Circulating bile acid profiles in Japanese patients with NASH

Sho-ichiro Yara1 | Tadashi Ikegami1 | Teruo Miyazaki2 | Masashi Murakami1 | Junichi Iwamoto1 | Takeshi Hirayama1 | Motoyuki Kohjima3 | Makoto Nakamuta3 | Akira Honda1,2

1Division of Gastroenterology and Hepatology, Department of Medicine, Tokyo Medical University Ibaraki Medical Center, Ibaraki, Japan
2Joint Research Center, Tokyo Medical University Ibaraki Medical Center, Ibaraki, Japan
3Department of Gastroenterology, National Hospital Organization Kyushu Medical Center, Fukuoka, Japan

Correspondence
Tadashi Ikegami, Division of Gastroenterology and Hepatology, Department of Medicine, Tokyo Medical University Ibaraki Medical Center, 3-20-1 Chuo, Ami-machi, Inashiki-gun, Ibaraki, 300-0395 Japan.
Email: ikegamit@tokyo-med.ac.jp

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Summary

Background: Nonalcoholic steatohepatitis (NASH) is an evolitional pattern of non-alcoholic fatty liver disease with inflammation and fibrosis. Although pharmacological options for treating NASH are limited, derivatives of bile acids and compounds that influence bile acid-related signalling pathways are emerging as potentially useful therapeutic agents.

Methods: To characterise bile acid profiles in relatively 'lean' Japanese patients, we analysed serum bile acid concentrations in patients with biopsy-proven NASH (n = 34) and healthy controls (n = 38) using liquid chromatography-tandem mass spectrometry.

Results: Mean total serum bile acid concentration in the NASH group was significantly higher compared with controls (P < .0001), and the higher level did not depend on the progression of hepatic fibrosis. Four characteristic bile acid-related features were observed in patients with NASH: significantly decreased ratio of cholic acid + deoxycholic acid / chenodeoxycholic acid + lithocholic acid (P < .05), taurine-conjugated bile acids to glycine-conjugated bile acids (P < .05), unconjugated bile acids to total bile acids (P < .05) and secondary bile acids to total bile acids (P < .05). Furthermore, significantly elevated farnesoid X receptor-affinity indices (P < .05) and marginally decreased serum 7α-hydroxy-4-cholesten-3-one concentration (P = .071) without changes in the bile acid hydrophobicity index suggested that farnesoid X receptor tended to be activated in patients with NASH.

Conclusion: These observations may help understand the pathogenesis of NASH regarding its association with altered bile acid metabolism.
INTRODUCTION

Estimation by recent epidemiologic studies revealed that 25%-30% of the general population is affected with nonalcoholic fatty liver disease (NAFLD). Majority of NAFLD patients remain simple hepatic steatosis, however, part of them progress to its chronic evolution patterns: nonalcoholic steatohepatitis (NASH) histologically characterised with inflammation and fibrosis. Currently, lifestyle intervention in diet and exercise habits as well as control of comorbidities such as type-2 diabetes mellitus and dyslipidaemia are limited strategy in managing NASH. Due to the lack of pharmacological options for treating NASH and increasing number of patients who need specific treatments, extensive exploitation of candidate drugs has been continued in pharmaceutical industries. Among these pharmacological approaches, derivatives of bile acids and compounds that influence bile acid-related signalling pathways including are emerging as potentially useful therapeutic agents for treating NASH.

Bile acids are amphipathic molecules that facilitate absorption of dietary fat and lipophilic vitamins in the small intestine. Bile acids also act as signalling molecules controlling glucose, lipid and energy homeostasis by activating farnesoid X receptor (FXR) and Takeda G-protein receptor-5 (TGR5). Bile acids have differing potencies for activating FXR and TGR5. When individually tested, Chenodeoxycholic acid was the strongest FXR agonist compared with other bile acids (chenodeoxycholic acid > cholic acid > deoxycholic acid > lithocholic acid, in order of decreasing potency). Different bile acids also activate TGR5 with differing potencies (lithocholic acid > deoxycholic acid > cholic acid > chenodeoxycholic acid). Changes in the bile acid pool size as well as the composition of bile acids in the pool are thought to impact the regulation of glucose, lipid and energy expenditure, and finally, progression of NAFLD. Bile acids are synthesised in the hepatocytes as primary bile acids (cholic acid and chenodeoxycholic acid in humans), which are transformed to secondary bile acids (deoxycholic acid and lithocholic acid) with multistep 7α-dehydroxylation by intestinal bacteria (Figure 1). The human gut microbiome may have an impact on FXR/TGR5 signalling by manipulating the availability and strength of bile acid ligands through bile acid metabolism.

