



Liver, Pancreas and Biliary Tract

Contribution of a genetic risk score to clinical prediction of hepatic steatosis in obese children and adolescents

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ABSTRACT

Background: Nonalcoholic fatty liver disease (NAFLD) is the commonest liver disease in children and adolescents in Western countries. Complex traits arise from the interplay between environmental and genetic factors in the pathogenesis of NAFLD.

Aims: We examined the association between NAFLD and eleven single nucleotide polymorphisms (SNPs) at genetic loci potentially associated with liver damage (*GCKR*, *MBOAT7*, *GPR120*), oxidative stress (*SOD2*), lipid metabolism (*PNPLA3*, *TM6SF2*, *LPIN1*, *ELOVL2*, *FADS2*, *MTTP*) and fibrogenesis (*KLF6*) in a paediatric population. A genetic risk score (GRS) was performed taking into account both these SNPs and clinical risk factors.

Methods: We recruited a cohort of 514 obese children and adolescents (mean age [\pm SD]: 11.2 \pm 2.8 years, z-BMI 3.3 \pm 0.8). NAFLD was identified by ultrasonography. Genotyping was performed by TaqMan-based RT-PCR system.

Results: The overall prevalence of NAFLD was 67.5% (347 patients). Among the eleven genotyped SNPs, the genetic variants in *TM6SF2* rs58542926 (OR = 4.13, p = 0.002), *GCKR* rs1260326 (OR = 1.53, p = 0.003), *PNPLA3* rs738409 (OR = 1.58, p = 0.004) and *ELOVL2* rs2236212 (OR = 1.34, p = 0.047) were significantly associated with a higher risk of NAFLD. Addition of a 11-polymorphism GRS to established clinical risk factors significantly (albeit modestly) improved the discriminatory capability of the regression model for predicting the risk of NAFLD (with SNPs C-statistic 0.81 [95%CI 0.75–0.88] vs. 0.77 [0.70–0.84] without SNPs; p = 0.047).

Conclusions: NAFLD was strongly associated with three genetic variants, *TM6SF2* rs58542926, *PNPLA3* rs738409 and *GCKR* rs1260326, and more slightly with *ELOVL2* rs2236212, in obese children and adolescents. Addition of a 11-polymorphism GRS to clinical risk factors improved the predictability of NAFLD.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common hepatic diseases in adolescents in developed countries [1].

Paediatric NAFLD has become a major public health problem worldwide with important implications for future development of liver dysfunction, type 2 diabetes and other cardiometabolic complications [2]. However, if detected early, the progression of NAFLD might be slowed and its extra-hepatic complications might be also prevented.

Obesity and a sedentary lifestyle, together with metabolic syndrome and ethnicity, contribute to the risk of NAFLD [3,4]. In recent

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years, a number of studies have suggested that genetic susceptibility also plays an important role in NAFLD development and progression in childhood [5,6]. Multiple genetic *loci* associated with the presence and the severity of NAFLD both in adults and in adolescents have been identified [7–9]. Although these *loci* account for only a small fraction of a total heritability of NAFLD, it is possible that they may be combined into a genetic risk score (GRS) for enhanced detection of people at higher risk of developing liver disease [10–17]. As recently reported, in NAFLD the gene-environmental synergy is more important than either single factor alone [18], and combining genetic and clinical information might be useful for risk stratification in clinical practice [19]. Several genetic models for NAFLD prediction have already been investigated in adults [5,17]. However, there remains a paucity of such models in the paediatric population. Analyzing genetic risk scores in paediatric populations could be useful since children and adolescents are less affected by comorbidities than adults, thereby limiting the effect of potential confounders for NAFLD in these subjects.

Therefore, the aim of this study was to test whether addition of genetic variants to established clinical risk factors improved risk prediction for NAFLD in a large sample of children and adolescents with obesity. The genetic variants included in our genetic risk score were 11 single nucleotide polymorphisms (SNPs) chosen on the basis of their involvement in lipid handling, insulin signaling, oxidative stress or hepatic fibrogenesis; all of which are potential contributing pathways affecting the development and progression of NAFLD.

