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ORIGINAL RESEARCH

CHDH-PNPLA3 Gene–Gene Interactions Predict Insulin Resistance in Children with Obesity

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Introduction: Insulin resistance plays a major role in metabolic syndrome and is recognized as the most common risk factor for non-alcoholic fatty liver disease (NAFLD). Identifying predictors for insulin resistance could optimize screening and prevention.

Purpose: To evaluate the contribution of multiple single nucleotide polymorphisms across genes related to NAFLD and choline metabolism, in predicting insulin resistance in children with obesity.

Methods: One hundred fifty-three children with obesity (73 girls), aged 7–18 years, were evaluated within the NutriGen Study (ClinicalTrials.gov-NCT02837367). Insulin resistance was defined by Homeostatic Model Assessment for insulin-resistance cut-offs that accommodated pubertal and gender differences. Anthropometric, metabolic, intake-related variables, and 55 single nucleotide polymorphisms related to NAFLD and choline metabolism were evaluated. Gene–gene interaction effects were assessed using Multiple Data Reduction Software.

Results: Sixty percent (93/153) of participants showed insulin resistance (58.7% of boys, 63% of girls). Children with insulin resistance presented significantly higher values for standardized body mass index, triglycerides, transaminases and plasma choline when compared to those without insulin resistance. Out of 52 single nucleotide polymorphisms analysed, the interaction between genotypes *CHDH*(rs12676) and *PNPLA3*(rs738409) predicted insulin resistance. The model presented a 6/10 cross-validation consistency and 0.58 testing accuracy. Plasma choline levels and alanine aminotransferase modulated the gene interaction effect, significantly improving the model.

Conclusion: The interaction between genotypes in *CHDH* and *PNPLA3* genes, modulated by choline and alanine aminotransferase levels, predicted insulin-resistance status in children with obesity. If replicated in larger cohorts, these findings could help identify metabolic risk in children with obesity.

Keywords: insulin-resistance, obesity, gene–gene interaction, *CHDH-PNPLA3*, choline, children

Introduction

Obesity prevalence is increasing worldwide, in both adults and children,[1,2] concomitantly escalating the risk for type 2 diabetes and cardio-metabolic diseases.[3] Insulin resistance plays a major part in both metabolic syndrome and type 2 diabetes mellitus.[4] Furthermore, insulin resistance is recognized as the most common risk factor for non-alcoholic fatty liver disease (NAFLD) development and progression in adults.[5,6] NAFLD has rapidly evolved into becoming the most common liver disease in the paediatric population in the United States, an inducer of insulin resistance, and being associated with increased adiposity.[7] Evidence

suggests that NAFLD is associated with hepatic and non-hepatic morbidity and mortality, and could progress to cirrhosis and end-stage liver disease.[8] Identifying predictors of insulin resistance could help optimize screening and prevention.

Insulin resistance in children is influenced by several factors including degree and disposition of adiposity, gender, pubertal stage, diet, lipid metabolism and genetic predisposition.[9,10] Single Nucleotide Polymorphisms (SNPs) in several genes, including *PNPLA3, TM6SF2, MBOAT7* and *GCKR*, have been identified to predict the development and severity of NAFLD in relation to insulin resistance in adults.[10–12] Another study identified SNPs related to choline metabolism genes including *PNPLA3, CHDH, PEMT, ABCB4, MTHFR*, and *SLC44A1*, which were associated with liver steatosis in adults with obesity.[13] Choline was identified as an essential nutrient for liver, muscle, and brain function, having a key role in the synthesis of acetylcholine, methylation, gene expression and lipid metabolism.[14] However, the potential roles of genetic variations on the risk of paediatric NAFLD are currently not well established.[11] This study aimed to evaluate the contribution of multiple SNPs across genes related to NAFLD and choline metabolism, and their interactions, in predicting insulin resistance in children with obesity.

Participants and Methods

Participants and Samples

Two hundred children (95 males, 105 females) aged 7–18 years, with obesity defined using the World Health Organization 2007 reference, if more than +2 Standard Deviation (SD),[15] were evaluated within the NutriGen Study protocol. The trial is registered at ClinicalTrials.gov, NCT02837367. Clinical evaluation was performed in a paediatric hospital in Timisoara, Romania. Exclusion criteria were previously described elsewhere[16] and included diagnosis of cancer, or medical history of cancer; auto-immune disease; psychiatric disorder; blood coagulation disorders; history of drug abuse; familial hypercholesterolemia; endocrine-induced obesity, hypothalamus-induced obesity, genetic syndromes; deposition diseases; personal history for: convulsive disorders, nephrotic syndrome, or asthma that required corticoid treatment. Subjects with incomplete sequencing data (1 SNP missing) were excluded from this study (n=47). Consequently, the present analysis included 153 children (80 boys, 73 girls). The term "children" will be used in this manuscript to also include adolescents.

The study was approved by the Ethics Committee of the "Victor Babes" University of Medicine and Pharmacy (6/20.06.2016), Timisoara, Romania, and conducted in accordance with the Declaration of Helsinki. Participants and their parents or legal guardians were informed about the aims and methods of the study. Written consent was signed by parents or legal guardians of the participants and children provided verbal consent to be included in the study.

