Gut Microbiota and Genetic Polymorphisms Appear to Drive Disease Expression of Nonalcoholic Fatty Liver Disease in Lean Individuals

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Background/Objectives: **There are very few comparative studies worldwide between 'lean' and 'nonlean/obese nonalcoholic fatty liver disease (NAFLD)' patients analyzing the differences in gut microbiome, genotype, and serum bile acids. Our aim was to compare the genotype, gut microbiome, bile acid profile, and metabolic patterns of lean NAFLD and obese NAFLD patients with special reference to hepatic fibrosis.** ***Methods:*** **Both lean and obese NAFLD patients diagnosed by ultrasonography along with matched controls were included. Genotyping, fecal microbiome analysis and estimation of serum total bile acid levels were done for patients as well as controls.** ***Results:*** **Biochemical and metabolic patterns of lean and obese NAFLD patients were comparable. Lean NAFLD patients had lower fasting plasma glucose (FPG) and homoeostasis model assessment-insulin resistance (HOMA-IR), although the proportions of patients having elevated HOMA-IR and metabolic syndrome (MS) were comparable. Noninvasive scores of liver fibrosis were also comparable. A greater proportion of lean NAFLD patients had the PNPLA3 rs738409 (G/G) genotype. However, there was no association of genetic polymorphisms with steatosis or fibrosis. Nonlean and lean NAFLD patients had comparable serum total bile acid levels. On microbiome analysis, lean NAFLD patients were found to have distinct expression of bacterial species while beta diversity was found to be significantly different across all groups.** ***Conclusion:*** **Lean NAFLD patients were found to have the PNPLA3 rs738409 (G/G) genotype. Lean NAFLD patients were also found to have unique gut microbial signatures, while beta diversity significantly differed across all groups. Differential expression of gut microbiota and genetic polymorphisms could underlie the pathogenesis of lean NAFLD. (J Clin Exp Hepatol 2025;15:102503)**

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Abbreviations: ALP: Alkaline Phosphatase; ALT: Alanine Aminotransferase; APRI: AST to Platelet Ratio Index; AST: Aspartate Aminotransferase; ATP III: Adult Treatment Panel III; AUROC: Area Under Receiver Operator Curve; BA: Bile Acid; BMI: Body Mass Index; CAP: Controlled Attenuation Parameter; CRN: Clinical Research Network; CVD: Cardiovascular Disease; EDTA: Ethylene Diamine Tetraacetic acid; FGF 19: Fibroblast Growth Factor 19; FIB-4: Fibrosis 4; GGT: Gamma Glutamyl Transpeptidase; HDL: High Density Lipoprotein; HOMA: Homoeostasis Model Assessment; IR: Insulin Resistance; LDL: Low Density Lipoprotein; LSM: Liver Stiffness Measurement; MS: Metabolic Syndrome; NAFLD: Nonalcoholic Fatty Liver Disease; NAS: NAFLD Activity Score; NASH: Nonalcoholic Steatohepatitis; NHANES: National Health and Nutrition Examination Survey; NPV: Negative Predictive Value; PCR: Polymerase Chain Reaction; PNPLA3: Patatin-like phospholipase domain-containing protein 3; PPV: Positive Predictive Value; PXR: Pregnane X Receptor; RFLP: Restriction Fragment Length Polymorphism; SD: Standard Deviation; SNP: Single Nucleotide Polymorphism; TBA: Total Bile Acid; TG: Triglyceride; TGR5: Takeda G protein-coupled receptor 5; TM6SF2: Transmembrane 6 Superfamily 2; VLDL: Very low-density lipoprotein; WHR: Waist-Hip ratio

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The growing epidemic of nonalcoholic fatty liver disease (NAFLD) has prompted investigators to examine various aspects of the entity in order to better delineate the pathophysiology and devise effective management strategies. The entity 'lean NAFLD' has emerged which has been reported to involve a younger and predominantly male population and has been associated with lower fasting glucose, glycated hemoglobin, and blood pressure values.[1] A meta-analysis has put the prevalence of lean NAFLD at 11.2% in the general population.[2] Although lean NAFLD patients seem to have a healthier metabolic profile at presentation, it has also been reported that such patients have a higher risk for mortality compared to overweight and obese patients.[3] Lean NAFLD has been considered a distinct pathophysiological entity based on the finding that a disproportionate number of such patients have nonalcoholic steatohepatitis (NASH).[4] Fecal microbiota profiles have been shown to display different patterns between obese and lean NAFLD patients.[5] Higher serum secondary bile acid (BA) and fibroblast growth factor-19 (FGF-19) levels and reduced 7-alpha-hydroxy-4-cholesten-3-one (C4) levels have also been reported to characterize 'lean NAFLD' patients.[6] Further, the association of PNPLA3 rs738409 with NAFLD has been observed to be more pronounced among lean individuals.[7]

However, the distinctiveness of 'lean NAFLD' can only be established by a comparative study between individuals with 'lean NAFLD' and 'obese NAFLD' with respect to insulin resistance, bile acid profile, gut microbiota, and genetic components. We therefore felt an urgent need to compare 'lean NAFLD' and 'obese NAFLD' patients based on these parameters, which would further our understanding of the pathophysiology of NAFLD.