2. Materials and methods

2.1. Subjects

We consecutively recruited unrelated obese children and adolescents at their first visit at the obesity outpatient clinic of the Pediatric Diabetes and Metabolic Disorders Unit of the University Hospital of Verona (Italy). Inclusion criteria were age between 6–18 years, European ancestry, Italian family origin and body mass index (BMI) greater than the age- and sex-specific BMI cutoff for obesity (using the World Health Organization BMI cutoffs as reference) [20,21]. Exclusion criteria were secondary obesity (e.g., Prader–Willi syndrome, Cohen syndrome, mutation in the *MC4R* gene), known chronic hepatic diseases (e.g., hepatitis by virus, drugs, autoimmunity, hemochromatosis, Wilson's disease), alcohol abuse (≥ 140 g/week), and chronic use of any drugs. Detailed information on these exclusion conditions and current use of medications was collected in all patients by structured interviews during medical examination. Based on these criteria, a total of 514 children and adolescents with obesity were included in the final analysis. Informed consent was obtained from children and their parents. The protocol of the study was approved by the local Ethics Committee of the University Hospital of Verona.

2.2. Anthropometric and biochemical data

At recruitment, anthropometric, clinical and biochemical data were collected for all children. BMI was calculated as body weight (in kilograms) divided by body height (in meters) squared. BMI values were standardized (z-BMI) using age- and sex-specific median, standard deviation and power of the box-cox transformation (least mean square method) based on WHO norms [20]. Waist circumference was measured as the minimal circumference measurable on the horizontal plane between the lowest portion of the rib cage and the iliac crest. Waist-to-height ratio (WHtR) was calculated and used as an index of body fat distribution, as previously described [22].

Venous blood samples were collected in the morning after an overnight fast of at least 8 h. Serum liver enzymes [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], glucose, lipids and other biochemical blood measurements were determined using standard laboratory procedures at the central Laboratory of our hospital. Low-density lipoprotein (LDL)-cholesterol was calculated using the Friedewald's equation. Hemoglobin A1c (HbA1c) was measured by a high-performance liquid chromatography analyzer on Tosoh-G7 automated analyzer (Tosoh Bioscience Inc., Japan).

2.3. Liver ultrasonography

At recruitment, as part of the routine investigation of children in our obesity outpatient clinic, two experienced radiologists (who were blinded to the clinical details of participants) performed ultrasonography in all children. No information was available on estimates of intra- or inter-rater reliability of ultrasonography. Hepatic steatosis was diagnosed according to ultrasonographic characteristics, including diffuse hyper-echogenicity of the liver relative to the kidneys, ultrasonography beam attenuation, or poor visualization of the intrahepatic vessel borders and diaphragm [23]. Liver ultrasonography has a good sensitivity and specificity for detecting moderate and severe hepatic steatosis, and traditionally its sensitivity is thought to be poor when $>20\%$ – 30% of hepatocytes are steatotic. In this study, a semi-quantitative ultrasonographic scoring of the degree of hepatic steatosis was not available.

2.4. Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes using salting-out procedures. Genotyping was carried out by pre-designed TaqMan probes (Applied Biosystem, USA), according to the manufacturers' protocol. Polymorphism genotyping was performed using 7900 HT Real Time PCR (Applied Biosystem, USA). We genotyped eleven genetic variants that have been previously robustly associated with NAFLD onset or progression: rs738409 (*PNPLA3*), rs58542926 (*TM6SF2*), rs1260326 (*GCKR*), rs2236212 (*ELOVL2*), rs116454156 (*GPR120*), rs1535 (*FADS2*), rs13412852 (*LPIN1*), rs641738 (*MBOAT7*), rs1800591 (*MTTP*), rs3750861 (*KLF6*) and rs4880 (*SOD2*). To estimate the overall contribution of the genetic variants under study on the risk of NAFLD and to test the diagnostic accuracy of this approach, a weighted genetic risk score (wGRS) was also derived from the SNP genotypes.

2.5. Statistical analysis

Data are expressed as means \pm SD, medians (inter-quartile ranges, IQR) or proportions. The unpaired Student's t-test (for normally distributed variables), the Mann–Whitney U-test (for non-normally distributed variables; i.e., z-BMI, HbA1c and serum aminotransferase levels) and the chi-squared test (for categorical variables) were used to test differences in clinical and biochemical characteristics between children with, and without NAFLD. Individual SNPs and wGRS associations with categorical variables were tested using logistic regression analyses, whereas associations with continuous variables were tested by generalized linear regression analyses; the aforementioned statistical tests were performed both in unadjusted models and in models adjusted for age, sex and z-BMI; the presence of a significant interaction with z-BMI was also tested in these regression models. The Benjamini–Hochberg (BH) step-up procedure was applied for multiple test adjustment [24]. Covariates included in multivariable regression models were selected as potential confounding factors based on their biological plausibility.