Recent evidence shows a role for the gut microbiome in insulin resistance, obesity and associated metabolic disturbances, raising interest in the gut microbiome’s relationship with NAFLD/NASH pathogenesis. However, there are several difficulties when studying the pathogenesis of NAFLD/NASH in association with bile acid metabolism and the gut microbiome. First, patient cohorts in previous NASH studies had a variety of coexisting metabolic disorders that could affect bile acids and the gut microbiome, such as type-2 diabetes mellitus and obesity. Additionally, body mass index > 35 kg/m², which is seen in Western patients, is rarely seen in Japanese patients; therefore, ethnic differences based on genetic and environmental factors should be considered. Second, because of differences in bile acid metabolism among different species, it is unknown whether bile acid- and gut microbiome-related pathophysiological mechanisms revealed using animal models are consistent with human NASH.

In the present study, we determined serum bile acid profiles in Japanese patients with biopsy-proven NASH, and compared these profiles with similar data from healthy controls. The study cohort consisted of ‘non-obese’ patients (majority of patients’ BMIs are below 30); which is different from previous studies, therefore, we could exclude the possible impact of obesity itself on serum bile acid profiles.

MATERIAL AND METHODS

2.1 Patients and sample collection

Fasting blood samples were obtained in the morning from 38 healthy controls (21 men and 17 women; mean age ± standard deviation: 56.9 ± 2.3 years) and 34 patients with NASH (22 men and 12 women; mean age: 59.6 ± 2.6 years). Serum from healthy controls was collected from patients undergoing regular health checkups or from volunteers. In the control group, we excluded patients classified as obese (body mass index > 25) and those with diabetes mellitus, dyslipidaemia or liver dysfunction.

All enrolled patients were pathologically diagnosed as having NASH by two independent pathologists based on NASH Clinical Research Network criteria; fibrosis stage was defined according to the criteria as stage 0-4 (cirrhosis). We excluded patients with other causes of chronic liver disease such as chronic viral hepatitis, autoimmune hepatitis or primary biliary cholangitis. In all patients, current and past daily alcohol consumption was <20 g per day; detailed information regarding alcohol consumption was obtained independently by at least two physicians and confirmed by close family members. Patients with clinically diagnosed cancers including hepatocellular carcinoma, and those taking medications that affect bile acid pool size and composition (eg ursodeoxycholic acid or anion exchange resin) were also excluded. Sera were stored at –20°C until analysed.

2.2 Determination of serum bile acid profiles

Serum bile acid concentrations were quantified according to the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method described previously.

2.3 Calculation of FXR affinity and bile acid hydrophobicity indices

FXR affinity indices were calculated using the concentration (µmol/L) or composition (%) and relative potency of each bile acid as an FXR agonist, as reported by Zhang et al. We used the following formula to calculate the indices: FXR affinity index = cholic acid (µmol/L or %) × 0.81 + chenodeoxycholic acid × 1 + deoxycholic
Acid × 0.4 + lithocholic acid × 0.04. The bile acid hydrophobicity index was calculated according to the method reported by Heuman.14

2.4 | Analysis of biomarkers for bile acid biosynthesis

Serum concentrations of 7α-hydroxy-4-cholesten-3-one (C4), a biomarker of cholesterol 7α-hydroxylase (CYP7A1) activity, were also determined using LC-MS/MS as described previously.15

2.5 | Statistical analysis

Data are reported as the mean ± standard error. The statistical significance of differences between results in patients with NASH and controls was evaluated by Student’s two-tailed t test or the nonparametric Mann-Whitney test. In all statistical tests, significance was accepted at P < .05 (JMP Pro 13 software, SAS institute Inc, Cary, NC, USA).

