RESEARCH PAPER

Study of Family Clustering and *PNPLA3* Gene Polymorphism in Pediatric Non Alcoholic Fatty Liver Disease

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Objectives: To find association of pediatric NAFLD with metabolic risk factors, and *Patatin-like phospholipase domain-containing protein 3 (PNPLA3)* gene polymorphism.

Design: Cross-sectional study

Setting: Pediatric Hepatology unit of a tertiary care hospital

Participants: Overweight/obese children (<18 years) with (69 patients) or without (30 patients) NAFLD (ultrasonography based), and their parents.

Intervention: Metabolic screening, *PNPLA*3 gene polymorphism, and transient elastography

Outcome measure: Association of pediatric NAFLD with parental metabolic risk factors and *PNPLA*3 gene polymorphism.

Results: In the NAFLD group, there was high parental incidence of metabolic diseases, fatty liver (80%) and low high-density lipoproteins levels (84%). Family history of NAFLD (in any parent), higher alanine aminotransferase levels and higher total cholesterol levels in the child independently predicted possibility of NAFLD, but similar results could not be replicated for *PNPLA*3 gene polymorphism. Controlled attenuation parameter measurement (by transient elastography) had high sensitivity and specificity to diagnose steatosis.

Conclusion: There is high familial incidence of metabolic diseases in children with NAFLD. Controlled attenuation parameter can be useful as a non-invasive modality to screen fatty liver in children.

Keywords: *Metabolic syndrome, Obesity, Transient Elastography*.

Nonalcoholic fatty liver disease (NAFLD) is a spectrum characterized by hepatic fat accumulation which ranges from simple steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis [1]. NAFLD is potentially one of the most common causes of liver disease worldwide both in adults and children [2-4]. Considering its ever increasing and epidemic proportions, its timely identification and optimum management should be a priority in the present times [5].

It is established that NAFLD is multi-factorial with a substantial genetic component. Familial and genetic factors (metabolic syndrome and *Patatin-like phospholipase domain-containing protein 3* or *PNPLA*3 gene polymorphism) are a major determinant of whether an individual will have NAFLD or not [6-10]. Thus, children with family history of NAFLD should be considered at high risk for NAFLD and *vice versa*. Several studies have shown that single-nucleotide polymorphisms (SNPs), especially in *PNPLA*3 gene (coding for a protein adiponutrin which plays a role in hepatic triglyceride hydrolysis) may influence hepatic steatosis and its progression in both adult and pediatric populations [11-14]. Thus, this *PNPLA3* gene polymorphism could be used as a genetic marker for assessing risk of early hepatic damage thus providing a window of opportunity to intervene at a pre-symptomatic stage, especially in children with additive familial risk factors.

Since no data on family clustering and *PNPLA*3 polymorphism in pediatric population is available from Indian subcontinent, where metabolic risk factors are highly prevalent [15], this study was planned with an aim to study parental metabolic disorders and *PNPLA*3 polymorphism as possible risk factors for Pediatric NAFLD in overweight/obese children.

METHODS

This prospective observational study was undertaken in the departments of Hepatology and Pediatric Hepatology in a tertiary care institute. The study was approved by the Institutional Review Board. The patients were enrolled after informed consent and assent was obtained from the parent(s) and patients. The duration of the study was from Ist October 2014 to 31st December 2016. Inclusion

criteria included: (*i*) all overweight/obese children (aged 8-18 years, overweight defined as a body mass index or BMI ≥85th percentile to <95th percentile, and obesity defined as a BMI ≥95th percentile, for children and teens of the same age and sex) and their parents, and (*ii*) adults (along with their spouses) suffering from diabetes mellitus (DM), obesity, dyslipidemia, metabolic syndrome or NAFLD having overweight/obese progeny (aged 8-18 years). Exclusion criteria included (*i*) history suggestive of acute hepatitis in last 6 months, (*ii*) abnormal thyroid profile, (*iii*) Wilson disease (≥1 of following- low ceruloplasmin/increased urinary copper/Kayser Fleischer ring +ve), (*iv*) hepatitis B/C infection, (*v*) concomitant liver diseases, (*vi*) severe malnutrition, (*vii*) ongoing total parenteral nutrition/jejunal-ileal bypass, (*viii*) alcohol intake of more than 20g/week, (*ix*) syndromic obesity, (*x*) medication use like steroids, estrogens, Amiodarone, Methotrexate, Tamoxifen and antitubercular therapy.