Anthropometric Measurements

Anthropometric measurements were performed following international guidelines,[17] as described previously.[16] Standardized BMI-for-age z-scores (zBMI) were calculated according to the World Health Organization guidelines taking into account the age and gender of the child.[15] Waist circumference was measured with an inextensible anthropometric tape, by a trained person, to the nearest 0.1 cm, in standing position, at the midpoint between the end of the rib cage and the top of the iliac crest. Hip circumference was measured around the widest portion of the hip.[18] Waist to hip ratio (WHR) was calculated by dividing waist circumference (cm) to hip circumference (cm).

Food and Drink Intakes

Food and drink intakes were evaluated using 5-pass 24-h dietary recalls as previously described.[19] In short, four recalls were performed on each participant, if older than 13 years of age, or to both a parent and the child, if the participant was younger. The declared amounts for each day investigated (foods and drinks) were converted to energy and nutrient intakes using a web-application (Nutritio, Bucharest, Romania, https://nutritioapp.com), using the United States Department of Agriculture Food and Nutrient Database for Dietary Studies and other databases for local foods, and with appropriate adaptations described elsewhere.[19] Resting energy expenditure (REE), validated in children with obesity, was computed using the formula proposed by Lazzer et al,[20] in order to compare food and drink intakes across different ages and genders. Energy intake and macronutrient percentage (carbohydrate and fat) were calculated as means of the four 24 h recalls for each child. The fraction of energy intake from REE was computed as energy intake (kcal)/REE (kcal) and further used as a variable.

Participants' intakes were evaluated during the period when they followed medical recommended diet and supervision, following recommendations from Endocrine Society Clinical Practice 2017 Guideline.[3] In brief, the main recommendations were to reduce portion size, decrease consumption of fast foods, high-fat, high-sodium, processed foods, added table sugar and the elimination of sugar-sweetened beverages.

Biochemistry

Blood samples were collected in the morning, following overnight fasting (for at least 8 h), in EDTA sterile vacutainers. Total plasma concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and glucose were performed using its standardized reagents and following the manufacturer's protocols, as presented previously.[16] Insulin measurements were performed using ELISA method, as shown.[16]

Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated using the formula: fasting insulin (mIU/L) × fasting glucose (mmol/L)/22.5. In order to account for differences between boys and girls and for the physiological insulin resistance during puberty,[21] different HOMA-IR cut-offs were used to define insulin resistance, stratified by gender and by age groups. For boys 13 years old or younger, HOMA-IR above 2.67 was used to define insulin resistance, while for boys older than 13 years, insulin resistance was defined if HOMA-IR was higher than 5.22. For girls, 11 years old or younger, HOMA-IR above 2.22 was used to define insulin resistance, while for girls older than 11 years, insulin resistance was defined if HOMA-IR was higher than 3.82, similarly to that described by Kurtoğlu et al 2010.[22]

Fatty Acids Quantification

Fatty acids quantification from red blood cell (RBC) membrane was performed by high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS), using an adaptation of a previously described protocol.[16]

Quantification of Plasma Choline and Betaine

Choline and betaine were measured from plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a previously described method,[23] with adaptations for the laboratory specifics. Method is described in the supplementary material (Table S1 and supplementary "Quantification of plasma choline and betaine" section).

Genetic Analysis of Single Nucleotide Polymorphisms (SNPs)

Genotyping was performed on a MiSeq sequencer (Illumina, San Diego, CA, USA) using a custom-made hotspot sequencing kit for 55 SNPs within 14 genes involved in choline/1-carbon metabolism,[16] selected based on their previous association with increased lipids, non-alcoholic fatty liver, or cardiovascular disease.[13] Three SNPs (rs12103822 in *PEMT*, rs7525338 and rs868014 in *MTHFR)* were excluded from analysis due to lack of variation in the participants (only homozygous status was identified).

Statistical Analysis

Data analysis was performed using IBM-SPSS version 25 (IBM, Armonk, New York, U.S.A.). Descriptive statistics for numerical variables included means and standard deviations. For categorical variables, frequency as percentage (%) and/or count (n) were included. The *t*-test with two-factor comparisons was used for variables assuming normal distribution. For variables with non-parametric distribution, the Mann–Whitney test was used. Normal distribution was assessed with the Kolmogorov–Smirnov test. Chi-square was used for proportion comparison. The multiple comparisons were adjusted for false discovery rate (FDR), using an online tool (https://tools.carbocation.com/FDR). This method adjusted the p values obtained by statistical tests to the number of tests per each research hypothesis. The threshold of statistical significance for adjusted p-values was $p<0.05$. Gene-gene interaction effects were assessed using Multifactor Dimensionality Reduction (MDR) version 3.0.2 (http://epistasis.org/). MDR software was used to evaluate the influence of the SNPs tested and/or their interactions on the insulin resistance, set as categorical outcome (no/yes), using the HOMA-IR (gender- and puberty-specific) cut-offs. Choline levels and ALT levels were grouped in tertiles and introduced in the model as covariates.