3 | RESULTS

3.1 | Patients’ profiles

As shown in Table 1, sera obtained from 38 controls and 34 Japanese patients with biopsy-proven NASH (22 men and 12 women) were used in the present study. Compared with controls (mean age: 56.9 ± 2.3 years), the mean age of the NASH group (59.6 ± 2.6 years) was not significantly different. Serum alanine transaminase (ALT) as well as γ-glutamyl transpeptidase (GGT) levels were significantly higher in the NASH group (ALT: 77.4 ± 14.9 /L, GGT: 154.1 ± 43.3 /L) compared with controls (ALT: 18.8 ± 4.4 /L, GGT: 63.2 ± 12.3 /L). The FIB-4 index, calculated from data obtained at sample collection, was significantly higher in the NASH group (2.37 ± 0.32) compared with controls (0.88 ± 0.42). In the NASH group, the numbers of patients with fibrosis scores of 0, 1, 2, 3 and 4 defined by the NASH Clinical Research Network criteria10 were 5, 15, 4, 6 and 4 respectively. We then assigned patients with a fibrosis score <2 (n = 20) to a ‘marginal fibrosis’ (F < 2) group, while the remaining patients were classified as the ‘significant fibrosis’ (F ≥ 2) group (n = 14).
TABLE 1 Patients’ clinical characteristics

|                  | Controls (n = 38) | NASH (n = 34) |
|------------------|------------------|--------------|
| Age (Mean ± SEM) | 56.9 ± 2.3       | 59.6 ± 2.6   |
| Gender (M:F)     | 21:17            | 22:12        |
| BMI (kg/m²)      | 22.3 ± 2.2       | 27.5 ± 0.9'  |
| AST (U/L)        | 17.2 ± 5.5       | 77.1 ± 19.8**|
| ALT (U/L)        | 18.8 ± 4.4       | 77.4 ± 14.9**|
| GGT (U/L)        | 63.2 ± 12.3      | 154.1 ± 43.3*|
| Alb (g/L)        | 47.0 ± 5.0       | 45.0 ± 6.0   |
| Platelet (10^9/L)| 252.0 ± 33.0     | 241.0 ± 14.0 |
| HbA1c (%)        | 5.61 ± 0.33      | 6.18 ± 0.21  |
| FIB-4 index      | 0.88 ± 0.42      | 2.37 ± 0.32**|
| Fibrosis score (0:1: 2:3: 4) | NA | 5:15: 4:6: 4 |

Abbreviations: AST, aspartate aminotransferase; Alb, albumin; ALT, alanine transaminase; BMI, body mass index; F: female; FIB-4, fibrosis-4 index; GGT, γ-glutamyl transpeptidase; HbA1c, haemoglobin A1c; M: male; NA, not applicable; NASH, nonalcoholic steatohepatitis; SEM, standard error of the mean.

*P < .05, significantly different from controls.
**P < .01, significantly different from controls.

3.2 Serum bile acid concentrations in patients with NASH

Figure 2 shows the fasting absolute concentrations of serum bile acids. The mean total serum bile acid concentration in the NASH group (8.2 ± 1.0 µmol/L) was significantly higher (P < .0001) compared with controls (4.0 ± 0.3 µmol/L) (Figure 2A), and this higher level in the NASH group did not depend on the progression of hepatic fibrosis (F < 2 vs F ≥ 2.8 ± 1.4 µmol/L vs 8.0 ± 1.4 µmol/L). The unconjugated fraction of total bile acids was not significantly higher (Figure 2B), but glycine- (Figure 2D) and taurine- (Figure 2E) conjugated fractions were significantly higher in patients with NASH. We also determined the unconjugated minor bile acid components including epi- and dehydro-bile acids and compared results between the NASH group and controls, but the concentrations were not significantly different (Figure 2C).

When we represented the bile acid concentration data as proportions, we saw increased cholic acid and decreased deoxycholic acid (Figure 3B, D, and E) as well as increased glycine- and taurine-conjugated fractions (Figure 3A). The sum of each bile acid (conjugated + unconjugated) was calculated and compared between controls and the NASH group. As shown in Figure 4, cholic acid, chenodeoxycholic acid and ursodeoxycholic acid concentrations were significantly higher in the NASH group in contrast to deoxycholic acid and lithocholic acid concentrations, which were not increased.