All patients (*i.e.*, including atleast the index child and both parents) underwent screening evaluation including detailed family history, baseline evaluation – vitals, anthropometry (body mass index, waist circumference, waist- hip ratio) and metabolic screen (liver function tests, fasting lipid profile, fasting blood sugar, serum insulin, and HbA1C), ultrasonography (USG) of abdomen, *PNPLA3* I148M polymorphism (nonsynonymous rs738409 SNP), transient elastography (TE) (for liver stiffness measurement (LSM); and controlled attenuation parameter (CAP) measurements for steatosis assessment) and liver biopsy (in NAFLD children, as applicable) (***Web Annexure* 1**). Diagnosis of NAFLD was based on ultrasonography of abdomen.

Detailed nutritional counseling was conducted in consultation with trained nutritionist to target weight loss of 5-10 %. Hobby development to burn calories in terms of any sports, whatever preferred and available. An individualized diet chart and exercise regimen (including sports and games) were prepared and explained to the whole family. Healthy dietary habits including reduced intake of saturated fats/fructose or sugar rich products and increased intake of polyunsaturated fats and fibres was emphasized. Vitamin E capsules 400 IU once daily was prescribed if elevation of transaminases was persistent despite adequate dietary compliance and weight loss after 3 months.

Statistical analysis: The mean differences between the groups were tested by independent sample t-test. The chi square (or Fisher's exact) test was used to compare differences between the groups for categorical variables. Variables affecting Family Clustering were analyzed using univariate and multivariate analysis. Data was analyzed by using SPSS 22 version. Probability of Y (Outcome, for *e.g*, Pediatric NAFLD) is predicted by: Probability (Y) = $1/1 + e^{-(b0 + b1X1 + b2X2...)}$ where *P*(*Y*) is the probability of *Y* occurring, *e* is the base of natural logarithms, X1/X2/X3 etc are predictor variables, bo/b1/b2 etc are Beta Coefficients [16].

RESULTS

A total of 99 overweight and obese children were included in the study, with 69 subjects in NAFLD group ***(Fig.* 1**). In the NAFLD group, there were 59 boys (85.5%) with median age of 13.1 years, while in the non- NAFLD group, there were 21 boys (70%) with a median age of 11.1 years. There was high incidence of metabolic diseases in the families having children with NAFLD where more than 3/4th of the families had atleast one parent with either fatty liver (80%) or low HDL levels (84 %). Similarly there was high incidence (>2/3rd of families) of insulin resistance, hypertension and high triglycerides in atleast one parent in the NAFLD group. In the NAFLD group, homozygosity (GG status) and heterozygosity (CG status) for *PNPLA*3 polymorphism was seen in 24 (34.8%) and 23 (33.3%) overweight/obese children respectively. In the non-NAFLD group, only 1 subject had homozygous mutation, while heterozygous status was found in 8 (26.7%).

Analysis of presence or absence of family history of metabolic risk factors (NAFLD, hypertension, insulin resistance/IR, type 2 diabetes mellitus, dyslipidemia and presence of metabolic syndrome) in the parents showed that presence of NAFLD in any one parent (OR 3.9, 95% CI

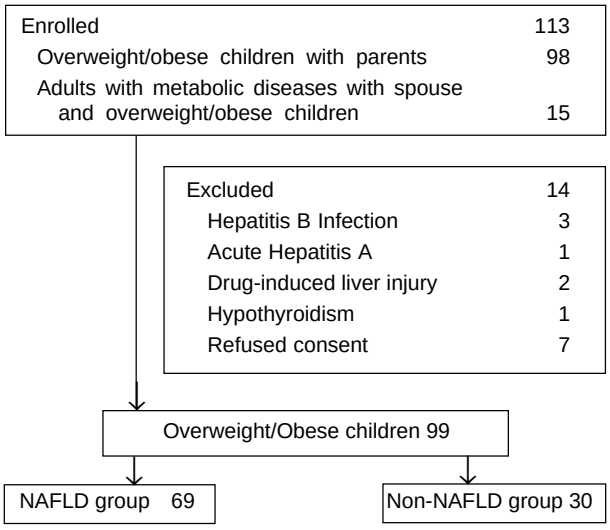

FIG. 1 *Flowchart depicting patient selection in the study. (*NAFLD diagnosis based on ultrasonographic evidence of fatty liver).*

