Use of noninvasive serum glycan markers to distinguish nonalcoholic steatohepatitis from simple steatosis

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Abstract

Background: The aims of this study were to investigate whole glycan expression in patients with nonalcoholic fatty liver diseases and to evaluate the potential use of glycan profiles as new clinical biomarkers to distinguish nonalcoholic steatohepatitis (NASH) from simple steatosis (SS).

Methods: We collected sera from 42 histologically-proven NASH and 15 SS patients prior to treatment. Serum glycan profiles were measured by comprehensive, quantitative, high-throughput glycome analysis, and diagnostic values of serum glycans for NASH prediction were examined.

Results: Among the 41 serum glycans examined, the expression levels of 8 glycans in NASH were significantly higher than those of SS. Out of these 8 glycans, three glycans (*m/z* 1955, 2032, and 2584) showed high areas under the receiver operating characteristic curve (0.833, 0.863, and 0.866, respectively) for distinguishing NASH from SS. In multivariate analyses with clinical parameters and serum glycans, these three glycans were significant predictive factors for distinguishing NASH from SS. The odds ratio of *m/z* 1955, 2032, and 2584 were 48.5, 6.46, and 11.8, respectively. These glycans also correlated significantly with lobular inflammation, ballooning, and fibrosis, but not with steatosis.

Conclusions: We clearly demonstrated whole serum glycan profiles in NASH patients, and the feasibility of serum glycans (*m/z* 1955, 2032, and 2584) as new noninvasive biomarkers for distinguishing NASH from SS.

Keywords: nonalcoholic steatohepatitis, serum glycan, diagnosis

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common etiologies of chronic liver disease in many countries all over the world [1, 2]. The incidence of NAFLD in the general population has increased recently, and, in many countries, NAFLD is a critical health problem that is highly correlated with insulin resistance [3, 4]. The definition of NAFLD ranges from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH), which is a condition that causes inflammation and fibrosis. Although SS is a benign condition that follows a nonprogressive clinical course, NASH is a potentially serious condition that can develop into cirrhosis and hepatocellular carcinoma (HCC). Thus, the diagnosis of NASH is very important to determine potential risk of the progression of liver disease [4, 5].

Although liver biopsy is the current gold standard for diagnosing NASH, it is an invasive, high-risk procedure. Furthermore, liver biopsy is limited by cost, and sampling errors are unavoidable. Thus, noninvasive procedures that accurately distinguish NASH from SS are needed urgently. Several noninvasive biomarkers and scoring systems, such as cytokeratin-18 fragments and NAFIC score, using a combination of ferritin, fasting insulin, and type IV collagen 7S, for diagnosing NASH have been reported previously. However, sensitivity and specificity for diagnosis by

these markers are limited, so that new modalities are needed to overcome these problems [6-9].

Recently, serum glycans were reported to be potential diagnostic and therapeutic biomarkers for many diseases; their expression levels change in various conditions, such as inflammation, fibrosis, and cancer progression [10-13]. Regarding NASH, an increase in serum fucosylated haptoglobin was reported as a novel diagnostic biomarker for predicting hepatocyte ballooning and NASH [14]. Although glycan changes in particular proteins in NASH have become clear, the full picture of glycan profile alterations in patients with NAFLD including SS and NASH has not been elucidated.

A new technology for glycan-specific enrichment, the “glycoblotting” method, was developed recently [15]. This method enables comprehensive, high-throughput quantitative analysis of serum glycans and has been applied to construct new diagnostic and pathophysiological markers for pancreatic cancer and Crohn’s disease, and to predict a prognosis for patients with hepatocellular carcinoma [10, 11, 16].

The aims of this study were to investigate whole glycan expression in patients with NAFLD and NASH using this method and to evaluate the potential use of glycan profiles as new clinical biomarkers to distinguish NASH from SS.

Methods

Patients

Between September 2005 and April 2013, we enrolled 62 consecutive patients with liver biopsy-proven NAFLD who were admitted to Okayama University Hospital. Three patients with hepatic tumors including HCC or focal nodular hyperplasia, and two patients who accumulated hepatic fat secondary were excluded. Thus, a total of 57 patients remained eligible for this retrospective cohort study, and serum glycans of these patients were analyzed before treatment.

Written informed consent for using the serum and clinical data was obtained from all patients. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by the institutional review board (authorization number #1919).