3.3 Characteristic features of serum bile acid profiles in the NASH Group

Bile acids are synthesised as conjugated primary bile acids in the liver and transformed into unconjugated secondary bile acids by the gut microbiome (Figure 1). To clarify differences in hepatic and intestinal bile acid metabolism between the NASH group and controls, we calculated the following ratios: First, to compare the activities of cholic acid and chenodeoxycholic acid biosynthetic pathways, we calculated the ratio of total cholic acid + total deoxycholic acid/total chenodeoxycholic acid + total lithocholic acid. As shown in Figure 5A, the ratio was significantly lower in the NASH group. Second, the ratio of taurine conjugation/glycine conjugation was also significantly lower in the NASH group (Figure 5B). Third, to examine the activities of deconjugation and 7α-dehydroxylation of bile acids by the gut microbiome, we calculated the ratio of unconjugated bile acids/total bile acids and secondary bile acids/total bile acids. As shown in Figure 5C and D, both ratios were significantly lower in the NASH group. We also compared these ratios between patients in the NASH group with marginal vs significant fibrosis, and found that changes in the ratios did not depend on the progression of hepatic fibrosis.

3.4 Effects of bile acid profile on FXR affinity and bile acid hydrophobicity indices

As shown in Figure 6A and B, both indices were significantly increased in the NASH group compared with controls. In contrast, the bile acid hydrophobicity index did not differ significantly between the NASH group and controls (Figure 6C).

3.5 Determination of a serum surrogate marker for bile acid biosynthesis

FXR was suggested to be activated in Japanese patients with NASH. FXR activation inhibits bile acid biosynthesis through downregulation of CYP7A1, the rate-limiting enzyme of bile acid synthesis.16 Therefore, we measured serum concentrations of C4, a surrogate marker of CYP7A1,15 to prove that FXR is activated in NASH. As shown in Figure 6D, C4 level tended to be lower in the NASH group, but the difference was not statistically significant (P = .071).

4 DISCUSSION

The elevated total serum bile acid concentrations we saw in the NASH group in this study were consistent with previous reports showing increased total serum bile acid concentration in patients with obesity, diabetes, metabolic syndrome,17-19 and NASH.20 The reason for the elevation is undetermined, but because the half-life of serum bile acids is only a few minutes, increased intestinal absorption and/or decreased hepatic uptake of bile acids appear to contribute to the elevation.21 In addition, serum levels of fibroblast growth factor-19, which is mainly secreted by the intestine in the presence of bile acids, were reported to be low or unchanged in patients with NASH,22-24 suggesting that the rate of bile acid absorption from the terminal ileum may not be higher in NASH. Therefore, strong evidence suggests that elevated serum bile acids in NASH are caused
by reduced net hepatic uptake, as reported in studies showing decreased protein levels in hepatic bile acid transporters such as Na/taurocholate cotransporting polypeptide, in NASH. Previous studies including human and animal models, suggested that increased deoxycholic acid might have a causative role in hepatic carcinogenesis. Deoxycholic acid is a hydrophobic bile acid, which is known to cause DNA damage. The role of deoxycholic acid in promoting colon carcinogenesis is well known. Moreover, recent studies using mice models demonstrated that deoxycholic acid, which is an obesity-induced gut microbial metabolite, may contribute to the development of liver cancer through senescence-associated secretory phenotype expression in hepatic stellate cells. In NAFLD patients, one study showed that serum deoxycholic acid levels were disproportionately elevated compared to all primary and secondary bile acid levels. However, in our study, serum deoxycholic acid concentration was not increased, and deoxycholic acid proportions were significantly lower in patients with NASH, raising the question whether deoxycholic acid is truly related to hepatocarcinogenesis in NASH.

The lower proportions of unconjugated bile acids (Figure 5C) and secondary bile acids (Figure 5D) seen in our study are consistent with previous reports, which suggest an altered gut microbiome in patients with NASH. Additionally, an increased Firmicutes/Bacteroidetes ratio has been reported in patients with NAFLD, NASH and diabetes. Because deconjugation of glycine and taurine is caused by many bacteria including Bacteroides, Clostridium, Lactobacillus, Bifidobacterium, Eubacterium, Peptostreptococcus and Streptococcus, it is possible that total bacterial numbers in the gut microbiome are decreased in NASH. In addition, dehydroxylation of the C-7α position in bile acids occurs exclusively with specific subclusters of Clostridium; therefore, these specific clostridial subclusters should be particularly reduced in patients with NASH.