Results

Sixty percent (93/153) of the children with obesity (58.7% of boys and 63% of girls) included in the present study presented insulin resistance, defined by HOMA-IR cut-offs that accommodate for pubertal and gender differences (see Materials and Methods). None of the children

presented type 2 diabetes mellitus. Proportions of increased ALT levels (>40 U/L) were similar between children with or without insulin resistance 20.0% (12), respective 33.3% (31), p=0.073. Descriptive statistics presented in Table 1 show the mean ± SD values of anthropometric, metabolic and intake-related variables, overall and separately for gender and insulin-resistance status. Between-gender comparisons showed higher mean values of zBMI, WHR and fraction of energy intake form REE for boys, as compared to girls. Participants with insulin resistance (defined by HOMA-IR status) presented significantly higher values for zBMI, triglycerides, AST, ALT and choline, compared to those without insulin resistance.

Age distribution of HOMA-IR values depicted as boxplot separately for boys and girls in Figure 1, showed a peak value of 5.25 at age 12 years in girls and a peak value of 6.67 at age 14 years in boys. The number of children in each age group is presented in supplementary material (Table S2).

The frequency of the 52 SNPs studied in our cohort of 153 children with obesity is presented in Supplementary Material (Table S3). Using 52 SNPs, in a multifactor dimensionality reduction statistical model, the interactions between genotypes in rs12676 (*CHDH*) and rs738409 (*PNPLA3*) were identified as the best predictor for insulin resistance. The nine interactions between the two SNPs were grouped in high and low risk, as shown in Table 2 and further used in Table 3. The high and low risk for insulin-resistance model presented a cross-validation consistency of 6/10 and a testing accuracy of 0.58, which exceeded the threshold 0.5 expected under the null hypothesis. Considering that, *CHDH* and *PNPLA3* genes are involved in choline metabolism, and that choline plasma concentrations were different between participants with and without insulin resistance, we further evaluated the modulating effect of choline on the model. When using choline as covariate, an improvement in the model's cross-validation consistency (9/10) and testing accuracy (0.63) was detected. When using ALT as covariate, the model has improved even further with cross-validation consistency (10/10) and testing accuracy (0.69).

Comparisons of anthropometric, metabolic and intake-related variables between high and low risk for insulin resistance, classified by the interaction between rs12676 and rs738409, are presented in Table 3. The only significant difference was for HOMA-IR levels, with higher values in the high-risk group.

Discussion

Obesity is increasingly prevalent in adults and children.[1,2] Obesity-related complications, such as cardiovascular disease and type 2 diabetes are also rising, and are frequently diagnosed in the paediatric population with obesity.[1] In Romania, almost one in four children, aged 6–19 years, was either overweight or with obesity, in a pooled analysis performed between 2006 and 2015.[24] Insulin resistance was recognized as the most common risk factor for non-alcoholic fatty liver disease (NAFLD), in the setting of excess adiposity, in adults and children.[7] Recognizing the predictors of insulin resistance is crucial for optimal screening. However, considering normal physiological changes that occur in children during puberty, it is difficult to establish a standard definition for insulin resistance across paediatric age groups.

Defining Insulin Resistance

The gold standard to determine insulin resistance is the euglycemic-hyperinsulinemic clamp study. However, this invasive method is not routinely used in daily clinical practice. HOMA-IR is the most widely used surrogate measure for insulin resistance.[25] In a systematic review of 298 articles, 51 different HOMA-IR cut-off values were used to classify patients as having insulin resistance.[25] The authors indicated that 85.6% of studies used a predetermined fixed cut-off value, with the most frequently used HOMA-IR cut-offs of 3.16 and 2.5.[25] Nonetheless, children normally experience transient insulin resistance at puberty.[22] This circumstance needs to be accounted for in defining insulin resistance in children. A systematic review, summarizing population-based studies on the epidemiology of insulin resistance during childhood, showed that prevalence rates vary widely due to the variety of definitions used.[26] A European study identified insulin resistance (using HOMA-IR>3.4) in 16.6% of prepubertal children (<10 years) with obesity and 47.3% of adolescents (11–18 years) with obesity,[4] comparable to the prevalence identified by this study.

In this study, HOMA-IR distribution across ages and genders was similar to that identified by Kurtoğlu et al 2010.[22] Notably in this study, the age of the HOMA-IR peak was higher in boys and girls when compared to Kurtoğlu et al 2010.[22] We defined insulin resistance using HOMA-IR cut-offs that accommodated gender and puberty,[22] in order to avoid classifying physiological pubertal values as pathologic status. Insulin resistance in our cohort was observed in 60.7% of the children with obesity

Table 1 Descriptive Statistics (Mean ± SD) of Anthropometric, Biochemical and Intake-Related Variables in 153 Children with Obesity and Comparatively in Boys and Girls