METHODS

Study Setting and Patients

The study was conducted at a tertiary care center in Eastern India from January 2020 to December 2021. Consecutive outpatients ≥18 years of age who had normal general health and normal findings on clinical history, physical examination, blood count, and had ultrasonographic evidence of fatty liver with no other abnormal ultrasonographic findings were included in this study. Both controls as well as subjects with NAFLD were teetotalers and did not consume any amount of alcohol. Both lean and nonlean subjects without ultrasonographic evidence of fatty liver and without any abnormal findings on clinical history, physical examination, and blood count were included as controls. Controls were matched for age, sex, and geography. Details of inclusion and exclusion criteria are mentioned in **Supplementary Digital Content**. The cutoff BMI for considering individuals as 'lean' was taken as 23 kg/m^2.[8] The study protocol was approved by the institutional ethics committee (IEC Application No. 293/26.08.2020) and was conducted in accordance with the 1964 Helsinki declaration and its later amendments. All subjects provided written consent for participation in the study.

The procedural details of the noninvasive methods and liver biopsy are provided in **Supplementary Digital Content**. Procedure for stool sample collection and methods of gut microbiome analysis, genotyping, and bile acid analysis are provided in the **Supplementary Digital Content**.

Statistical Analysis

Sample size was calculated based on the estimated prevalence of significant fibrosis in lean NAFLD subjects (around 5%) compared with nonlean subjects (around 30%). In order to be 80% certain (i.e. $1-\beta = 0.8$) of detecting a prevalence ratio of RR = 0.30/0.05 = 3 using a 0.05 level of significance (i.e. $\alpha = 0.05$) with an equal number of recruited lean and nonlean NAFLD subjects, the study needed to enroll 36 lean and 36 nonlean NAFLD subjects.

Normality of the distribution of data was measured using the Kolmogorov-Smirnov test of normality. A chi-square test was performed for categorical variables expressed in number and percentage. Student's t-test, analysis of variance (one-way ANOVA) was applied for normally distributed continuous variable reported in mean ± SD. Non-normally distributed continuous data were analyzed with Mann–Whitney U test. Bivariate analysis was done using the Chi-squared test while multivariate analysis was done using binary logistic regression. A *P* value of less than 0.05 was considered significant. Receiver operating characteristic (ROC) curves were made and the area under the ROC (AUROC) was estimated for predictors. All analyses were done using IBM SPSS Statistics (SPSS version 25.0, SPSS Inc., Chicago, IL, USA). Microbiome data was analyzed using R statistical software.

RESULTS

One hundred fifty-seven NAFLD patients were evaluated, out of which 71 patients were enrolled in the study. 86 patients were excluded due to missing data and nonavailability of matched controls. Out of 71 NAFLD patients, 38 patients were obese and 33 were lean. Stool samples of 52 subjects: 16 from Lean NAFLD, 16 from obese NAFLD, 10 from each control group were analyzed. NAFLD patients were predominantly males (n = 48, 67.6%), the gender distribution across the two groups was: lean NAFLD (Male-72.7%, Female-27.27%) and Obese NAFLD (Male-63.15%, Female-36.84%). Mean age of presentation in males was 37.33 ± 8.39 years while in females it was 44.7 ± 10.56 years. The mean age of lean NAFLD patients was 39.76 ± 10.89 years compared to 39.69 ± 9.88 years in lean controls (p value = 0.9). Similarly, the mean

age of obese NAFLD patients was 39.87 ± 10.50 years compared to 39.34 ± 8.08 years in obese controls (p value = 0.8).

Anthropometric Characteristics of NAFLD Patients and Controls

The anthropometric characteristics of NAFLD patients and controls are summarized in Supplementary Tables 1 and 2. The anthropometric characteristics between lean NAFLD patients and lean controls were comparable. Obese NAFLD patients had significantly greater weight ($p < 0.01$) and systolic ($p < 0.01$) and diastolic blood pressures ($P < 0.05$) compared to obese controls.

Demographic Characteristics, Dietary Patterns, and Dietary Habits

The demographic characteristics, dietary patterns, and dietary habits of lean NAFLD patients compared to obese NAFLD patients are summarized in Supplementary Table 3. Lean NAFLD and obese NAFLD patients did not differ significantly with respect to demographic patterns and dietary habits.

Anthropometric Characteristics and Metabolic Parameters of Lean NAFLD Patients and Obese NAFLD Patients

The anthropometric characteristics and metabolic parameters of lean NAFLD patients as compared to those of obese NAFLD patients are presented in Table 1. As expected, lean NAFLD patients had lower waist circumference, lower waist hip ratio, and lower waist-height ratio compared to obese NAFLD patients. However, liver biochemistries were comparable between the two groups. Lean NAFLD patients had lower serum total cholesterol and low density lipoprotein-cholesterol compared to obese NAFLD patients but serum high density lipoprotein (HDL) cholesterol and serum triglyceride levels were comparable between the two groups. Further, the fasting plasma glucose and HOMA-IR index were higher in the obese NAFLD group compared to lean NAFLD group, although the proportion of patients having metabolic syndrome (MS) was comparable across both the groups (see Table 2).

Noninvasive Markers of Liver Fibrosis

The mean APRI, FIB-4, and transient elastography scores were comparable between lean NAFLD and obese NAFLD groups (APRI - 0.458 ± 0.25 vs 0.497 ± 0.30, $P > 0.05$, FIB-4 - 1.11 ± 0.61 vs 1.21 ± 0.89, $P > 0.05$, TE - 6.64 ± 2.15 kPa vs 7.42 ± 3.81 kPa, $P > 0.05$).

