.8	0.46	1.71	0.3	1–3.11	0.63	0.72
rs4823173	0.8	0.5	1.57	0.28	1–2.75	0.63	0.72
rs2072906	0.8	0.5	1.57	0.28	1–2.75	0.63	0.72
rs2076213	1	0.59	1.68	0.16	1.23–2.3	0.16	1
rs141106484	1	0.6	1.65	0.15	1.22–2.22	0.11	1
rs138736228	–	0.63	–	–	–	–	1
g.44322818	1	0.62	1.61	0.15	1.21–2.14	0.05	1

The relative risk of developing MAFLD in the presence of a combination of these four SNV *PNPLA3* genotypes increased by 1.2 times among patients with MUO. A direct correlation was found between the association of SNV rs738409 C/G and the combination of rs738408 C/T, rs4823173 G/A, and rs2072906 A/G in patients with MAFLD (r=0.74; p<0.05).

Discussion

This work is devoted to the search for genetic determinants of MAFLD by carefully studying the target group of patients with MUO, in which, according to Murag the risk of this disease is 70-90%.[49] According to our results, MAFLD occurs in 66% of children with MUO. In previous studies, a stable association of the SNV *PNPLA3* gene with MAFLD (RR> 1.6) was found accompanied by an increase in TG and a decrease in HDL.[7,50] Unlike the study by Lee et al. our work demonstrates a greater likelihood of MAFLD occurrence among girls than among boys, and confirms the relationship of the onset of the disease with adolescence (RR=3.11), levels of basal insulinemia (RR=9.33); and to a lesser extent with a decrease in HDL levels (RR=1.87), p<0.05 compared with the results obtained by Gloudemans et al.[7,23]

In this work, we first determined the contribution of 14 SNV *PNPLA3* to the formation of various obesity phenotypes in children and the risk of MAFLD in MUO/MHO and presented SNV *PNPLA3* is not available in the dbSNP core database (NC_000002.11:g.44322818 C>A), whose role remains to be explored in a larger sample of patients.

We found that the RR of MUO occurrence doubled in the presence of the rs139051 A/G *PNPLA3* genotype and was higher compared to the rs141106484 A/G (RR=1.3), rs738409 C/G (RR=1.2), rs738408 C/G genotypes (RR=1.2).

At the same time, we did not reveal the contribution of SNV rs139051 A/G *PNPLA3* to the formation of MAFLD, as well as the research group of Ragab et al.[20] According to their results, this genetic variant was equally common in both MAFLD patients (82.5%) and healthy individuals (85%). And according to some authors, it even reduced the risk of MAFLD by 0.58 times (95% CI: 0.342–0.984; p=0.04).[51] At the same time, other researchers indicate that SNV rs139051 *PNPLA3* is significantly correlated with persistent meta-inflammation[51] and the level of basal insulinemia (p=0.04)[52] demonstrating its pathological role based on the modulation of the phospholipid metabolite profile and the formation of insulin resistance in MUO.

We also demonstrated the contribution of SNV rs141106484 of the *PNPLA3^{B162M}* gene to the formation of MAFLD in children, in contrast to the only work by Gerhard, which determined the pathogenic risk (0.974) of SNV rs141106484 of the *PNPLA3^{B162M}* gene in liver cirrhosis.[53]

Najafi et al. also considered the combined contribution of the G/C rs738409 and T/C rs738408 genotypes to the development of MAFLD (p=0.004).[52] The combination of these SNV *PNPLA3* was associated with an earlier onset of MAFLD in non-obese patients.[17] Qin Pang et al. also indicated a higher likelihood of developing MAFLD, NASH, and liver fibrosis in the presence

of a combination of the following SNVs: rs738409 G allele (OR=2.77, 95% CI: 1.18-6.54; p=0.02); rs4823173 A allele (OR=2.73, 95% CI: 1.16-6.44; p=0.02), and rs2072906 G allele (OR=3.05, 95% CI: 1.28-7.26; p=0.01) but in adult patients with chronic viral hepatitis B.[54]

We have for the first time revealed an increase in RR MAFLD in the presence of a combination of four SNV *PNPLA3* genotypes (rs738409 C/G, rs738408 C/T, rs4823173 G/A and rs2072906 A/G) by 1.2 times among children with MUO. We found a strong direct correlation between the SNV rs738409 C/G association and the combination of rs738408 C/T, rs4823173 G/A, and rs2072906 A/G genotypes in patients with MAFLD (r=0.74; p<0.05).

Conclusion

The presence of the following SNV *PNPLA3* genotypes predetermines the development of MUO: rs139051 G/G (RR=2; PC=3); rs141106484 A/G (RR=1.3; PC=1.2); rs738409 C/G (RR=1.2; PC=1); rs738408 C/T (RR=1.2; PC=1), p<0.05.

The combination of four SNV *PNPLA3* genotypes (rs738409 C/G, rs738408 C/T, rs4823173 G/A and rs2072906 A/G) among children with MUO increases the risk of development by 1.2 times.

The presence of SNV rs738409 and rs738408 *PNPLA3* affects both the occurrence of MUO and MAFLD in children.

Declarations

Author contributions
Conceptualization, A.A. and A.N.; Methodology, A.A.; Software, A.A.; Validation, A.A. and A.N.; Formal Analysis, A.A. and A.N.; Investigation, A.A. and A.N.; Resources, A.A. and A.N.; Data Curation,A.N.; Writing – Original Draft Preparation, A.A. and A.N.; Writing – Review & Editing, A.A. and A.N.; Visualization, A.A. and A.N.; Supervision, A.A. and A.N.; Project Administration, A.A.; Funding Acquisition, A.A. and A.N.

Conflicts of interest
The authors declare no competing interests.

Data availability
The datasets used and/or analyzed during the current study are open from the corresponding author on reasonable request.

Ethical approval
Participants provided written informed consent, and research protocols and procedures were approved according to the ethical standards of the Helsinki Declaration 2013 and by the Human Research Ethics Committee of Dnipro State Medical University (ethical approval DSMU/EC/19/1107).

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