Table 1
Clinical and biochemical characteristics of children and adolescents stratified by NAFLD status.

	With NAFLD (n = 347)	Without NAFLD (n = 167)	p-Value ^a
n (M/F)	204/143	75/92	0.003
Age (years)	11.3 ± 2.7	10.8 ± 3.0	0.042
Weight (kg)	73.3 [58–91]	62.1 [49.6–76]	<0.0001
z-BMI (kg/m ²)	3.42 [3.01–3.73]	2.95 [2.49–3.61]	<0.0001
Waist circumference (cm), n = 412	94 [87–104]	85 [78–93]	<0.0001
WhtR, n = 412	0.64 ± 0.07	0.58 ± 0.07	<0.0001
Systolic blood pressure (mmHg)	118 [110–125]	114 [107–122]	0.007
Diastolic blood pressure (mmHg)	68 [60–75]	66 [60–73]	0.351
Total cholesterol (mmol/L)	4.03 [3.54–4.56]	3.97 [3.53–4.55]	0.684
LDL cholesterol (mmol/L)	2.41 ± 0.6	2.39 ± 0.6	0.939
HDL cholesterol (mmol/L)	1.10 [0.9–1.28]	1.18 [1.1–1.38]	0.001
Triglycerides (mmol/L)	1.02 [0.7–1.43]	0.86 [0.6–1.28]	0.006
ALT (U/L)	28.5 [20–43]	22.0 [17–29]	<0.0001
AST (U/L)	24.0 [19–29]	21.0 [18–26]	0.003
Fasting glucose (mmol/L)	4.61 [4.3–4.9]	4.60 [4.3–4.9]	0.971
HbA _{1c} DCC ^T (%)	5.5 [5.4–5.7]	5.4 [5.3–5.7]	0.257
HbA _{1c} IFCC (mmol/mol)	37 [36–39]	36 [34–39]	0.257
Fasting insulin (mU/L)	23.5 [13.4–37.3]	14.0 [8.7–24.6]	<0.0001
wGRS (11 variants on training subset)	12.02 ± 3.08	10.27 ± 2.40	<0.009
wGRS (11 variants on validation subset)	11.62 ± 2.77	10.59 ± 3.03	0.007

Sample size, n = 514, unless otherwise indicated. Data are expressed as means ± SD, medians and interquartile range [IQR] or proportions.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; z-BMI, z-score body mass index; HbA_{1c} DCC^T, Diabetes Control and Complication Trial-Aligned Haemoglobin A1c; HbA_{1c} IFCC, International Federation of Clinical Chemistry-Aligned Haemoglobin A1c; HbA_{1c}, hemoglobin A1c; WhtR, waist circumference to height ratio; wGRS, weighted genetic risk score.

^a Differences between the two groups of individuals were tested by the unpaired Student's t-test for normally distributed variables, the Mann–Whitney U-test for non-normally distributed variables or the chi-squared test for categorical variables, respectively.

We also created two subsets (i.e. training and validation subsets) from the target sample, randomly splitting our cohort. The training subset (n = 251) was used to build four logistic regression models for predicting NAFLD: model M1, a clinical model based on established clinical predictors of NAFLD and including age, sex, z-BMI, log-transformed serum triglycerides, ALT, fasting glucose and insulin levels; model M2, a clinical + genetic model in which the three major NAFLD-related SNPs (rs58542926, rs738409, rs1260326) were included; model M3 that added also rs2236212 (ELOVL2) to model M2; and, finally, a model M4, a clinical plus genetic model in which all 11 genotyped SNPs were added to the clinical model. The resulting predictive equations were applied to the validation subset of children (n = 263) and compared to each other in terms of predictive accuracy. To evaluate the model accuracy, receiver operating characteristic (ROC) curves, plotting sensitivity against 1–specificity, were also constructed for all possible cut-offs for the aforementioned logistic regression models. The area under the ROC curve (AUC), or C-statistic, was estimated as a measure of the probability that a randomly selected individual had NAFLD.

This analysis was also performed in a subgroup of patients to predict the presence of NAFLD plus high serum ALT levels; however, in such case, we did not split our cohort into training and validation subsets.

A p-Value <0.05 was considered statistically significant. Statistical analysis were performed using Plink [25], SPSS v22.0 software package (SPSS Inc., Chicago, USA) and STATA software 14.2.