Liver Histopathology

Histological features of patients in both groups are shown in Table 3. Altogether, only 24 patients out of 71 lean and obese NAFLD patients underwent liver biopsy–12 patients in each group. The proportion of patients having significant fibrosis and NASH was comparable between both the groups. However, the mean NAS in the lean NAFLD group was significantly lower compared to that in the obese NAFLD group. Presence of NASH and hypertension were found to be the only predictors of significant fibrosis on univariate analysis but not on multivariate analysis (Supplementary Table 4).

Genetic Polymorphisms

The distribution of PNPLA3 rs738409 genotypes and TM6SF2 rs58542926 genotypes in NAFLD patients were analyzed**.** 12/33 lean NAFLD patients had the GG genotype compared to 5/38 obese NAFLD patients ($P < 0.05$) (Table 4). However, PNPLA3 GG and TM6SF2 CT genotypes did not have any significant association with hepatic steatosis or fibrosis (Supplementary Table 5). Only age was significantly associated with the presence of hepatic steatosis (P value < 0.05). After adjustment, there was no significant association of age, BMI, sex or the presence of PNPLA3 GG and TM6SF2 CT genotypes with hepatic steatosis and fibrosis. There was no significant association of the SNP rs738409 in PNPLA3 (G allele) with either steatosis or fibrosis on univariate and multivariate analysis. There was no significant association of the SNP rs738409 in PNPLA3 (G allele) and SNP rs58542926 in TM6SF2 (T allele) with F2 fibrosis on univariate and multivariate analysis (Supplementary Table 6).

Serum Bile Acid Levels

The serum total bile acid levels did not significantly differ between the lean NAFLD group and the lean control group. However, the serum total bile acid level of the obese NAFLD group was significantly higher compared to the obese control group (mean rank, 13.30 vs 7.70 of control subjects, $U = 22.0$, $P < 0.05$). The serum total bile acid level of the lean NAFLD group was comparable to the obese NAFLD group (mean rank, 8.20 vs 12.80, $U = 27.0$, $P > 0.05$), while that of patients with significant fibrosis was significantly higher compared to those without significant fibrosis (mean rank, 12.71 vs 7.1, $U = 21.5$, $P < 0.05$). The differences in serum bile acid levels among the different groups are summarized in Supplementary Tables 7 and 8.

Bioinformatic Analysis of Microbiome Data

The relative abundance of bacterial taxa at the phylum level in four groups is shown in compositional plots (Figure 1A). We also determined the microbiome composition at a lower taxonomic level–such as the top 20 highly abundant species (Figure 1B, Supplementary Figure 1). *Prevotella copri* was the predominant species across all 4 groups (Supplementary Figure 1). There was a significant

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Table 1 Comparison of Anthropometric Characteristics and Metabolic Patterns Between Lean NAFLD and Obese NAFLD Patients.

Anthropometric parameters	Lean NAFLD (n = 33)	Obese NAFLD (n = 38)	*P* value
Weight (kg) Mean ± SD	60.7 ± 6.68	77.39 ± 9.52	<0.005
Height (cm) Mean ± SD	166.12 ± 7.56	164.44 ± 6.56	0.322
BMI (kg/m^2) Mean ± SD	21.90 ± 1.36	28.67 ± 3.42	<0.005
Waist circumference (cm) Mean ± SD	81.06 ± 4.51	88.77 ± 5.91	<0.005
Hip circumference (cm) Mean ± SD	91.48 ± 5.65	93.84 ± 5.44	0.078
Waist hip ratio Mean ± SD	0.88 ± 0.03	0.94 ± 0.05	<0.005
Waist-height ratio (Mean ± SD)	0.48 ± 0.03	0.54 ± 0.04	<0.005
SBP (mm Hg)	121.88 ± 13.55	123 ± 11.11	0.703
DBP (mm Hg)	77.09 ± 6.71	77.00 ± 5.61	0.951
Serum total bilirubin (mg/dL) Mean ± SD	0.65 ± 0.32	0.63 ± 0.34	0.779
AST (IU/L) Mean ± SD	40.50 ± 18.76	43.66 ± 23.50	0.542
ALT (IU/L) Mean ± SD	42.94 ± 25.28	51.24 ± 30.98	0.225
ALP (IU/L) Mean ± SD	179.97 ± 67.30	200.45 ± 61.29	0.184
GGT (IU/L) Mean ± SD	32.88 ± 10.38	32.68 ± 11.02	0.939
Serum albumin (gm/dL) Mean ± SD	4.16 ± 0.3	4.11 ± 0.28	0.474
Serum cholesterol (mg/dL) Mean ± SD	163.88 ± 53.98	189.55 ± 37.29	0.021
Serum triglyceride (mg/dL) Mean ± SD	173.33 ± 117.69	218.92 ± 93.54	0.074
Serum HDL cholesterol (mg/dL) Mean ± SD	44.17 ± 9.44	43.68 ± 6.25	0.797
Serum LDL cholesterol (mg/dL) Mean ± SD	101.82 ± 42	118.89 ± 20.56	0.030
Serum VLDL cholesterol (mg/dL) Mean ± SD	33.55 ± 10.12	34.79 ± 9.73	0.600
Fasting plasma glucose (mg/dL) (Mean ± SD)	94.52 ± 12.75	105.32 ± 29.23	<0.05
Postprandial plasma glucose (mg/dL) (Mean ± SD)	131.36 ± 32.15	145.61 ± 50.6	0.169

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Table 1 (*Continued*)

Anthropometric parameters	Lean NAFLD (n = 33)	Obese NAFLD (n = 38)	*P* value
Fasting serum insulin (U/L) (Mean ± SD)	10.45 ± 7.96	14.65 ± 12.79	0.107
HOMA-IR (Mean ± SD)	2.39 ± 2.10	4.09 ± 4.34	0.044
HOMA-IR > 2 [n]	13	24	0.05
Metabolic syndrome (MS) [n]	6	13	0.3

NAFLD, nonalcoholic fatty liver disease; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transpeptidase; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HOMA-IR, homoeostasis model assessment; IR, insulin resistance.