1.5 to 10.6; *P*=0.008) or both parents (OR 6.7, 95 % CI 1.4 to 30.6; *P*=0.009) and presence of insulin resistance in any parent (OR 3.6, 95 % CI 1.1 to 12.0; *P*= 0.009) or both parents (*P*=0.01) was significantly associated with occurrence of NAFLD in the progeny **(*Table* I)**. Amongst the clinical parameters, presence of acanthosis and higher mean BMI significantly differentiated NAFLD from non-NAFLD group **(*Table* II)**. In the laboratory features, presence of higher mean serum aspartate aminotransferase (AST) levels, higher mean serum alanine aminotransferase (ALT) levels, higher mean uric acid levels, higher mean cholesterol levels, high mean fasting insulin levels, higher Homeostasis model assessment of insulin resistance-1 (HOMA-1) index, higher HOMA-2 index and lower Quantitative insulin sensitivity check index (QUICKI), presence of insulin resistance and presence of homozygosity of *PNPLA3* polymorphism predicted occurrence of fatty liver disease in children **(*Table* II)**.

Only 11 of the total NAFLD group gave consent for liver biopsy. The results showed simple steatosis in 5 and presence of NASH (with NAS score ≥5) in 6 children. In the 6 patients with NASH, 1 patient had stage 3 fibrosis, 3 patients had stage 2 fibrosis while 2 patients showed stage 1 fibrosis. Due to limited number of subjects, no statistical analysis could be performed.

On comparing transient elastography features (LSM and CAP), higher controlled attenuation parameter (CAP) values significantly differentiated NAFLD from non NAFLD group while there was no significant difference on LSM between two groups **(*Table* II)**. At a

TABLE I COMPARISON OF DEMOGRAPHIC FEATURES OF THE NAFLD VERSUS NON-NAFLD GROUP

Parameter	*NAFLD group (n= 69)*	*Non-NAFLD group (n= 30)*	*Effect Size (95 % CI)*[#]	*P value*
Age (y)[*]	13.4 (2.9)	12.2 (2.9)	1.2 (- 5.2, 2.9)	0.170
Males gender	59 (85.5 %)	21 (70 %)	0.4 (0.1, 1.2)	0.130
Family history (abdominal obesity)				
Any parent	69 (100%)	30 (100%)	NC	NC
Both parents	58 (86%)	21 (70%)	1.8 (0.6, 5.4)	0.360
NAFLD				
Any parent	55 (79.7%)	12 (40%)	3.9 (1.5, 10.6)	0.008
Both parents	26 (37.7%)	02 (6.7%)	6.7 (1.4, 30.6)	0.009
Hypertension				
Any parent	49 (71%)	17 (56.7%)	1.0 (0.4, 2.8)	1.000
Both parents	17 (24.6%)	03 (10%)	2.3 (0.6, 8.6)	0.260
Insulin resistance				
Any parent	45 (65.2%)	08 (26.7%)	3.6 (1.1, 12.0)	0.009
Both parents	15 (21.7%)	0	NC	0.010
Diabetes mellitus				
Any parent	30 (43.5%)	09 (30%)	1.3 (0.5, 3.3)	0.640
Both parents	07 (10.1%)	02 (6.7%)	1.2 (0.2, 6.4)	1.000
High triglycerides				
Any parent	43 (62.3%)	15 (50%)	0.9 (0.4, 2.6)	1.000
Both parents	12 (17.4%)	01 (3.3%)	4.8 (0.6, 39.4)	0.170
Low HDL				
Any parent	58 (84.1%)	18 (60%)	1.8 (0.6, 5.4)	0.360
Both parents	28 (40.6%)	07 (23.3%)	1.7 (0.6, 4.5)	0.460
Metabolic syndrome				
Any parent	36 (52.2%)	14 (46.7%)	0.8 (0.3, 2.0)	0.642
Both parents	19 (27.5%)	08 (26.7%)	0.8 (0.3, 2.1)	0.609

*Values in Number (%) or *mean (SD); [#]Mean Difference (95 % CI-Lower Limit,Upper Limit) for comparison of means and OR (95% CI-Lower Limit,Upper Limit) for comparison of proportions, NAFLD: Non-alcoholic fatty liver disease; NC: Not computable, HDL: High Density Lipoprotein.*