Variables Mean ± SD		Boys			Girls			All Children			p value Boys vs Girls	p value Insulin Resistance No vs Yes
		Insulin Resistance			Insulin Resistance			Insulin Resistance				
		No (n=33)	Yes (n=47)	All Boys (n=80)	No (n=27)	Yes (n=46)	All Girls (n=73)	No (n=60)	Yes (n=93)	All (n=153)		
Anthropometric	Age (years)	12.4 ± 3.5	11.5 ± 2.7	11.9 ± 3.1	12.8 ± 2.8	11.4 ± 3.0	12.0 ± 3.0	12.6 ± 3.2	11.5 ± 2.8	11.9 ± 3.0	0.493 *	0.021 *
	zBMI	3.39 ± 0.84	3.91 ± 1.48	3.70 ± 1.28	2.61 ± 0.78	3.36 ± 1.05	3.08 ± 1.02	3.04 ± 0.90	3.64 ± 1.31	3.40 ± 1.20	**<0.001 ****	**0.002 ***
	Waist to hip ratio	0.98 ± 0.15	0.98 ± 0.06	0.98 ± 0.11	0.95 ± 0.11	0.95 ± 0.09	0.95 ±0.10	0.97 ± 0.13	0.97 ± 0.08	0.97 ± 0.10	**0.007 ****	0.199 **
Metabolic	HOMA-IR	2.2 ± 1.3	6.1 ± 3.2	4.5 ± 3.2	2.2 ± 1.2	5.8 ± 3.2	4.5 ± 3.2	2.2 ± 1.2	5.9 ± 3.2	4.5 ± 3.2	0.968 **	**<0.001 ***
	Cholesterol (mg/dl)	171.1 ± 39.7	188.8 ± 49.5	181.5 ± 46.3	169.6 ± 36.2	170.0 ± 32.7	169.8 ± 33.8	170.4 ± 37.9	179.5 ± 42.9	175.9 ± 41.1	0.076 *	0.184 *
	Triglycerides (mg/dl)	128.5 ± 71.6	165.4 ± 80.3	150.1 ± 78.6	118.0 ± 56.8	148.7 ± 70.0	137.3 ± 66.7	123.8 ± 65.1	157.1 ± 75.4	144.0 ± 73.2	0.415 **	**0.001 ****
	HDL cholesterol (mg/dl)	48.8 ± 11.0	50.7 ± 15.0	50.0 ± 13.4	50.7 ± 16.6	45.4 ± 10.9	47.4 ± 13.4	49.7 ± 3.7	48.1 ± 13.3	48.7 ± 13.5	0.074 **	0.408 **
	AST (U/L)	31.4 ± 9.1	37.7 ± 17.6	35.1 ± 15.0	29.2 ± 9.6	41.5 ± 46.9	36.9 ± 38.0	30.4 ± 9.3	39.6 ± 35.2	36.0 ± 28.3	0.222 **	**0.040 ****
	ALT (U/L)	34.1 ± 16.6	45.5 ± 28.1	40.8 ± 24.6	28.6 ± 12.3	41.7 ± 36.6	36.9 ± 30.5	31.6 ± 15.0	43.6 ± 32.5	38.9 ± 27.6	0.106 **	**0.002 ****
	Choline (µmol/L)	8.0 ± 2.5	9.4 ± 3.1	8.8 ± 2.9	7.5 ± 2.8	8.7 ± 2.8	8.2 ± 2.8	7.8 ± 2.6	9.1 ± 2.9	8.5 ± 2.9	0.211 *	**0.006 ***
	Betaine (µmol/L)	21.1 ± 11.6	22.2 ± 9.6	21.7 ± 10.4	21.9 ± 10.5	24.2 ± 10.3	23.3 ± 10.4	21.4 ± 11.0	23.2 ± 9.9	22.5 ± 10.4	0.347 *	0.312 *
	ALA RBC (µmol/L)	1.5 ± 0.7	2.1 ± 1.4	1.8 ± 1.2	1.4 ± 0.7	1.6 ± 0.8	1.5 ± 0.8	1.4 ± 0.7	1.8 ± 1.2	1.7 ± 1.0	0.064 **	0.080 **
	DHA RBC (µmol/L)	171.7 ± 73.7	197.8 ± 96.8	187.2 ± 88.6	168.5 ± 94.8	193.0 ± 158.9	183.9 ± 138.5	170.2 ± 83.3	195.4 ± 130.5	185.7 ± 114.9	0.324 **	0.325 **
	LA RBC (µmol/L)	217.0 ± 104.7	259.7 ± 113.8	242.4 ± 111.6	212.1 ± 90.0	220.5 ± 99.3	217.4 ± 95.4	214.8 ± 97.5	240.3 ± 108.1	230.4 ± 104.5	0.136 **	0.168 **
	AR ARBC (µmol/L)	348.1 ± 113.2	419.2 ± 179.6	390.4 ± 159.2	355.3 ± 165.1	374.9 ± 166.2	367.6 ± 164.9	351.4 ± 138.1	397.3 ± 173.6	379.5 ± 161.9	0.236 **	0.323 **
	EPA RBC (µmol/L)	3.1 ± 1.9	3.9 ± 2.2	3.6 ± 2.1	3.3 ± 2.2	4.0 ± 6.4	3.7 ± 5.3	3.2 ± 2.0	3.9 ± 4.8	3.7 ± 3.9	0.085 **	0.234 **
Intake related	Percentage of energy intake from carbohydrates	46.1 ± 7.7	44.0 ± 8.2	44.9 ± 8.0	47.8 ± 6.2	46.6 ± 5.8	47.0 ± 5.9	46.9 ± 7.1	45.3 ± 7.2	45.9 ± 7.2	0.062 *	0.172 *
	Percentage of energy intake from fat	31.4 ± 7.2	34.0 ± 7.0	32.9 ± 7.2	32.5 ± 5.8	32.1 ± 5.6	32.2 ± 5.6	31.9 ± 6.6	33.0 ± 6.4	32.6 ± 6.5	0.496 *	0.291 *
	Fraction of energy intake from REE	0.69 ± 0.26	0.72 ± 0.26	0.71 ± 0.26	0.64 ± 0.16	0.60 ± 0.16	0.61 ± 0.16	0.67 ± 0.22	0.66 ± 0.23	0.66 ± 0.22	**0.026 ****	0.575 **