Table 2 Comparison of Histological Characteristics Between Lean NAFLD and Obese NAFLD Patients.

Histological feature	Lean NAFLD (n = 12)	Obese NAFLD (n = 12)	*P* value
NASH (n)	5	9	0.24
Definite NASH (n)	1	4	0.36
NAS (mean ± SD)	1.58 ± 1.83	3.50 ± 1.93	<0.05
Significant fibrosis (n)	2	3	0.86

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NAS, NAFLD activity score.

Table 3 Distribution of PNPLA3 and TM6SF2 SNPs in NAFLD Patients.

Genotype	Lean NAFLD (n = 33)	Obese NAFLD (n = 38)	*P* value
SNP PNPLA3 rs738409			
G allele (n)	21	18	0.23
G/G (n)	12	5	0.02
SNP TM6SF2 rs58542926			
T Allele (n)	4	11	0.14
C/T (n)	1	7	0.06

SNP, single nucleotide polymorphism; NAFLD, nonalcoholic fatty liver disease.

difference between obese control and obese NAFLD groups in terms of their F/B ratio (Supplementary Figure 2A). No significant difference was observed for the lean control vs lean NAFLD, lean NAFLD vs obese NAFLD and lean control vs obese control groups.

Relative abundance in percentage was also calculated for the top 10 most abundant species in the NAFLD group, based on genotype (PNPLA3 and TM6SF2), for both the lean and obese NAFLD groups (Supplementary Figure 3 A, B, C, and D).

Table 4 Lean and Obese NAFLD Dominant Species in the Community.

Bacterial species in lean NAFLD	Degree centrality	Community
Shigella flexneri	0.4	4
Klebsiella pneumoniae	0.4	0
Streptococcus alactolyticus	0.39	2
Melissococcus plutonius	0.39	5
Collinsella aerofaciens	0.38	3
Streptococcus agalactiae	0.38	6
Bacterial species in obese NAFLD		
Lactobacillus delbrueckii	0.44	4
Bulleidia moorei	0.43	6
Lactobacillus helveticus	0.41	0
Eubacterium dolichum	0.41	1
Leuconostoc mesenteroides	0.40	5
Weissella cibaria	0.38	3

NAFLD, nonalcoholic fatty liver disease.

Comparison Among Lean NAFLD, Lean Control, Obese NAFLD, and Obese Control Groups

Differences in alpha diversity between the groups are shown in Supplementary Figure 4. No significant difference in alpha diversity was seen between lean control and lean NAFLD groups, between obese control and obese NAFLD groups, and between lean and obese NAFLD groups. Beta diversity was significantly different between lean control and lean NAFLD groups ($P < 0.05$) and between lean and obese control groups ($P < 0.05$) (Figure 2A, Figure 2B). However, when comparing all four groups together, we observed significant difference in beta diversity ($P = 0.023$) (Figure 2C). We observed a significant difference ($P = 0.03$) in beta diversity when plotting the genotype (TM6SF2) in lean NAFLD and obese NAFLD groups (Figure 2D).

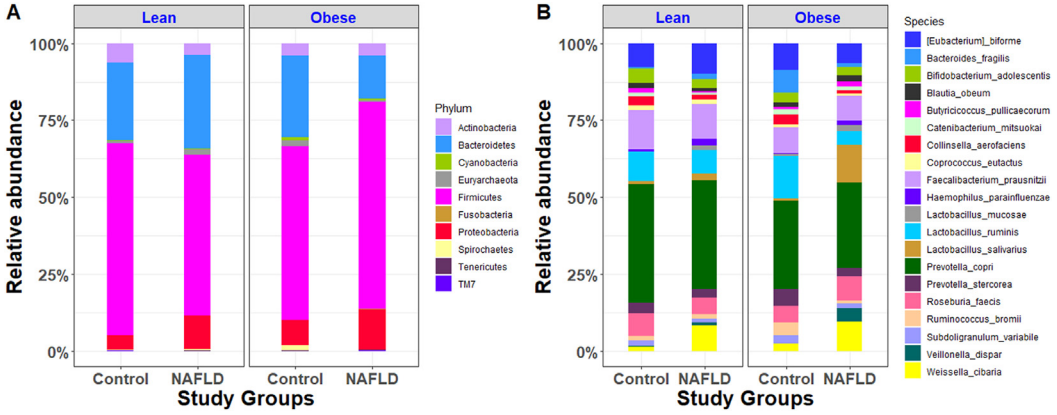

Figure 1 Microbial composition of OTUs at A) phylum level B) species level.