TABLE II COMPARISON OF CLINICAL AND LABORATORY FEATURES OF NAFLD *VERSUS* NON-NAFLD GROUP

Parameter	*NAFLD group (n=69)*	*Non-NAFLD group (n=30)*
Weight (kg)	65.6 (15.8)	56.3 (20.7)
BMI* (kg/m^2)	26.7 (3.6)	24.5 (4.2)
Waist circumference (WC, cm)	87.6 (8.6)	85.1 (10.2)
Waist/Hip ratio	0.9 (0.05)	0.9 (0.04)
Obesity (BMI based) (%)	58	53.3
Abdominal obesity (WC based) (%)	78.3	83.3
Acanthosis* (%)	52.2	13.3
Pre-hypertension (%)	24.6	6.7
Hypertension (%)	11.6	0
Serum AST* (IU/L)	57.2 (48.9)	34.2 (14.1)
Serum ALT* (IU/L)	89.1 (78.6)	28.4 (8.4)
Serum uric acid* (mg/dL)	5.7 (1.5)	4.7 (0.95)
Total cholesterol* (mg/dL)	162.9 (38.1)	135.0 (35.7)
Serum triglycerides (mg/dL)	138.5 (43.3)	136.3 (75.7)
Serum HDL (mg/dL)	36.3 (9.1)	38.2 (10.7)
FBS (mg/dL)	92.9 (27.2)	87.2 (7.2)
Serum insulin* (mIU/mL)	11.2 (5.3)	7.9 (3.6)
HOMA 1 Index*	2.6 (1.5)	1.7 (0.8)
HOMA 2 Index*	1.5 (0.7)	0.9 (0.3)
QUICKI*	0.3 (0.02)	0.3 (0.012)
IR (HOMA-1 >2.5)* (%)	34.8	10
LSM (K Pa or Kilopascals)	5.3 (1.6)	4.9 (1.2)
CAP (db/m)*	285.3 (26.6)	225.3 (30.9)
Homozygous *PNPLA3* polymorphism* (%)	34.8	3.3

*Values in mean (SD) unless specified; *P<0.05; NAFLD: Non-alcoholic fatty liver disease, BMI: Body mass index, ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; HDL: High Density Lipoprotein; FBS: Fasting blood sugar; HOMA-IR: Homeostasis model assessment of Insulin Resistance, LSM: Liver stiffness measurement; CAP: Controlled attenuation parameter; QUICKI: Quantitative insulin sensitivity check index; PNPLA3: Patatin-like phospholipase 3.*

cut-off of 259.5 dB/m, CAP could predict presence of NAFLD in children with 88.4 % sensitivity and 100 % specificity and AUROC of 0.965 (95 % CI 0.931 to 1.000) **(*Fig.* 2**).

Multivariate binary logistic regression analysis showed that family history of NAFLD (in any parent), higher ALT levels and higher total cholesterol levels may independently predict presence of NAFLD **(*Table* III**). Only ALT levels reached significance on calculating area under receiver operating characteristic (AUROC) curve,

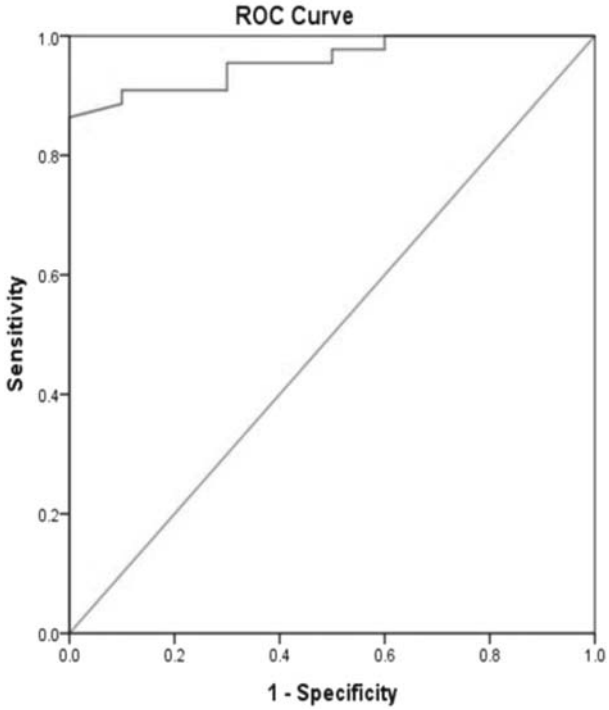

FIG. 2 *Receiver operating characteristic (ROC) curve for Controlled Attenuation Parameter (by transient elastography) for NAFLD prediction.*

where in an overweight/obese child, ALT levels > 31.5 IU/L predicted presence of NAFLD with 80.6% sensitivity and 60.9% specificity with an AUROC of 0.825 (95 % CI 0.743 to 0.908).