Notes: *t-test, **Mann–Whitney test. All tests were adjusted for false discovery rate, p-values in bold denote statistical significance. Insulin resistance was defined: for boys ≤13 years old, if HOMA-IR >2.67; for boys > 13 years, if HOMA-IR > 5.22; for girls ≤11 years old, if HOMA-IR > 2.22; for girls > 11 years, if HOMA-IR > 3.82. See Materials and Method.

Abbreviations: zBMI, standardized body mass index (BMI) to account for age and gender; HOMA-IR, homeostatic model assessment for insulin resistance; HDL, high-density cholesterol; g, grams; mg, milligrams; µmol/L, micromoles/litre; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL cholesterol, high-density lipoprotein cholesterol; ALA, alfa-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; RBC, red blood cell membrane; REE, resting energy expenditure; SD, standard deviation; n, number.

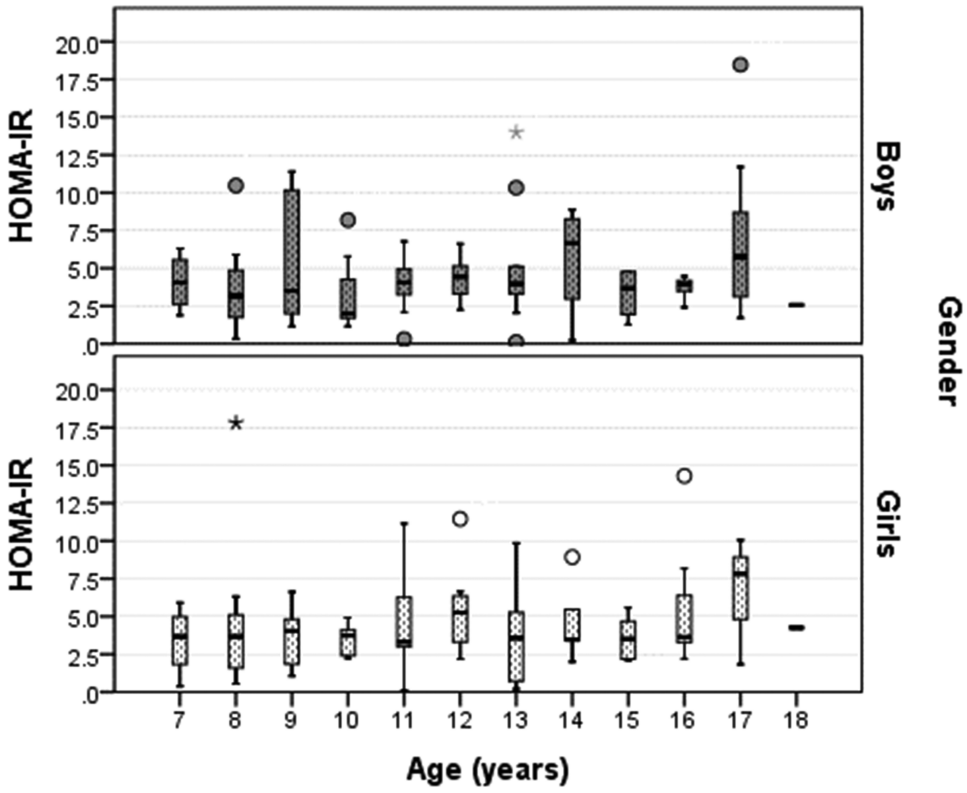

Figure 1 Distribution of HOMA-IR values depicted as boxplot separately for boys and girls, by age (rounded).
Notes: Horizontal lines, within each boxplot, indicate minimum, first quartile (Q1), median, third quartile (Q3), and maximum. Outliers marked with circles are cases with values between 1.5 and 3 times the IQ range, beyond the whiskers. Outliers marked with a star are cases with values more than 3 times the IQ range.

(58.7% of boys and 63% of girls) using HOMA-IR cut-offs that accommodate gender and pubertal differences.