Diagnosis

A diagnosis of NAFLD was established if the following clinical and histological features were present: (1) liver biopsy showing steatosis of hepatocytes; (2) exclusion of liver disease of other etiology, such as viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, hemochromatosis, Wilson's

disease, α-1-antitrypsin deficiency-associated liver disease, and other secondary hepatic fat accumulation disease; and (3) alcohol consumption level of less than 140 grams per week (less than 20 grams per day) over at least 5 years prior to entry into this study [5]. All patients enrolled in this study had undergone liver biopsy under ultrasonic or laparoscopic guidance. All liver specimens were fixed with formalin, embedded in paraffin, and examined using the following stains: hematoxylin and eosin, Masson's trichrome, and reticulin silver stain. Three experienced hepatologists (Y.I., F.I., and T.Y.) diagnosed and scored all liver specimens. All NAFLD patients were classified as either SS or NASH by liver histology according to the classification by Matteoni et al. [17] as follows: type 1: fatty liver alone, type 2: fat accumulation and lobular inflammation, type 3: fat accumulation and ballooning degeneration, or type 4: fat accumulation, ballooning degeneration and either Mallory-Denk body or fibrosis. Types 1 and 2 were classified as SS, and types 3 and 4 were classified as NASH. In addition, a histological scoring system for NAFLD was used to evaluate the histological features of the liver specimens [18].

Serum glycoblotting method

All serum samples from NAFLD patients were collected at the time of admission.

Glycoblotting was performed according to the procedure described by Nishimura et al. [15]. Briefly, 10-μL serum samples were applied to “Sweetblot” prototype 7 (System Instruments Co.), which is an automated machine for pretreatment and glycoblotting. After enzymatic cleavage from the proteins, glycans were captured on BlotGlyco H beads (Sumitomo Bakelite, Co.), and sialic acids were methyl-esterified. The processed glycans were tagged with benzyloxyamine (BOA) and released from the beads, followed by MALDI-TOF-MS detection (Ultraflex 3, Bruker, Germany).

Statistical analysis

Results are presented as the medians and ranges for continuous variables. The Chi-square test and Fisher’s exact test were used to compare categorical data, and the Wilcoxon’s rank sum test and Kruskal-Wallis test were used to compare continuous data. Diagnostic values of serum glycans to predict NASH were demonstrated by area under the receiver operating characteristic curve (AUROC). The odds ratio (OR) for discriminating NASH from SS was calculated by logistic regression, and factors exhibiting significance in a univariate analysis were further analyzed by multivariate analysis. In these analyses, cut-off values were set at the median. To avoid the effects of multicollinearity, the OR of each serum glycan was examined separately by

multivariate analysis.

The JMP (version 8) software package (SAS Institute, Cary, North Carolina, USA) was used for all statistical analyses, and a *P* value less than 0.05 was considered significant. The Bonferroni correction was used for multiple comparisons, and a *P* value less than 0.05/41 was considered significant for evaluation of differences between the expression of serum glycan in the SS patients and that in the NASH patients.

Results

Characteristics of NAFLD patients

Among the 57 patients enrolled in this study, 15 patients were classified into SS and the rest of the patients (n=42) were diagnosed as NASH. The clinical and histological characteristics of the patients are shown in Table 1. There were no differences in age or gender between the groups; however, the platelet number (PLT), prothrombin time (PT), and albumin (ALB) levels in SS patients were higher than those in the NASH patients, whereas aspartate aminotransferase (AST), homeostasis model assessment-insulin resistance (HOMA-IR) and immunoglobulin A were lower in the SS patients.

In histological findings according to the NAFLD activity score [18], the degree of lobular inflammation, ballooning, and fibrosis were significantly higher in the NASH patients. There were no differences in the degree of steatosis.

Changes in serum glycan profiles

Glycome analysis performed by Sweetblot revealed the expression levels of 41 BOA-labeled glycans with molecular weights (*m/z*) ranging from 1362.481 to 3865.407 kDa. Five glycans were classified as high-mannose type, six as hybrid type, and 30 as

complex type (of these, 18 were bi-antennary, 7 were tri-antennary, and 5 were tetra-antennary).

Among the 41 glycans examined, the expression levels of 8 glycans in the NASH patients were significantly higher than those in the SS patients after Bonferroni correction, (Table 2). These glycans consisted of 3 hybrid type, 3 bi-antennary bisected, and 2 tri-antennary. AUROCs of three glycans (glycan *m/z* 1955, 2032, and 2584) for discriminating NASH from SS were above 0.83 (0.833, 0.863, and 0.866, respectively). The expression levels of these three glycans are shown in Figure 1.