Based on the logistic regression model of Pediatric NAFLD in overweight/obese children, probability of developing Pediatric NAFLD was derived by the equation: P (Y) = 1/1 + e $^{-}$ [-7.088 + (2.935 × Family History of NAFLD in any parent) + (0.075 × ALT) + (0.045 × Cholesterol)] where 'Family History of NAFLD in any parent' is 0 or 1 when absent or present, respectively. The above equation leads us to a probability of developing NAFLD in overweight/obese children as 92.4 % based on the sample data.

TABLE III RESULTS OF MULTIVARIATE ANALYSIS (NAFLD *VERSUS* NON NAFLD)

Parameter	*P*	*Adj OR (95% CI)*
*Family history of NAFLD	0.004	18.81 (2.55, 138.94)
Alanine aminotransferase	0.014	1.08 (1.02, 1.15)
Total cholesterol	0.012	1.05 (1.01, 1.08)

**(Any parent)*; *NAFLD*: *Non-alcoholic fatty liver disease; HDL-C: High Density Lipoprotein Cholesterol.*

WHAT IS ALREADY KNOWN?

- Familial/genetic factors are known risk factors for development of nonalcoholic fatty liver disease (NAFLD).

WHAT THIS STUDY ADDS?

- Presence of family history of NAFLD and abnormal laboratory profile (high alanine aminotransferase and cholesterol levels) in an overweight/obese child can predict presence of NAFLD.
- Homozygosity for PNPLA3 polymorphism may not have an independent effect on NAFLD causation.

DISCUSSION

In this study, we found that family history of NAFLD (in any parent), higher ALT levels and higher total cholesterol levels in children may independently predict presence of NAFLD. Homozygosity for *PNPLA*3 polymorphism did not have an independent effect on NAFLD causation. Also, CAP measurement by TE, had high sensitivity and specificity to predict steatosis in children.

This study is limited by small sample size as well as with lack of biopsy proven NAFLD in majority of the patients, since we had based diagnosis of NAFLD on ultrasonography only. This was based on the universal acceptance of USG as the first line screening modality for pediatric NAFLD including parental preference, considering its non-invasive nature.

There is limited literature stressing the significance of familial clustering of NAFLD [6-10]. This study revealed high familial incidence of metabolic diseases. Similar results were found in another study [9], where fatty liver was present in 17% of siblings and 37% of parents of non-NAFLD group against siblings (59%) and parents (78%) of NAFLD (biopsy-proven) group [9]. Thus, children with family history of NAFLD may be considered at higher risk for NAFLD. Presence of insulin resistance and diabetes mellitus in first degree relatives as a predictor of NAFLD was seen in another familial aggregation analysis [8]. Thus, overweight/obese children with parental history of NAFLD may constitute a high-risk group for early targeted interventions to prevent future development of NAFLD.

rs738409 SNP in gene *PNPLA*3 is associated with hepatic steatosis in adult and pediatric populations [11-14]. In the present study, independent effect of homozygosity for PNPLA3 polymorphism on NAFLD causation could not be confirmed on logistic regression analysis. This may be due to the fact that NAFLD is a multifactorial disease, where environmental (dietary habits, physical activity), genetic (*PNPLA*3 polymorphism) and metabolic risk factors play a role in tandem to affect its causation. Thus, a single risk factor like *PNPLA*3 polymorphism alone, may not affect the NAFLD causation in an individual child.

Higher ALT levels and higher total cholesterol levels also independently predicted pediatric NAFLD in the present study. In the present study, each 10 unit increase in ALT (in IU/L) and each 20 unit increase in total cholesterol (in mg/dL) increased the risk of pediatric NAFLD by approximately 1.5 times and 2 times, respectively. In previous studies, serum ALT had been used as a screening tool for NAFLD in children [17-20]. In the present study, 28.9 % children with NAFLD had normal ALT values. We had used the adult cut off values (≥40 IU/L) for defining normal ALT values to allow comparison with available literature. If we use the proposed normal 'pediatric' ALT values (*i.e* 25.8 U/L in boys and 22.1 U/L in girls), frequency of abnormal ALT increased from 71% to 88.4% (61 out of 69 children) [21]. Thus, though ALT independently predicted NAFLD, this limitation highlights the importance of not depending upon ALT alone to diagnose NAFLD since we may miss upto 12-29 % children. Similarly, Schwimmer, *et al*. [22] had also found that children with NAFLD had significantly higher serum total cholesterol, fasting glucose, insulin, low density lipoprotein (LDL), and triglycerides (TG) levels and significantly lower HDL than those without NAFLD. Huang, *et al*. [23] had also shown that higher body mass index (BMI) and ALT levels were significant independent predictors of pediatric NAFLD.