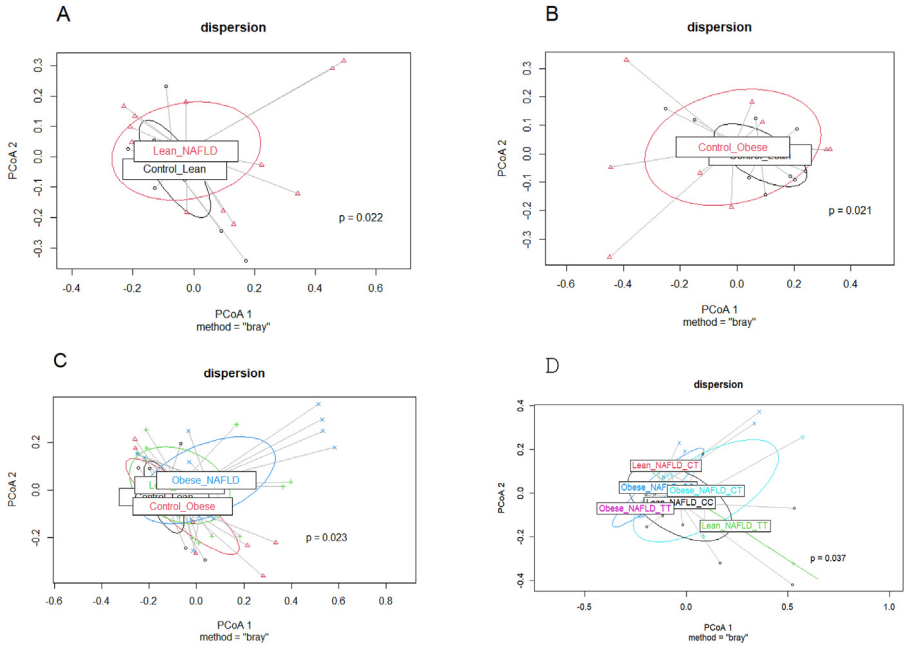

Figure 2 Beta diversity between groups – A) Lean control and lean NAFLD B) lean control and obese control C) all four groups D) genotype based (TM6SF2) beta diversity in lean NAFLD and obese NAFLD. NAFLD, nonalcoholic fatty liver disease.

There was a significant difference in alpha diversity between subjects with and without fibrosis, as revealed by Shannon, Simpson's indices and Pielou's evenness (Figure 3). There was also a significant difference in beta diversity ($P < 0.05$) between NAFLD subjects with and without fibrosis (Figure 4A). Influential species responsible for the difference in beta diversity are shown (Figure 4B).

Indicator Species Analysis

Streptococcus anginosus and Rothia mucilaginosa were found to be indicators of lean NAFLD whereas *Veillonella dispar* was found to be an indicator of obese NAFLD group (Supplementary Figure 5). In PNPLA3 genotype (CC) from lean NAFLD, *Lactobacillus salivarius* and *B. fragilis* were found as indicators (Supplementary Figure 6A), while in the TM6SF2 genotype CC + CT, *Fecalibacterium prausnitzii* was found as an indicator species (Supplementary Figure 6B). In PNPLA3 genotype (GG) from obese NAFLD, *Roseburia faecis* (Supplementary Figure 6C) and in TM6SF2 genotype TT, *C. bartlettii, D. longicatena, S. variabile*, *Collinsella aerofaciens* were found as indicator species (Supplementary Figure 6D). *P. distantosis, L. salivarius, B. fragilis* were observed as indicators in the PNPLA3 genotype CC group from lean NAFLD, whereas in TM6SF2 genotype TT + CT group from lean NAFLD (Supplementary Figure 6E), *Rothia lactaris, Rothia bromii, F. prausnitzii, Butyricicoccus pullicaecorum, Bulleidia p 1630 c5* were the indicators (Supplementary Figure 6F).

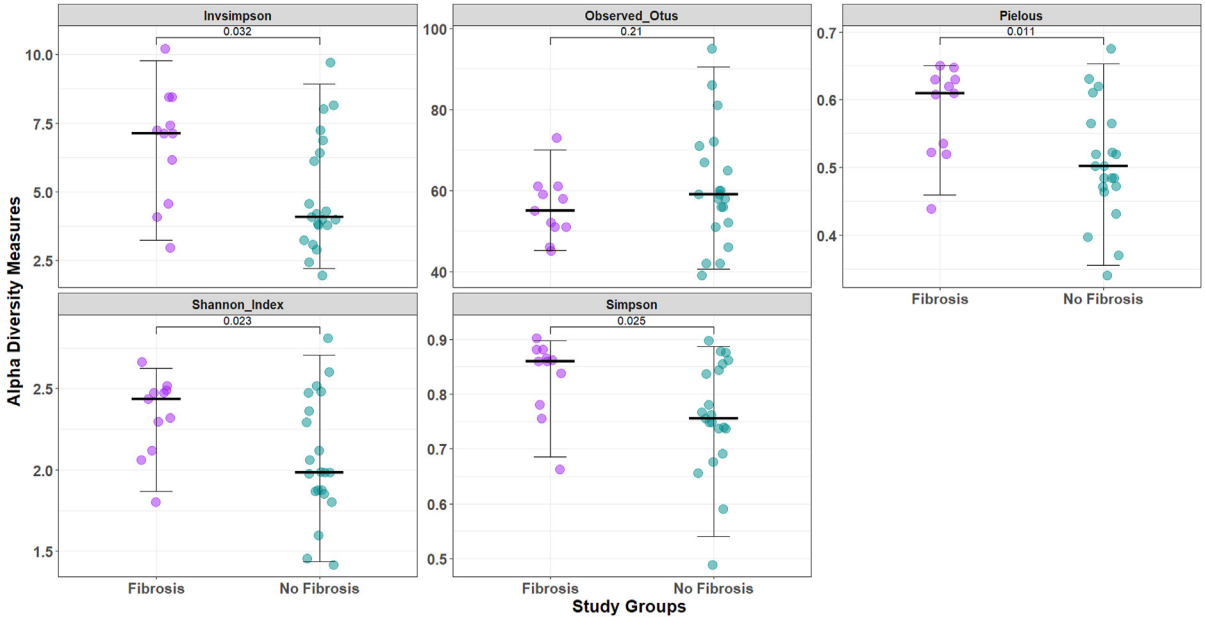

Figure 3 Alpha diversity indices between NAFLD subjects with and without fibrosis. NAFLD, nonalcoholic fatty liver disease.