Gender Differences in Insulin Resistance

Gender differences in body composition and energy balance are well known.[27] In this study, boys presented higher standardized BMI score when compared to girls (Table 1), in accordance with other studies from Romania regarding children with obesity.[24] Boys presented higher mean values of waist-to-hip ratio when compared to girls, similar to other studies in this age group, showing differences in adiposity disposition between genders, especially after

Table 2 Description of Interaction Groups Formed by Genotypes in rs12676 in *CHDH* and rs738409 in *PNPLA3* Gene to Predict Insulin Resistance Using the Gender and Puberty-Specific HOMA-IR Cut-Offs

HOMA-IR Mean ± SD, n		**rs12676 CHDH Gene**		
		AA	**AC**	**CC**
rs738409 PNLAP3 gene	CC	High risk 5.8 ± 4.3 n=7	Low risk 3.9 ± 2.1 n=31	High risk 4.1 ± 2.6 n=41
	CG	High risk 6.5 ± 3.1 n=4	High risk 6.8 ± 4.8 n=11	Low risk 2.4 ± 2.0 n=14
	GG	High risk 6.2 ± 3.1 n=6	Low risk 3.9 ± 2.6 n=22	High risk 5.7 ± 4.3 n=17

Table 3 Comparison Between Anthropometric, Biochemical and Intake-Related Variables in 153 Children with Obesity, Classified by the Interaction Between Rs12676 and Rs738409, to Predict High/Low Risk for Insulin Resistance Using the Gender and Puberty-Specific HOMA-IR Cut-Offs

Variables Mean ± SD		Interaction Groups		p-values
		Low Risk n=67	High Risk n=86	
Anthropometric	Age (years)	12.2 ± 3.1	11.7 ± 3.0	0.289 *
	zBMI	3.30 ± 0.94	3.49 ± 1.36	0.341 *
	Wait to hip ratio	0.96 ± 0.09	0.98 ± 0.11	0.401 **
Metabolic	HOMA-IR	3.6 ± 2.3	5.2 ± 3.6	**0.001 ***
	Cholesterol (mg/dl)	174.9 ± 41.0	176.7 ± 41.4	0.787 *
	Triglycerides (mg/dl)	150.9 ± 84.2	138.7 ± 63.3	0.691 **
	HDL cholesterol (mg/dl)	46.3 ± 10.6	50.6 ± 15.1	0.200 **
	AST (U/L)	34.5 ± 15.0	37.1 ± 35.4	0.800 **
	ALT (U/L)	36.4 ± 18.9	40.9 ± 32.7	0.935 **
	Choline (µmol/L)	8.5 ± 2.9	8.6 ± 2.9	0.721 *
	Betaine (µmol/L)	21.7 ± 10.3	23.1 ± 10.4	0.387 *
	ALA RBC (µmol/L)	1.6 ± 0.8	1.7 ± 1.2	0.564 **
	DHA RBC (µmol/L)	176.4 ± 89.4	193.0 ± 131.6	0.589 **
	LA RBC (µmol/L)	221.9 ± 94.2	237.1 ± 112.1	0.605 **
	ARA RBC (µmol/L)	369.2 ± 150.6	387.5 ± 170.7	0.765 **
	EPA RBC (µmol/L)	3.3 ± 2.2	3.9 ± 4.9	0.187 **
Intake related	Percentage of energy intake from carbohydrates	46.1 ± 7.3	45.7 ± 7.1	0.711 *
	Percentage of energy intake from fat	32.5 ± 6.4	32.6 ± 6.5	0.911 *
	Fraction of energy intake from REE	0.66 ± 0.18	0.66 ± 0.25	0.932 *

Notes: *t-test, **Mann–Whitney test. All tests were adjusted for false discovery rate, p-values in bold denote statistical significance.
Abbreviations: zBMI, standardized body mass index (BMI) to account for age and gender; HOMA-IR, homeostatic model assessment for insulin resistance; HDL, high-density cholesterol; g, grams; mg, milligrams; µmol/L, micromoles/liter; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL cholesterol, high-density lipoprotein cholesterol; ALA, alfa-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; RBC, red blood cell membrane; SD, standard deviation; n, number of subjects in a group.

puberty.[28] The mean fraction of energy intake from REE was higher for boys when compared to girls. In the same way, a large study on 6553 children aged 9–11 years from 12 countries has shown that boys had a lower compliance with dietary recommendations as compared to girls.[29] These findings may provide insight into the pathogenesis of insulin resistance, visceral adiposity and obesity, with the potential to guide gender-tailored interventions for prevention and treatment.

Metabolic Predictors for Insulin Resistance

Participants with insulin resistance in our cohort presented significantly higher values for standardized BMI, triglycerides, AST and ALT, as compared to those without insulin resistance. Similar findings were observed in many studies,[4,5,30,31] reflecting the metabolic risk in children with obesity and insulin resistance.[9] Furthermore, insulin resistance, dyslipidaemia and ALT are the recommended screening makers for NAFLD in all children with obesity or overweight starting at the age of 9–11 years, according to the NASPGHAN 2017 guidelines.[7] Based on these recommendations, the children with insulin resistance in our cohort showed an increased risk for NAFLD.

No significant differences regarding the prevalence of insulin resistance between girls and boys were noted in this study, in the context of the variable cut-offs used, which were tailored for gender and pubertal status.