Though the present study had limited histological data, it still suggested that NAFLD in children may, like adult counterparts, also progress to advanced hepatic fibrosis stages. This implies that it is imperative to carefully follow pediatric NAFLD patients for disease progression and that benign prognosis should not be automatically ascribed to them.

The present study also found measurement of CAP, by TE, as a useful parameter to predict steatosis. TE is a technique where shear wave velocity is correlated with

the stiffness or elasticity of the underlying liver. One of its parameter, CAP, has been recently validated as a non-invasive tool that can detect and quantify steatosis in adults [24], though pediatric literature is still limited [25,26]. If CAP can be further validated in prospective pediatric studies, it may prove to be an ideal non-invasive and painless alternative to liver biopsy to predict steatosis and prognosticate NAFLD cases.

We conclude that there is high familial incidence of metabolic diseases in the NAFLD population. Presence of family history of NAFLD (in any parent), and abnormal laboratory profile (higher ALT and higher total cholesterol levels) in an overweight/obese child can predict presence of NAFLD. Homozygosity for *PNPLA*3 polymorphism in children could have a potential to be an independent predictor of pediatric NAFLD, but could not be proven in the present study. CAP can be useful as a non invasive modality to screen fatty liver in children. Large multicenter biopsy-proven studies among pediatric NAFLD can strengthen the evidence.

Contributors: VS, RK, DS: contributed in compiling clinical and laboratory information and writing the initial draft; SS: supervised the genetic testing; SA, SKS: conceptualised the study, and supervised the editing and revision; VS: finally drafted the article. All authors are in agreement with the content of the manuscript.

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REFERENCES

1. Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: A global perspective. Semin Liver Dis. 2008;28:339-50.
2. Day CP. Non-alcoholic fatty liver disease: a massive problem. Clin Med. 2011;11:176-8.
3. Matthiessen J, Velsing Groth M, Fagt S, Biltoft-Jensen A, Stockmarr A, Andersen JS, *et al*. Prevalence and trends in overweight and obesity among children and adolescents in Denmark. Scand J Public Health. 2008;36:153-60.
4. Ji CY, Cooperative Study on Childhood Obesity: Working Group on Obesity in China (WGOC): The prevalence of childhood overweight/obesity and the epidemic changes in 1985-2000 for Chinese school-age children and adolescents. Obes Rev. 2008;9:78-81.
5. Feldstein AE, Charatcharoenwitthaya P, Treeprasertsuk S, Benson JT, Enders FB, Angulo P. The natural history of non-alcoholic fatty liver disease in children: a follow-up study for up to 20 years. Gut. 2009;58:1538-44.
6. Struben VM, Hespenheide EE, Caldwell SH. Nonalcoholic steatohepatitis and cryptogenic cirrhosis within kindreds. Am J Med. 2000;108:9-13.
7. Willner IR, Waters B, Patil SR, Reuben A, Morelli J, Riely CA. Ninety patients with non- alcoholic steatohepatitis: insulin resistance, familial tendency, and severity of disease. Am J Gastroenterol. 2001;96:2957-61.
8. Abdelmalek MF, Liu C, Shuster J, Nelson DR, Asal NR. Familial aggregation of insulin resistance in first-degree relatives of patients with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol. 2006;4:1162-9.
9. Schwimmer JB, Celedon MA, Lavine JE, Salem R, Campbell N, Schork NJ, *et al*. Heritability of non-alcoholic fatty liver disease. Gastroenterology. 2009;136:1585-92.
10. Loomba R, Abraham M, Unalp A, Wilson L, Lavine J, Doo E, *et al*. and the Nonalcoholic Steatohepatitis Clinical Research Network. Association between diabetes, family history of diabetes, and risk of nonalcoholic steatohepatitis and fibrosis. Hepatology. 2012; 56:943-51.
11. Krawczyk M, Portincasa P, Lammert F. PNPLA3-Associated Steatohepatitis: Toward a Gene-Based Classification of Fatty Liver Disease. Semin Liver Dis. 2013;33:369-79.
12. Bhatt SP, Nigam P, Misra A, Guleria R, Pandey RM, Pasha MA, *et al*. Genetic variation in the patatin-like phospholipase domain-containing protein-3 (PNPLA-3) gene in Asian Indians with nonalcoholic fatty liver disease. Metab Syndr Relat Disord. 2013;11:329-35.
13. Valenti L, Alisi A, Galmozzi E, Bartuli A, Del Menico B, Alterio A, *et al*. I148M patatin-like phospholipase domain-containing 3 gene variant and severity of pediatric non-alcoholic fatty liver disease. Hepatology. 2010;4:1274-80.
14. Lin YC, Chang PF, Hu FC, Yang WS, Chang MH, Ni YH. A common variant in the PNPLA3 gene is a risk factor for non-alcoholic fatty liver disease in obese Taiwanese children. J Pediatr. 2011;158:740-4.
15. Misra A, Khurana L. The metabolic syndrome in South Asians: epidemiology, determinants, and prevention. Metab Syndr Relat Disord. 2009;7:497-514.
16. Field, Andy. Discovering Statistics Using SPSS, 3rd ed. London: SAGE Publications; 2009.
17. Fraser A, Longnecker MP, Lawlor DA. Prevalence of elevated alanine aminotransferase among US adolescents and associated factors: NHANES 1999-2004. Gastroenterology. 2007;133:1814-20.
18. Wiegand S, Keller KM, Röbl M, L'Allemand D, Reinehr T, Widhalm K, *et al*; APV-Study Group and the German Competence Network Adipositas. Obese boys at increased risk for nonalcoholic liver disease: evaluation of 16,390 overweight or obese children and adolescents. Int J Obes. 2010;34:1468-74.
19. Welsh JA, Karpen S, Vos MB. Increasing prevalence of nonalcoholic fatty liver disease among United States adolescents, 1988-1994 to 2007-2010. J Pediatr. 2013;162):496-500.
20. Yang HR, Yi DY, Choi HS. Comparison between pediatric health promotion center and pediatric obesity clinic in detecting metabolic syndrome and nonalcoholic fatty liver disease in children. J Korean Med Sci. 2014;29:1672-7.
21. Schwimmer JB, Dunn W, Norman GJ, Pardee PE, Middleton MS, Kerkar N, *et al*. SAFETY study: Alanine amino transferase cutoff values are set too high for reliable detection of pediatric chronic liver disease. Gastroenter-