3. Results

3.1. Subjects characteristics

We studied a cohort of 514 (54.3% male) Italian obese children and adolescents, who consecutively underwent ultrasonography for detecting hepatic steatosis. Their mean (±SD) age was 11.2 ± 2.8 years (range: 6–18 years), mean z-BMI 3.3 ± 0.8 and mean waist circumference 92.1 ± 14 cm, respectively. The overall prevalence of NAFLD was 67.5% (347 children). In the entire cohort, 80% of children had serum aminotransferase levels within the reference

values, whereas nearly 20% of them had slightly elevated (>40 IU/L) ALT or AST levels, with only 1.5% (n = 8) with serum ALT or AST levels ≥100 IU/L.

The main clinical and biochemical characteristics of participants stratified by presence or absence of NAFLD are shown in Table 1. Compared to those without NAFLD, children with NAFLD were more likely to be boys, older and with higher BMIs. They also had significantly higher values of systolic blood pressure (SBP), serum triglyceride, AST and ALT concentrations (although most of these children had fairly normal values), but lower HDL-cholesterol levels. In contrast, diastolic blood pressure (DBP), total cholesterol, LDL-cholesterol, fasting glucose, 2-h OGTT glucose and hemoglobin A1c levels did not significantly differ between the two groups. When we compared wGRS, both in the training and validation subsets, children with NAFLD had a significantly greater wGRS than those without the liver disease.

3.2. Associations of genetic variants with NAFLD

The call rate exceeded 99% for all SNPs and genotypes' distributions were in Hardy–Weinberg Equilibrium (HWE). Only four out of 11 SNPs reached a statistical power of at least 80% (rs1260326, rs58542926, rs738409 and rs2236212). Table 2 shows HWE p-Values and the results of the association between NAFLD and the genotyped SNPs. The frequency distributions of *PNPLA3* rs738409, *GCKR* rs1260326, *ELOVL2* rs2236212, and, especially, *TM6SF2* rs58542926 variants remained significantly different between children with and without NAFLD even after adjustment for age, sex and z-BMI. In all these adjusted regression models, no significant interactions with z-BMI were observed in the associations between individual SNPs and NAFLD (p-Value ≥0.20 for the interaction term in all regression models). These results did not change even after further adjustment for WhtR, a measure of body fat distribution, which was available in a subgroup of 412 children (data not shown). As also shown in the table, when an adjustment for multiple tests (by using the Benjamini–Hochberg step-up procedure) was made, only the genetic variants in *TM6SF2* rs58542926, *GCKR* rs1260326 and *PNPLA3* rs738409 remained independently associated with NAFLD.

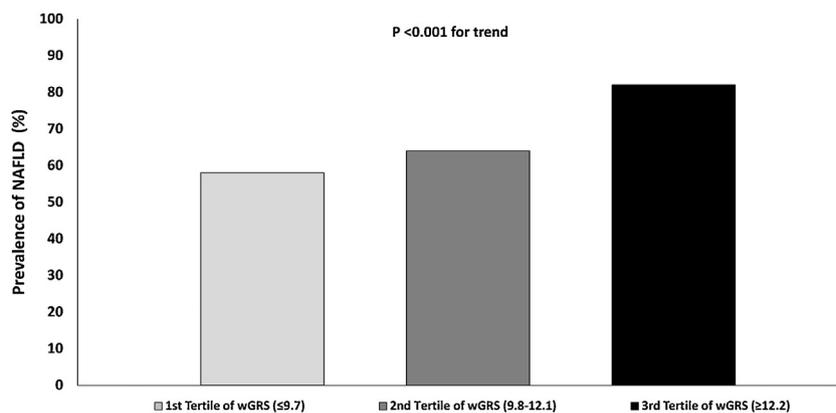
Table 2

Unadjusted and age-, sex- and z-BMI-adjusted associations between nonalcoholic fatty liver (NAFLD) and each of the eleven genotyped single nucleotide polymorphisms.