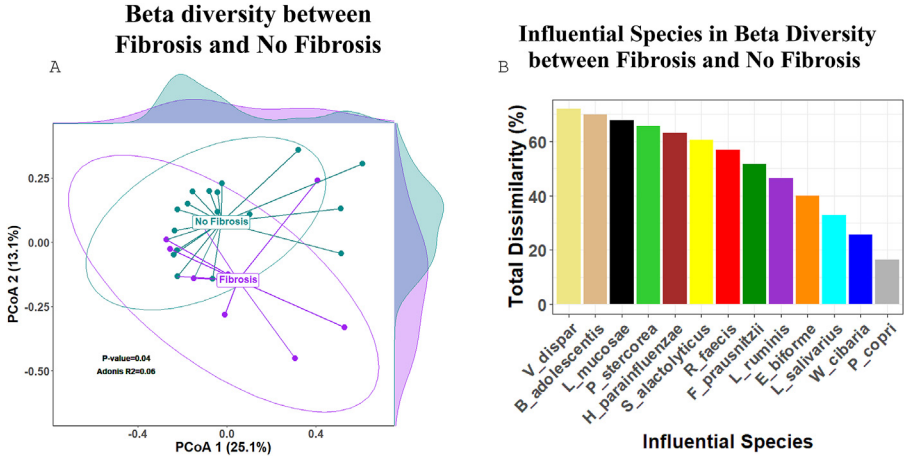

Figure 4 Beta diversity indices between NAFLD subjects with and without fibrosis. NAFLD, nonalcoholic fatty liver disease.

Differential Abundance Analysis

Obese NAFLD group showed significantly higher abundance ($P < 0.05$) of *H. parainfluenzae*, *Rothia mucilaginosa*, *Veillonella dispar, Veillonella parvula*, *Weissella cibaria*, and *Weissella hellenica* compared to their control counterparts (Supplementary Figure 7A). In the lean NAFLD group, *Lactobacillus mucosae*, *R. mucilaginosa, S. anginosus, Veillonella dispar, V. parvula* were significantly different compared to the controls (Supplementary Figure 7B). Further, *Butyricicoccus pullicaecorum* and *Clostridium celatum* were found to be higher in controls. By comparing only NAFLD groups, we found higher abundance of *Coprococcus eutactus* in the lean group, while *Streptococcus luteciae* was higher in the obese group. We also compared the differential abundance of species between lean and obese NAFLD groups based on genotype (Results are provided in Supplementary Figure 8 A, B, C, D).

Regression Analysis

Regression analysis was performed to establish a relationship between clinical parameters, including liver stiffness on transient elastography at a cut-off value for fibrosis 7.5 (independent variables) and microbiome (dependent variables) (Supplementary Figures 9 and 10). There was a significant difference in gut microbiome between lean and obese NAFLD subjects with respect to serum triglyceride levels while between NAFLD subjects with and without fibrosis, there was a significant difference in gut microbiome with respect to serum alanine aminotransferase (ALT) levels (Figure 5). We also performed Spearman

correlation between the influential species and clinical parameters in the various groups–lean NAFLD, obese NAFLD, subjects with and without fibrosis [Results are provided in Supplementary Figure 11(A,B), 12(A,B), 13(A,B), and 14(A,B)].

Species Network

Species networks using clinical parameters and the microbiome of lean and obese NAFLD patients were constructed [Supplementary Figure 15(A,B), 16(A,B), 17(A,B) and 18(A,B)]. Based on this, we plotted the difference of microbial species between the groups according to degree centrality (Supplementary Figures 19,20,21,22). **We observed that between lean and obese NAFLD patients, based on degree centrality**, *Shigella flexneri, Klebsiella pneumoniae, Streptococcus alactolyticus, Melissococcus plutonius, C. aerofaciens, and Streptococcus agalactiae* were the dominating species in the community in lean NAFLD patients (Table 4). We also observed that between NAFLD subjects with and without fibrosis, based on degree centrality, *Bifidobacterium breve, Bifidobacterium bifidum, S. agalactiae, Blautia obeum,* and *Streptococcus equi* were the differentiating species.