The primary bile acids synthesised in the liver are cholic acid and chenodeoxycholic acid. Cholic acid is converted to deoxycholic acid, and chenodeoxycholic acid is converted to lithocholic acid in the intestines (Figure 1). Therefore, the ratio of cholic acid + deoxycholic acid/chenodeoxycholic acid + lithocholic acid represents changes in bile acid biosynthesis in the liver. Our data showed that this ratio

FIGURE 2 Serum bile acid (BA) concentrations in controls (n = 38) and patients with NASH (n = 34). Absolute serum concentration of total BA (A), unconjugated BA fraction (B), unconjugated epi- and dehydro-BA fraction (C), glycine conjugated (D), and taurine conjugated (E), fraction were demonstrated. NASH, nonalcoholic steatohepatitis; BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid. Prefixed ‘G’ to the name of each bile acid; glycine-conjugated, prefixed ‘T’ to the name of each bile acid; taurine-conjugated.
was significantly lower in the NASH group (Figure 5A) suggesting downregulated hepatic sterol 12α-hydroxylase (CYP8B1) activity in NASH as in a previous report. This relatively decreased production of cholic acid may contribute to the lower proportion of deoxycholic acid in NASH Clostridium subcluster XIVa (XIVa) is a major bacterial group producing secondary bile acids, and their number is influenced by the amount of intestinal cholic acid. In patients with liver cirrhosis, the cholic acid pool size is decreased; therefore, intestinal XIVa and deoxycholic acid are also decreased. In contrast, high-fat diets stimulate biliary secretion of cholic acid and increase
intestinal XIVA and deoxycholic acid. These results suggest that the pattern of bile acids to gut microbiome profiles in NASH is similar to that seen with liver cirrhosis rather than with high-fat diets.

In our study, the taurine-conjugated bile acid/glycine-conjugated bile acid ratio was significantly lower in the NASH group (Figure 5B). Because this ratio is influenced by hepatic taurine concentration, taurine may be decreased in Japanese patients with NASH.

It is not clear whether FXR is activated in patients with NASH. Jiao et al showed that increased deoxycholic acid was accompanied by a suppression of hepatic FXR-mediated and fibroblast growth factor receptor-4-mediated signalling in NAFLD. However, FXR may be activated in NASH because deoxycholic acid levels are relatively decreased. When we calculated FXR-affinity indices using bile acid concentration (μmol/L) and composition (%), both indices were significantly higher in patients with NASH compared with controls, suggesting that FXR is activated in NASH. FXR activation downregulates CYP7A1 and CYP8B1 activity. Several reports indicated unaffected bile acid synthesis in patients with NASH.
NAFLD/NASH, although other reports showed controversial results. Downregulation of CYP8B1 in patients with NASH is suggested by the low ratio of total cholic acid + total deoxycholic acid/total chenodeoxycholic acid + total lithocholic acid. In contrast, in our study, the levels of C4, a surrogate marker of CYP7A1, were not significantly different between the NASH group and controls; however, the mean C4 concentration tended to be lower in the NASH group (Figure 6D). It is important to note that CYP7A1 is controlled not only by FXR but also by the detergent properties of bile acids. Therefore, we also calculated the bile acid hydrophobicity index in our patients; however, the indices were not significantly different between the NASH group and controls (Figure 6C), suggesting that FXR was activated in NASH, in contrast to NAFLD.

In conclusion, decreased serum cholic acid + deoxycholic acid, taurine-conjugated bile acids, unconjugated bile acids and secondary bile acids were characteristics of Japanese patients with NASH, in our study. Our results may provide basic information to better understand the pathogenesis and treatment of NASH regarding changes in bile acid metabolism. In addition, these results raised further question whether therapeutic strategy by using agents modifying bile acid metabolism is effective to the same extent for all races and ethnic groups.

ETHICS STATEMENT
The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to and the approval from Ethics Committee of Tokyo Medical University Ibaraki Medical Center and the National Hospital Organization of Kyushu Medical Center has been received. Informed consent was obtained from all patients.

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CONFLICT OF INTEREST
The authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTIONS
SY and TI designed the study. SY, TM, JI, TH, MK and MN contributed to obtain clinical data, serum samples and informed consent from our patients. SY, TM, TI and AH contributed data analysis and implication. AH contributed to LC-MS/MS operation and data analysis. SY prepared the manuscript and all authors commented on the paper.

ORCID
Tadashi Ikegami https://orcid.org/0000-0002-9216-3186

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