Chr	SNP ID	Gene ID	Minor Allele	MAF	Risk allele	HWE <i>p</i> -value	Unadjusted OR [95% CI] ^a	<i>p</i> -Value ^a	Adjusted OR [95% CI] ^b	<i>p</i> -Value ^b
19	rs58542926	TM6SF2	A	0.04	A	0.410	4.15 [1.44–11.9]	0.008 ^c	4.13 [1.41–12.1]	0.002 ^c
2	rs1260326	GCKR	C	0.47	T	0.486	1.50 [1.14–1.96]	0.003 ^c	1.53 [1.15–2.03]	0.003 ^c
22	rs738409	PNPLA3	G	0.31	G	0.734	1.51 [1.12–2.03]	0.007 ^c	1.58 [1.15–2.18]	0.004 ^c
6	rs2236212	ELOVL2	C	0.45	C	0.398	1.35 [1.03–1.77]	0.032	1.34 [1.01–1.80]	0.047
10	rs116454156	GPR120	A	0.02	A	0.618	1.68 [0.61–4.63]	0.317	1.79 [0.58–5.56]	0.289
11	rs1535	FADS2	G	0.29	G	0.471	1.16 [0.87–1.55]	0.309	1.16 [0.85–1.57]	0.342
2	rs13412852	LPIN1	T	0.34	C	0.847	1.13 [0.86–1.48]	0.395	1.13 [0.84–1.50]	0.421
19	rs641738	MBOAT7	T	0.44	T	0.915	1.15 [0.88–1.49]	0.316	1.12 [0.85–1.49]	0.442
4	rs1800591	MTTP	T	0.28	T	0.068	1.02 [0.77–1.34]	0.916	1.08 [0.80–1.45]	0.625
10	rs3750861	KLF6	T	0.08	C	0.109	1.15 [0.73–1.81]	0.556	1.10 [0.68–1.79]	0.697
6	rs4880	SOD2	G	0.49	A	0.748	1.05 [0.80–1.36]	0.743	1.05 [0.80–1.39]	0.721

Sample size, *n* = 514. SNPs are ordered by *p*-values (see last column). Presence of NAFLD was the dependent variable in all logistic regression models.

Abbreviations: HWE, Hardy–Weinberg Equilibrium; z-BMI, z-score body mass index; Chr, chromosome position; SNP, single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

^a Data were tested by univariable (unadjusted) logistic regression analysis.^b Data were tested by multivariable logistic regression analysis after adjustment for age, sex and z-BMI.^c Significant *p*-Values after further adjustment for multiplicity (by using the Benjamini–Hochberg step-up procedure).**Fig. 1.** Prevalence of ultrasound-defined nonalcoholic fatty liver (NAFLD) in obese children and adolescents stratified by tertiles of weighted genetic risk score (wGRS).

Interestingly, wGRS, calculated on the validation subset, was significantly associated with presence of NAFLD (unadjusted OR of 1.14, 95% CI 1.03–1.25, $p=0.007$). This association remained statistically significant even after adjustment for age, sex, z-BMI, log-transformed serum triglycerides, ALT, fasting glucose and insulin levels (adjusted OR of 1.18, 95% CI 1.04–1.33, $p=0.010$). Additionally, as reported in Fig. 1, the prevalence of NAFLD increased progressively across wGRS tertiles.

3.3. Diagnostic accuracy of the genetic risk score

In the validation subset, we undertook a ROC curve analysis applying four different logistic regression models in order to identify subjects at higher risk of NAFLD. The clinical model (M1) including age, sex, z-BMI, log-serum triglycerides, ALT, fasting glucose and insulin levels showed an AUC = 0.77 (95%CI 0.70–0.84). In model M2, addition of the three major NAFLD-related SNPs slightly improved the risk prediction for NAFLD (AUC = 0.80; 95%CI 0.73–0.86; C-statistic $p=0.173$ for comparison to model M1). Similar finding was observed in model M3 that was computed as model M2 plus rs2236212 (ELOVL2) (AUC = 0.80; 95%CI 0.74–0.87; C-statistic $p=0.118$ for comparison to model M1). Finally, the model M4, a clinical + genetic model in which all 11 genotyped SNPs were added, showed an AUC = 0.81 (95% CI 0.75–0.88). Notably, compared to the clinical model alone (M1), this final regression model (M4) significantly (albeit modestly) improved risk prediction for NAFLD (C-statistic $p=0.047$) (Fig. 2).

When we performed the same ROC curve analyses, on the whole cohort, in order to identify the subgroup of children with both

NAFLD and elevated (>40 IU/L) serum ALT levels (i.e., a proxy of suspected steatohepatitis [NASH]), we found that the addition to the clinical M1 (not including serum ALT levels) of both the three or four major NAFLD-related SNPs (M2: AUC = 0.72; 95%CI 0.67–0.77; C-statistic $p=0.015$ vs. M1; M3: AUC = 0.72; 95%CI 0.68–0.77; C-statistic $p=0.015$ vs. M1) and all genotyped SNPs significantly improved the risk prediction of NAFLD plus elevated ALT levels (M4: AUC = 0.74; 95%CI 0.68–0.78; C-statistic $p=0.005$ vs. M1) (Fig. 2).