DISCUSSION

Previous studies have considered lean NAFLD as an entirely distinct entity.[6] However, our observations indicate that this assumption may not be correct. We observed that lean NAFLD patients and lean controls had comparable BMI and WC levels which contradict the results of previous studies.[9,10] Further, contrary to the observations made by other workers that lean NAFLD patients have significantly lower ALT levels compared to their nonlean counterparts,[6,10] we also did not observe any differences in serum ALT levels between lean and obese NAFLD patients. In addition, we did not find any differences in serum HDL cholesterol and triglycerides between lean and obese NAFLD patients. Importantly, these two parameters are important components of the metabolic syndrome as per NCEP ATP III criteria. We did observe, though, that lean NAFLD patients had lower levels of FPG and HOMA-IR score compared to obese NAFLD patients. However, the proportions of patients having elevated HOMA-IR and MS were comparable across both groups. The reasons for these apparent discrepancies between our study and the results of previous studies could be several. NAFLD, itself is a heterogenous entity and the pathophysiological details are yet to be completely explored across diverse populations. Given the multiple players involved in NAFLD pathogenesis, it is likely that there would be variations in the anthropometric, metabolic, and biochemical parameters across populations. Most of the data we have with us are from the West, while in India, the studies discussed earlier were conducted in North India.[6,9,10] Therefore, nationwide studies involving different population groups and subgroups are still required to fill up this knowledge gap.

Assessment of liver fibrosis by noninvasive methods did not show any significant differences between the two groups. There have been several studies evaluating the utility of these noninvasive tests to determine significant fibrosis.[11–13] However, none of these studies have mentioned the proportion of lean and obese NAFLD patients in their cohorts. It is obvious that there is a paucity of data regarding the accuracy of noninvasive scores to assess liver fibrosis in lean NAFLD patients. On histopathology, too, the proportion of patients having NASH and significant fibrosis were comparable across both the groups, although lean NAFLD patients had significantly lower mean NAS scores compared to their nonlean counterparts. These findings are in conformity with a similar study comparing liver histopathology between the two groups.[9] In our study, after adjusting for other variables, in multivariate analysis, neither the presence of NASH nor hypertension were found to significantly predict hepatic fibrosis. This indicates that it is important to determine predictors of hepatic fibrosis specifically in lean NAFLD patients by conducting studies on larger populations.

Hepatic steatosis, hepatocellular damage, and fibrosis in lean and nondiabetic adults suggest that alternative mechanisms of NAFLD exist that need to be uncovered. We examined the genetic profiles of these patients for single

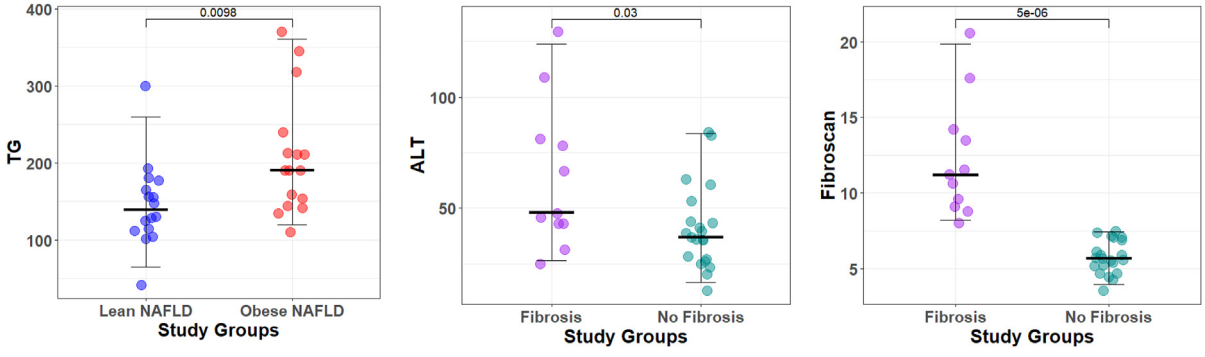

Figure 5 Comparison of clinical parameters between study groups.

nucleotide polymorphisms (SNP) for the two most implicated genes in NASH pathogenesis, the (PNPLA3) gene and the TM6SF2 gene. We observed that a significantly higher portion (12/33, $P < 0.05$) of the lean NAFLD patients had the pathogenic I148M variant compared to obese NAFLD patients (5/38). This corroborated several previous reports where the PNPLA3 I148M pathogenic variant was associated with NAFLD, steatohepatitis, and fibrosis severity but not metabolic syndrome. However, the exact mechanism of the PNPLa3 I148M variant in developing NAFLD and liver fibrosis is unclear. We also observed that a greater proportion of lean NAFLD patients were associated with PNPLA3 rs738409 (GG) genotype. However, there was no association between PNPLA3 and TM6SF2 polymorphisms and steatosis or fibrosis. Among Indian subjects, PNPLA3 rs738409 and TM6SF2 rs58542926 gene polymorphisms have been observed to increase the risk of NAFLD.[14,15] However, the patients included in these studies were obese NAFLD patients, while the controls had significantly lower BMI levels compared to the patients.

Recently, a study by Pirola and colleagues studied the association of liver bacterial taxa and various genetic variants such as PNPLA3, TM6SF2, MBOAT7, and HSD17B13 with NAFLD histological severity and found specific genera associated with particular variants.[16] This data suggested that host genetics influenced the liver microbial DNA composition and might have a role in the severity of NASH. Leinwand et al showed that gut microbes populate the liver microbiota and affect the liver's local inflammatory microenvironment.[17] Extrapolating this, we hypothesized that gut microbiota interacts with the host genetics to produce distinct outcomes in NAFLD. Firstly, we observed that the lean NAFLD patients had distinct bacterial species such as *S. flexneri, K. pneumoniae, S. alactolyticus, M. plutonius, C. aerofaciens, and S. agalactiae* as the dominant species in the community.