Predictive factors for discriminating NASH from SS

To identify nonhistological factors that can discriminate NASH from SS, univariate logistic analyses were conducted using the clinical parameters in Table 1 and serum glycans *m/z* 1955, 2032, and 2584. In the analyses, three clinical parameters, PT [OR, 0.22; 95% confidence interval (CI), 0.05–0.77], AST (OR, 5.88; 95% CI, 1.58–28.75), HOMA-IR (OR, 5.88; 95% CI, 1.58–28.75), and serum glycans *m/z* 1955 (OR, 10.5; 95% CI, 2.50–73.4), 2032 (OR, 9.55; 95% CI, 2.26–66.3), and 2584 (OR, 10.5; 95% CI, 2.50–73.4) were significant predictive factors for discriminating NASH from SS. Multivariate analyses with these three clinical parameters and serum glycan *m/z*

1955 revealed that high values of *m/z* 1955 (OR, 48.5) as well as PT and HOMA-IR were significant predictive factors for discriminating NASH from SS (Table 3). To avoid multicollinearity, serum glycans *m/z* 1955, 2032, and 2584 were examined separately. Similarly, serum glycans *m/z* 2032 (OR, 6.46) and 2584 (OR, 11.8) were significant predictive factors for discriminating NASH from SS.

Correlation between glycans and histological characteristics in NAFLD patients

The expression levels of glycans *m/z* 1955, 2032, or 2584 were compared with the degree of steatosis, lobular inflammation, ballooning, and fibrosis (Table 4). All three glycans showed positive correlations with the degree of lobular inflammation, ballooning, and fibrosis. No correlations were observed between glycan levels and the grade of steatosis.

Discussion

In this study, we first analyzed whole-serum *N*-glycan profiles in NAFLD patients using Sweetblot, a high-throughput and automated glycomics system that can measure glycans comprehensively and quantitatively. In NASH patients, the expression of 8 glycans among the 41 glycans examined were significantly higher than those in SS patients. In particular, we found that the levels of three glycans (*m/z* 1955, a bi-antennary bisected and fucosylated glycan; *m/z* 2032, a hybrid type; and *m/z* 2584, a tri-antennary fucosylated glycan) were predominantly higher in NASH than in SS patients. These glycans also correlated significantly with lobular inflammation and fibrosis, but not with steatosis.

As deterioration of liver functions affect the production, secretion, and degradation of glycoproteins, various changes in the levels of serum glycoproteins in patients with liver diseases have been observed [13]. In this study, out of 8 highly expressed glycans in NASH patients, three glycans were fucosylated by α1, 6 fucosyltransferase (α1, 6FT) [19]. Glycans *m/z* 1955 and 2584 belong to this group. Although we could not specify all carrier proteins of the fucosylated glycans, haptoglobin was considered to be one of them. Fucosylated haptoglobin was reported to increase in NASH patients and the bound glycans included *m/z* 2584 and 2890, which

were two of the four fucosylated glycans picked up in this study [12, 20–22]. There are several putative mechanisms of the increase in NASH-related serum fucosylated proteins. Fucosylated glycoproteins produced in normal hepatocytes are known to be secreted into the bile. In ballooning hepatocytes, microtubules and the cytoskeleton, the sorting machinery, are destroyed, and fucosylated glycoproteins was aberrantly transported in hepatocytes and secreted into the serum [14, 23]. The physiological roles of fucosylated proteins were also examined with an α1, 6FT transgenic mice model. The overexpression of α1, 6FT in mice caused steatosis in the liver [24]. Although the precise mechanism of steatosis has not been shown in humans, the overexpression of α1, 6FT might cause steatosis and NASH.

In this study, three hybrid glycans, including *m/z* 2032, were significantly increased in NASH. These glycans were also reported to increase in the nonimmunoglobulin fraction of the serum in patients with liver cirrhosis [25]. It is possible that rapid cell proliferation occurs in cirrhotic liver, resulting in the binding of hybrid glycans to proteins before maturation to multi-antennary glycans; however, the reasons why hybrid-type glycans are increased in NASH is not currently clear.

Among the 8 differentially expressed glycans, two, including *m/z* 2584, were tri-antennary glycans. Multi-antennary glycans are known to be elevated in

inflammatory diseases and advanced cancers, so that the elevation of glycans in NASH was considered to be the result of inflammation in the liver to some extent. In this study, *m/z* 2584 correlated with the presence of lobular inflammation, supporting this hypothesis.