4. Discussion

The main and novel finding of this study was that a genetic risk score, based on the combination of 11 genetic risk variants plus established clinical risk factors, improved risk prediction for NAFLD in obese children and adolescents by 5.2%, compared to risk prediction based on clinical factors alone. To our knowledge, this is one of the largest studies (involving a mono-ethnic cohort of Caucasian obese children and adolescents) that has examined a broad panel of genetic polymorphisms associated with susceptibility to NAFLD in paediatric obesity.

Our findings are consistent with similar studies that have been performed previously. Nobili et al. showed that addition of a 4-polymorphism risk score (including rs738409, rs4880, rs3750861 and rs13412852 variants) to a clinical model performed better than a clinical risk score alone (including age, diastolic blood pressure and serum AST levels) in predicting the risk of NASH in 152 obese children and adolescents with biopsy-proven NAFLD and elevated serum aminotransferase levels [17]. In our study, using a larger polymorphism genetic score (that also included the four aforemen-

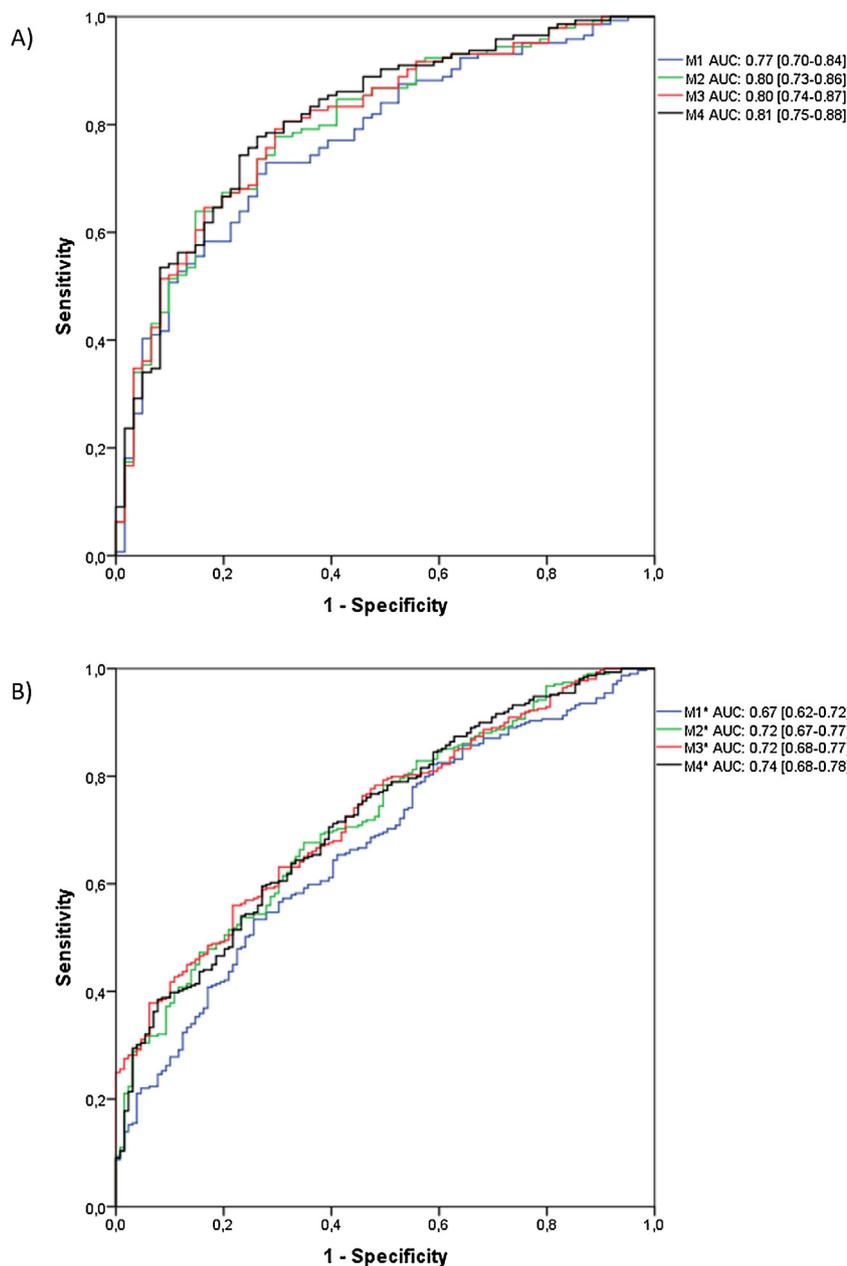


Fig. 2. Panel A. Receiver operating characteristic (ROC) curves with and without genotyped single nucleotide polymorphisms (SNP) in risk prediction models of nonalcoholic fatty liver (NAFLD) in obese children and adolescents. Model M1: clinical regression model including age, sex, z-BMI, serum triglycerides, alanine aminotransferase, glucose and insulin levels (area under curve [AUC]: 0.77, 95% CI 0.70–0.84). Model M2: regression model including the same variables of M1 plus the three major SNPs (*GCKR* rs1260326, *TM6SF2* rs58542926 and *PNPLA3* rs738409) associated with NAFLD (AUC: 0.80, 95% CI 0.73–0.86). Model M3: regression model including the same variables of M2 plus *ELOVL2* rs2236212 (AUC: 0.80, 95% CI 0.74–0.87). Model M4: regression model including the same variables of M1 plus all 11 genotyped SNPs (AUC: 0.81, 95% CI 0.75–0.88). Compared to the clinical regression model alone (M1), the model M4 significantly improved risk prediction for NAFLD ($p = 0.047$ by Harrell's C-statistic).

Panel B. Receiver operating characteristic (ROC) curves with and without genotyped SNPs in risk prediction models of suspected nonalcoholic steatohepatitis (defined as hepatic steatosis on ultrasound plus elevated serum alanine aminotransferase levels) in the whole cohort of obese children and adolescents. *In models M1–M4 were included the same list of variables of the aforementioned logistic regression models (except for exclusion of serum ALT in model M1). NB: in this analysis, we excluded those patients with NAFLD and normal serum ALT levels.

tioned genetic variants), we found that the addition of the GRS to the established clinical risk factors significantly improved the risk prediction of NAFLD in our cohort of obese children/adolescents and fairly normal serum aminotransferase levels.