ology 2010;138:1357-64.
22. Schwimmer JB, Zepeda A, Newton KP, Xanthakos SA, Behling C, Hallinan EK, *et al*; Nonalcoholic Steatohepatitis Clinical Research Network. Longitudinal assessment of high blood pressure in children with nonalcoholic fatty liver disease. PLoS One. 2014; 9;e112569.
23. Huang SC, Yang YJ. Serum retinol-binding protein 4 is independently associated with pediatric NAFLD and fasting triglyceride level. J Pediatr Gastroenterol Nutr. 2013; 56:145-50.
24. Chan WK, Nik Mustapha NR, Mahadeva S. Controlled attenuation parameter for the detection and quantification of hepatic steatosis in nonalcoholic fatty liver disease. J Gastroenterol Hepatol. 2014;29:1470-6.
25. Desai NK, Harney S, Raza R, Al-Ibraheemi A, Shillingford N, Mitchell PD, *et al*. Comparison of Controlled Attenuation Parameter and Liver Biopsy to Assess Hepatic Steatosis in Pediatric Patients. J Pediatr. 2016;173:160-4.
26. Cho Y, Tokuhara D, Morikawa H, Kuwae Y, Hayashi E, Hirose M, *et al*. Transient Elastography-Based Liver Profiles in a Hospital-Based Pediatric Population in Japan. PLoS One. 2015;10:e0137239.