Choline was significantly higher in children with insulin resistance when compared to those without. We did not identify other studies that investigated choline in children with obesity and in relationship with their insulin resistance. In adults, higher levels of serum choline were associated with a healthier body composition only in men.[32] Higher intakes of choline were found negatively associated with insulin resistance,[33] subclinical markers of cardiovascular disease and incidence of cardiovascular disease[34] in both genders and risk of type 2 diabetes in men.[35,36] Choline metabolism is involved in very low-density lipoprotein secretion in the liver, making this

nutritional pathway an important contributor to hepatic lipid homeostasis. It also intersects with multiple pathways that intervene in the deposition of lipids in the liver.[13] The contribution of choline in the complexity of insulin-regulated glycolytic and lipogenic homeostasis is insufficiently understood in children.

The fatty acid level of RBC could reflect their status in other organs and could potentially represent a proxy for the evaluation of polyunsaturated fatty acid homeostasis in the human body.[16] In our study, fatty acids measured in RBC were not significantly different between those with and without insulin resistance. However, alfa-linolenic acid (ALA, Table 1) was higher in those with insulin resistance, and close to significance (p=0.08), suggesting the possibility that, in a larger cohort, the difference could reach significance. A study in Danish adolescents (10–17 years old, out of which 8% with obesity) observed a tendency toward a significant association between HOMA-IR and RBC-DHA (0.052), but not with ALA.[37] Nonetheless, comparison is difficult as the population differed in obesity prevalence and feeding practices.

Genetic Predictors for Insulin Resistance

We used a machine-learning automatic approach, within a multifactor dimensionality reduction analysis to evaluate the association of 52 SNPs and/or their interactions with insulin resistance. Prediction for insulin resistance was identified for the interaction between genotypes rs12676 (*CHDH)* and rs738409 *(PNPLA3)* (shown in Table 2), which was further modulated by choline plasma levels and ALT (used as a proxy for NAFLD). These findings were not reported in other studies, although many nutrigenomic studies investigated the influence of single or multiple SNPs using multiple regression.[38,39] Regression is limited due to the complex evaluation of the million possible genetic interactions between various SNPs. The MDR overcomes this limitation, being a non-parametric machine-learning method, proposed in 2001, that classifies multi-dimensional genotypes into one dimensional, binary approach.[40,41] Thus, MDR is able to evaluate complex outcomes associated with multiple genetic and environmental factors alone, as well as with their interactions. MDR is increasingly popular and has been recognized as a robust methodology for the evaluation of gene–gene interaction effects.[42–44]

Different studies have chosen different SNPs to evaluate the association with insulin resistance, using a hypothesis-driven approach, as presented in the 2017 ISNN consensus regarding nutrigenetic, nutrigenomic and nutriepigenetic approaches for precision nutrition involving the prevention and management of chronic diseases associated with obesity.[39] Most of this research has used results from Genome Wide Association Studies (GWAS) to identify the effects of genetic variants on the disease risk. However, the identified SNPs have a modest effect, leading to the "missing heritability" problem. Many findings from GWAS are not replicated in smaller cohorts. For example, results on 53 loci, in several genes including *INSR, IRS1* and *PIK3R1* genes, selected based on genome-wide analyses of fasting insulin adjusted for BMI, did not identify loci with a primary effect on higher adiposity and insulin resistance in a large study on adults.[38] However, a genetic score, computed from the same 53 loci, was associated with insulin resistance, in children with overweight or obesity,[45] suggesting that genomic approaches need to be integrative, and also envisaging gene–gene interaction effects. SNPs identified to be associated with NAFLD were not included in the 53 SNPs studied in relation to insulin resistance. Nonetheless, other studies have investigated *PNPLA3, TM6SF2, MBOAT7, GCKR, CHDH, PEMT, ABCB4, MTHFR*, and *SLC44A1* genes and identified associations with the development and severity of NAFLD in relation to insulin resistance in adults.[10–13] Genes in choline and 1-carbon metabolism, including CHDH and PNLAP3, were shown to influence hepatic lipid balance. The unfavorable balance between lipid intake and output contributes to hepatic steatosis. Secretion of very low-density lipoproteins requires synthesis of a lipid cover enclosing apoproteins and phosphatidylcholine. Several gene products interplay in the lipid balance in the liver, including CHDH and PNLAP3. *CHDH* is an important gene for the pathway forming phosphatidylcholine, which is used to make very low-density lipoproteins, or can be hydrolyzed in a pathway implicating PNPLA3, or secreted in bile by a flippase encoded by *ABCB4* gene.[13]

The *PNPLA3* rs738409 polymorphism may be the most investigated SNP in relation to the accumulation of lipids in the liver.[10,13,39,46,47] Despite this, the variants' effects on the risk of paediatric NAFLD are currently not well established.[11] Findings from the current study bring additional clues into the elucidation of the insulin-regulated glucose and lipid metabolism in children with obesity.

A special discussion deserves the "U"-shaped association between the combination of genetic variants and the outcome (high-risk versus low risk for HOMA-IR values),

presented in Table 2. With the exception of the AA rs12676 genotype (*CHDH*), which in any association with the rs738409 (*PNLAP3*) genotypes, had a higher risk, other gene–gene interactions suggested a "U"-shaped effect, as the heterozygosity of any of the two variants associated to the outcome does not fit a hypothetical gene dosage effect. However, a lack of linearity in gene dosage has been previously described for gene–gene interactions (e.g.[48]). The non-linear association of complex genotypic traits with health outcomes has been previously discussed in the context of gene–environment interactions, including nutrition.[49] In the same context, the statistical approaches in analyzing gene–gene interactions have been also discussed in regard to why assumptions of linearity in gene–environment interactions, with consequences upon gene–gene interactions, are not necessarily always true.[50] Moreover, epigenetics is another factor that sometimes plays important roles in gene–environment interactions, and their association with defined phenotypes (e.g.[51]). The design of our study did not allow us to further explore such possible intricacies.