The addition of the GRS also significantly improved the predictability of NAFLD with coexistent elevated serum aminotransferase levels (i.e., a proxy of suspected NASH). Furthermore, we observed a graded relationship between increasing GRS and NAFLD prevalence, suggesting the potential clinical relevance of a combination of risk alleles of individually small effects (Fig. 1).

Overall, all of these findings partly reflect the fact that these genetic variants may act in an additive manner amplifying the phenotypic effect(s), and these data also support the potential contribution of a genetic score approach to improving identification of individuals at increased NAFLD risk [26]. Although in our study the addition of the eleven genotyped SNPs provided a significant, but modest, improvement in the performance of a prediction model for NAFLD, we believe that the potential future availability of new genetic loci of susceptibility to NAFLD might further improve the role of genetics in risk prediction for NAFLD in paediatric obesity. Furthermore,

it is likely that the progressive reduction in the costs of genotyping associated with the validation of its predictive power, will progressively promote the use of genetic scores in clinical practice.

In our study, only four out of the eleven SNPs included in the score (*TM6SF2* rs58542926, *GCKR* rs1260326, *PNPLA3* rs738409 and *ELOVL2* rs2236212) were significantly associated with an increased risk of NAFLD, independently of age, sex and z-BMI. Since, we did not find any significant association between NAFLD and other genotyped SNPs, it is plausible to assume that this could be due to the relatively low sample size and the low frequency of the risk alleles. However, since it is likely that most of our children and adolescents with ultrasound-defined hepatic steatosis and fairly normal serum aminotransferase levels had NAFL (defined as the presence of $\geq 5\%$ hepatic steatosis on histology, most likely $\geq 30\%$ as it was evident by ultrasonography [27,28]), and none (or very few) of them had advanced fibrosis or cirrhosis, we believe that other possible explanations for this finding could also be that the use of a more accurate method for measuring intra-hepatic fat content (by magnetic resonance imaging or spectroscopy) could allow detection of significant associations with some of genetic variants [29]. Moreover, the lack of significant associations of *SOD2* and *KLF6* genes with NAFLD traits might be partly due to the predominant role of these genes in the pathogenesis of advanced forms of NAFLD (NASH and fibrosis) [17]. With regards to *PNPLA3*, *TM6SF2* and *GCKR* genes, our findings corroborate the results of recently published studies showing that these three SNPs are strongly associated with the presence of NAFLD in obese children/adolescents of European ancestry [30–34].