WEB ANNEXURE 1

DETAILS OF SUBJECT EVALUATION

Evaluation	*Details*
Historical	• Clinical symptomatology (abdominal pain, satiety, vomiting, dyspepsia, jaundice, drug intake etc). • Family history of metabolic diseases
Physical	• Anthropometric parameters (all measured in triplicate and average taken) o Height and weight were measured using portable stadiometer and weighing scale to the nearest 1 mm and 100 grams, respectively. o Body mass index (BMI, as weight in kilograms/height in metres^2) percentile according to age and gender. o Waist Circumference (WC) measured in standing position with tape applied along a horizontal line just above the uppermost lateral border of the right ilium to nearest 1 mm. Hip circumference (HC) measured in centimeters (cms) at the largest diameter of the hips. Waist-Hip ratio was calculated as a ratio of WC and HC. o Overweight diagnosed if BMI between the 85th and <95th and obese if it ≥95th percentile for age and gender. For adults (≥18 years age), overweight and obese defined as BMI ≥23 and ≥25 kg/m^2 respectively. o Abdominal obesity- (*a*) WC ≥90 cm (males)/≥80 cm (females) for adolescents aged ≥16 yr or adults, (*b*) for children aged 10 - <16 years, WC ≥90th percentile or adult cut off if lower and (*c*) for children <10 years age, ≥90th percentile. WC percentiles based on published Indian data. o Blood pressure (BP) measured (in millimetres of mercury or mm Hg) in sitting position, with cuff at heart level using an aneroid sphygmomanometer with appropriate sized cuff (cuff bladder encircled at least 80% of the mid-upper arm) after 5 minutes of rest. § Systolic blood pressure (SBP) and/or diastolic blood pressure (DBP) <90th percentile were reported as normal. SBP and/or DBP between 90th and 95th percentile or BP ≥120/80 at any age was defined as prehypertension, Stage 1 HTN as >95th but <99th percentile plus 5 mmHg and stage 2 HTN as > 99th percentile + 5 mmHg. o Acanthosis nigricans defined as increased pigmentation of skin (darkening) and thickening (hyperkeratosis) of the skin, mostly in the nape of neck, the axilla and/or groin.
Laboratory	• After an overnight fast- aspartate aminotransferase and alanine aminotransferase (standard automated kinetic enzymatic assay), lipid profile (total cholesterol, high-density lipoprotein cholesterol or HDL, and triglycerides or TG, using enzymatic-calorimetric test), serum uric acid and fasting plasma glucose (glucose oxidase method) and insulin levels (immunoenzymometric assay). • Homeostasis model assessment 1- of IR (HOMA1-IR) calculated as fasting insulin (mIU/l) × fasting glucose (mg %)/405. HOMA1-IR >2.5 was taken as an evidence of Insulin Resistance (IR). The HOMA2-IR index was obtained by the program HOMA Calculator v2.2.2. The quantitative insulin sensitivity check index (QUICKI) calculated as 1/[log (Insulin ¼U/mL) + log (Glucose mg/dL)]. • Metabolic Syndrome (MS) defined as per standard criteria and divided according to the age. MS definition required presence of abdominal obesity (as per WC) plus ≥2 or more of other parameters (elevated TG, low HDL, high BP, and hyperglycemia). • In those patients fulfilling hyperglycemia definition (FBS ≥100 mg%), an oral glucose tolerance test (OGTT) (2-hour plasma glucose testing after 1.75 g/kg, maximum 75 g, glucose load dissolved in water) and fasting glycosylated hemoglobin (HbA1c) testing was done. Diabetes and pre-diabetes defined as per standard criteria. • Ultrasonography of abdomen was performed by trained operators using a scanner with a 3.5-MHz transducer. Standard grading system used. • Transient Elastography was done using Fibroscan apparatus (Echosens, Paris, France) by trained operators with experience of > 500 examinations. LSM and CAP were calculated based on principles and examination described previously. The LSM (as kilo pascals or K Pa) was considered reliable only if 10 successful acquisitions were obtained and success rate was >60% and, interquartile range was <30%. The CAP was measured on validated measurements as per LSM criteria with the final value as the median of

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Evaluation	*Details*
	individual ten CAP values (expressed as dB/m). As per manufacturer's guidelines, CAP measurements were done using the 'M' probe in the study subjects.
Liver biopsy	Percutaneous liver biopsy performed using the 18G trucut biopsy needle in those children in NAFLD group in whom there was persistent elevation of transaminases (ALT > 40 IU/L) despite atleast 3 months of diet control. NAFLD activity score (NAS) was used for evaluation activity in cases of NAFLD where a score ≥5 suggested NASH.

Details of *PNPLA3* Polymorphism Testing

For *PNPLA3* analysis, genomic DNA (gDNA) was extracted from peripheral blood by the phenol-chloroform method. The PNPLA3 rs738409 C>G SNP was identified following the polymerare chain reaction (PCR) using the forward primer 5'-TGGGCCTGAAGTCCGAGGGT-3' and reverse primer the 5'- CCGACACCAGTGCCCTGCAG-3'.