Notably, the observed gene–gene interactions in children might not be necessarily comparable with the effect observed in adults with obesity, as prolonged exposure to environmental factors and associated comorbidities might have more intricate effects in adults. Future studies are needed to examine the interaction between rs12676 in *CHDH* and rs738409 in *PNPLA3* genes, and the potential modulating role of plasma choline, in larger populations andadults. Potentially, these findings could be used as a clinical tool for the identification of children with an increased risk of insulin resistance, to ultimately prevent type 2 diabetes mellitus and cardiovascular disease.

Limitations

This study presents relevant metabolic and genetic predictors for insulin resistance in children with obesity, but with some limitations. Pubertal stage was not assessed using the Tanner method; however, age and HOMA-IR peaks were used as a proxy for puberty onset, in relation to other studies previously published.[25,52] We did not assess liver steatosis using ultrasound, as the NASPGHAN 2017 guidelines recommended against using it routinely for screening, and due to low sensitivity.[7] Conversely, we have used ALT as proxy for NAFLD, in order to identify children at risk.[7,10]

Notably, due to study design, intake assessments do not reflect children's usual diets, but rather the compliance to the dietary recommendations made by their doctors, in order to improve their body weight and metabolic status. Therefore, gene–diet interactions could not be addressed in our prediction model, as nutrition intakes would not necessarily be causal to the phenotype.

Another limitation of the study was the relatively small number of cases, diminishing statistical power and strongly suggesting that these results need to be replicated in larger studies.

Conclusion

Participants with insulin resistance and obesity presented significantly higher values for standardized BMI, triglycerides, transaminases, and choline when compared to those without insulin resistance, indicating increased risk for NAFLD. Out of 52 explored SNPs related to NAFLD, choline and 1-carbon metabolism, the interaction between rs12676 (*CHDH)* and rs738409 (*PNPLA3)* genotypes was identified to predict insulin resistance in children with obesity, using gender and puberty-specific HOMA-IR cut-offs. Plasma choline levels and ALT modulated the gene interaction effect, significantly improving the model's cross-validation consistency and testing accuracy. If replicated in larger cohorts, this gene–gene interaction could help identify the metabolic risk in children with obesity.

Abbreviations

µmol/L, micromoles/litre; AA, homozygous for one allele (generic); *ABCB4*, ATP binding cassette subfamily B member 4; AB, heterozygous (generic); ALA, alfa-linolenic acid, ALT, alanine aminotransferase; *APOC3*, apolipoprotein C3; ARA, arachidonic acid; AST, aspartate aminotransferase; BB, homozygous for the other allele (generic); BMI, body mass index; *CHDH*, choline dehydrogenase; *CHKB*, choline/ethanolamine kinase beta; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; *FADS2*, fatty acid desaturase 2; g, grams; HDL chol, high-density lipoprotein cholesterol, HDL, high-density lipoprotein cholesterol; HOMA-IR, Homeostatic model assessment for insulin resistance; LA, linoleic acid; LC-MS/MS, high-performance liquid chromatography–tandem mass spectrometry; NAFLD, non-alcoholic fatty liver disease; MDR, Multifactor dimensionality reduction (software); mg, milligrams; *MTHFD1*, methylenetetrahydrofolate dehydrogenase; *MTHFR*, methylenetetrahydrofolate reductase; *PCYT1A*, phosphate cytidylyltransferase 1, choline, alpha; *PCYT1B*, phosphate cytidylyltransferase 1, choline, beta; *PEMT*, phosphatidylethanolamine N-methyltransferase; *PNPLA3*, patatin-

like phospholipase domain containing 3; RBC, red blood cell membrane; REE, resting energy expenditure; *SCD*, stearoyl-CoA desaturase; SD, standard deviation; *SLC44A1*, solute carrier family 44 member 1; SNP, Single Nucleotide Polymorphism; *STAT3*, signal transducer and activator of transcription 3; zBMI, Standardized BMI-for-age z-scores; WHR, waist to hip ratio.

Data Sharing Statement

Raw data for variables of the cohort are available at request.

Ethics Approval

The study was approved by the Ethics Committee of the "Victor Babes" University of Medicine and Pharmacy (6/20.06.2016), Timisoara, Romania, and conducted in accordance with the Declaration of Helsinki.

Consent to Participate

Participants and their parents or legal guardians were informed about the aims and methods of the study. Written consent was signed by parents or legal guardians of the participants and children provided verbal consent to be included in the study.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed on the journal to which the article will be submitted; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

Mihai Dinu Niculescu is the founder and CEO of Advanced Nutrigenomics LLC. The authors declare no other potential conflicts of interest.

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