In our study, we also showed for the first time a potential involvement of the *ELOVL2* rs2236212 variant in paediatric NAFLD. *ELOVL2* gene encodes for elongase subtype 2, an enzyme involved in the endogenous synthesis of long-chain polyunsaturated fatty acids (n-3 PUFAs). In a large genome-wide association study, Lemaitre et al. reported a significant inverse association between polymorphisms in *ELOVL2* gene and plasma n-3 PUFA levels (mostly lower plasma docosahexaenoic acid levels) in populations of European ancestry, underscoring the possible role of the *ELOVL2* variant in phospholipid metabolism [35].

NAFLD is a common disorder and identifying new determinants, such as new genes, may improve understanding of mechanisms potentially implicated in NAFLD development and progression [3]. Moreover, by identifying individuals at higher risk of developing NAFLD based on their GRS, clinicians could more accurately assess a patient's unique risk of developing NAFLD and, therefore, recommend personalized interventions to modify that risk. Currently, no recommendations for the use of genetic screening have been made in scientific societies' guidelines [36–39]. Nevertheless, it has been suggested that genotyping could be useful to stratify the prognosis of NAFLD (risk of progression to cirrhosis and hepatocarcinoma) and the probability of responses to specific therapeutic approaches in selected patients [40,41]. However, further research is needed before the use of genetic testing can be routinely recommended for risk stratification of NAFLD patients in clinical practice [41].

Our study has some limitations that should be mentioned. First, the cross-sectional design of the study limits our ability to establish causality and temporality of the observed associations, although this limitation is mitigated by the fact that the genetic variants are inherited and therefore, reverse causality does not apply. We also could not control for the presence of lifestyle factors associated with NAFLD development, such as sweetened drinks intake and physical activity [42]. Second, the diagnosis of NAFLD was based on ultrasonography and exclusion of secondary causes of hepatic steatosis. Ultrasonography is the recommended first-line imaging method for detecting hepatic steatosis in clinical practice, and it enables a reliable and accurate detection of mild-to-moderate hepatic steatosis. Unfortunately, we did not perform a semi-quantitative

ultrasound score of the degree of hepatic steatosis, such as the ultrasonographic fatty liver indicator (US-FLI), which may detect more accurately mild steatosis (i.e. minimum amount 10% on histology [43]). Liver ultrasonography was undertaken by two experienced radiologists, but unfortunately, we are not able to comment on the intra- and inter-observer agreement as this data was not collected. In addition, we did not perform liver biopsy, which remains the current 'reference' method for diagnosing and staging NAFLD [23,27]. Our sample was composed mostly by children and adolescents with fairly normal serum aminotransferase levels and undertaking liver biopsies in these subjects is always far more challenging as sometimes it is necessary for the child to undergo a general anaesthetic for the procedure to be undertaken. Third, we did not have any data on vibration-controlled transient elastography (Fibroscan[®]) for measuring liver stiffness in order to non-invasively stage hepatic fibrosis. Fourth, ethnicity limited to Caucasian, which does not allow to export results to children and adolescents with other ethnic backgrounds. Finally, the lack of a validation cohort cautions against the generalizability of our findings to other paediatric groups.

Our study also has strengths. First, the study population; e.g. obese children and adolescents have much lower obesity-associated comorbidities than obese adults and, by implication this offers the opportunity to explore genetic associations with NAFLD with reduced influence from other potential confounding factors. Second, the sample size of the study was relatively large to explore associations with NAFLD, despite low allele frequencies and small gene-effect sizes. Third, we have included well-established clinical risk factors in the prediction risk score and adjusted for potential confounders.

In conclusion, in a large cohort of unrelated obese children and adolescents, our study has identified three polymorphisms, i.e. *TM6SF2* rs58542926, *PNPLA3* rs738409 and *GCKR* rs1260326, as major genetic variants that are strongly associated with NAFLD. Our data also suggests the potential involvement of the *ELOVL2* rs2236212 variant as this polymorphism was also independently associated with NAFLD. Furthermore, our data suggest that the addition of a 11-polymorphism risk score to multiple established clinical risk factors improved (albeit modestly) risk prediction of NAFLD. We suggest that further research is now needed to validate our findings in other cohorts of children and adolescents, and to examine whether these genetic variants may predict resolution (or progression) of NAFLD with lifestyle interventions or treatments.

Conflict of interest

None declared.

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