Interestingly, lean NAFLD patients had pathogenic bacteria dominating the gut microbiome compared to obese NAFLD patients. The dominant species in lean NAFLD were also distinct from the lean normal counterparts. The lean NAFLD patients had distinct gut microbial diversity and abundance. Secondly, in all patients with the pathogenic I148M variant, we observed the relative abundance of *P. copri, Faecalibacterium prausnitzii, L. salivarius, R. faecis, Eubacterium biforme, Lactobacillus ruminis, Bifidobacterium adolescentis, and Prevotella stercorea* were significantly different from those of obese NAFLD patients carrying the same variant and also significantly different from the lean normal counterparts. These data support our hypothesis and indicate that the gut microbiome composition can exacerbate the NAFLD pathogenesis in individuals with the PNPLA3 I148M variant, even without central obesity, diabetes, and other symptoms of the metabolic syndrome. This data is crucial in understanding the missing link in the pathogenesis of the genetic polymorphisms in NAFLD and the progression to steatohepatitis.

We observed significant differences in beta diversity between lean NAFLD and lean control groups, between lean control and obese control groups, and across all four groups. There was significant alpha and beta diversity between subjects with and without fibrosis. Although another recent study has shown no differences in diversity between subgroups of NAFLD.,[18] our findings lend credence to the hypothesis that there might be differential gut microbiota expression between lean and obese NAFLD patients.

Our observations regarding serum bile acids are different from what has been reported so far. We found that obese NAFLD patients and lean NAFLD patients had comparable serum total bile acid levels. It has been reported earlier that lean NAFLD patients have higher secondary bile acid levels compared to obese NAFLD patients.[6] Although there is some evidence that NAFLD is associated with perturbations in serum bile acid levels, the studies conducted so far have thrown up conflicting results. It is imperative therefore, to conduct more studies and characterize differences, if any, in serum primary and secondary bile acid levels between lean and obese NAFLD patients.

The strength of our study includes the analysis of multiple pathophysiological aspects of NAFLD, which has not been conducted earlier. Our study is limited by the lesser number of liver biopsies performed. It is possible that a larger sample size would have provided adequate power to the study and strengthened the validity of the results. The smaller sample size could have underpowered the study and hence larger, multicentric studies exploring these differences need to be carried out to confirm or refute our findings. We did not examine the biochemical parameters of lean controls and compare them to lean NAFLD patients. We did not compare the metabolic, gut microbiota and genetic parameters between subjects with NASH and those without NASH, considering the lesser number of biopsies proven NASH subjects. We did not analyze the profile of various bile acids in the NAFLD subjects and controls, and we could assess only the total bile acids. Analysis of primary and secondary bile acids in lean and obese NAFLD patients would have yielded more information. The cross-sectional nature of the study limits it from evaluating patient outcomes and treatment implications, a follow-up of this cohort is required to assess these parameters in the correct clinical context.

To conclude, lean NAFLD patients don't seem to be an entirely distinct pathophysiological entity as regards metabolic patterns, liver biochemistry, serum bile acid levels, and liver histology are concerned. However, a significantly higher proportion of the lean NAFLD patients had the pathogenic I148M variant compared to obese NAFLD patients while lean NAFLD patients seem to have a unique gut microbiome signature. Both gut microbiota expression and genetic polymorphisms could play significant roles in

driving disease pathogenesis of NAFLD in lean individuals. These aspects need to be closely looked at in larger longitudinal studies. The findings of our study throw new light on some of the pathophysiological aspects of NAFLD and may help unravel its complex pathogenesis. The development of steatotic liver disease in lean individuals needs further exploration while the findings of a unique gut microbiome profile of such individuals and their potential modulation might prove to be useful in ameliorating hepatic steatosis. As regards therapeutics of NAFLD is concerned; the findings of our study have the potential to contribute to better management, especially in lean NAFLD population.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Informed consent for each patient was taken through an informed consent form in a language understandable by the patient and signed by the patient himself/herself. The study was approved by the Institutional Ethics Committee of SCB Medical College, Cuttack, Odisha, India – 753007 (IEC Application No-293/26.08.2020) and was conducted in accordance with the 1964 Helsinki declaration and its later amendments.

AVAILABILITY OF DATA AND MATERIALS

Datasets created during and/or analyzed during the current study are available from the corresponding author on reasonable request.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Specific author contributions: Prajna Anirvan – Methodology, Data Curation, Investigation, Writing- Original draft preparation, Formal analysis, Validation, **Zaiba Hasan Khan** – Methodology, Formal analysis, Software, Writing- Original draft preparation, **Pallavi Bhuyan** – Methodology, Validation, Investigation, Resources, Formal analysis, **Sujata Dixit** – Investigation, Resources, Formal analysis, **Rishikesh Dash** – Formal analysis, Software, **Priyanka Mishra** – Data curation, Resources, Investigation, Software, **Giriprasad Venugopal** – Data curation, Resources, Investigation, **Gowri Manohari Balachander** – Methodology, Software, Investigation, **Pankaj Bharali** – Data curation, Resources, **Mrinal Gogoi** – Data curation, Resources, **Manas Kumar Panigrahi** – Methodology, Supervision, **Manoranjan Ranjit** – Formal analysis, Supervision, Resources, **Balamurugan Ramadass** – Methodology, Investigation, Formal analysis, Resources, Funding Acqusition, Writing-Reviewing & Editing, **Shivaram Prasad Singh** – Conceptualization, Methodology, Supervision, Visualization, Validation, Funding Acquisition, Resources, Writing-Reviewing & Editing, Project Administration.

All authors approved the final draft submitted.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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APPENDIX A

SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jceh.2025.102503.