We also examined the expression levels of these three glycans in the healthy controls who matched the NAFLD patients in terms of age and sex. Although the data were preliminary, the expression levels of m/z 1955 and 2032 in the serum of NAFLD patients were significantly higher than that of healthy controls after Bonferroni correction, so that they also could be biomarkers for the presence of steatosis (supplementary Table).

Blomme, et al. reported that the ratio of bi-antennary fucosylated glycan and bi-galacto bi-antennary glycan was a good biomarker for distinguishing NASH patients from SS patients [26]. Different from our study, a desialylation reaction was performed in the report so that the presence of sialic acid was not identified. Although it was difficult to compare the results directly, the ratio of these two glycans was also increased in NASH patients compared to SS patients in this study (data not shown).

To predict NASH, several biomarkers have been reported. The AUROCs of cytokeratin-18 was 0.83–0.88 [6, 7], Mac-2-binding protein, which is one of the major

fucosylated proteins, was 0.816 [27], fucosylated haptoglobin was 0.734 [14], and NAFIC was 0.782–0.851 [9]. Although we did not directly compare the diagnostic ability of glycan markers with all of these other markers, the AUROCs of cytokeratin-18 in this study population was 0.661 (data not shown), meaning that the AUROCs of glycans *m/z* 1955, 2032, and 2584 (0.83–0.86) are comparable to those of the other biomarkers. In addition, we measured the serum concentration of tumor necrosis factor-alpha and interleukin-6 that were reported to be markers for NASH; however, we could not differentiate SS from NASH with them in this study population.

Even though we successfully revealed the full picture of alterations of serum glycan profiles in patients with NAFLD and NASH, there are several limitations of this study. First, this study is a retrospective, single-center analysis with a small sample size, and the number of SS patients is small compared with that of NASH patients. Second, we did not prove the biological effects of these glycan alternations or the specific ligands of these glycans. Third, these results were not validated in other sets of NAFLD and NASH patients, and we need to establish specific cut-off values to identify NASH patients.

Despite these limitations, we clearly demonstrate whole serum *N*-glycan profiles in NAFLD patients and show the feasibility of using glycans *m/z* 1955, 2032, and 2584

as new biomarkers to distinguish NASH from SS. Further prospective studies are needed to confirm the usefulness of these results.

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Conflict of interest

Kazuhiro Nouso received a research grant from a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and a research grant from the Japan Science and Technology Agency (JST). Kazuhiro Nouso, Hideki Onishi, and Fusao Ikeda belong to a donation-funded department (Department of Molecular Hepatology, funded by MSD). Shin-Ichiro Nishimura received a research grant from the Japan Science and Technology Agency (JST) and the Ministry of Education, Culture, Science, and Technology, Japan, and serves as a consultant to Medicinal Chemistry Pharmaceuticals, Co., Ltd.

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Figure Legends

Figure 1. Box plots of the glycan levels of m/z 1955, 2032, and 2584 in simple steatosis (SS) and nonalcoholic steatohepatitis (NASH). The glycan expression levels in NASH patients were significantly higher than those in SS patients.

Figure 1

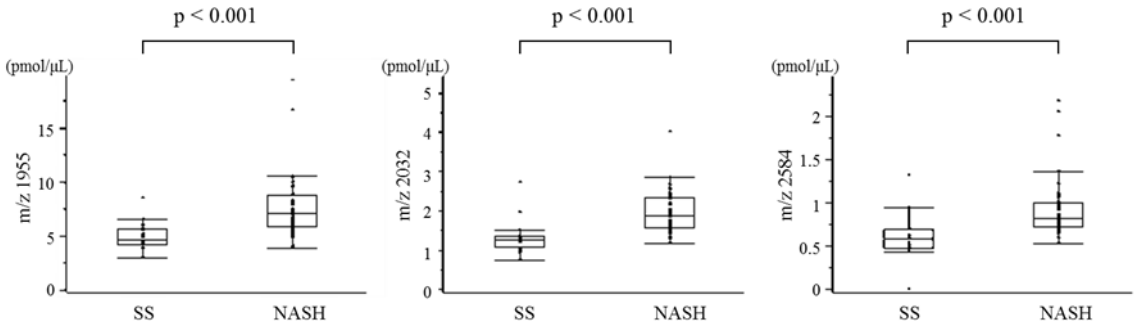


Table 1. Characteristics of the patient population

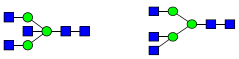
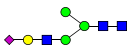
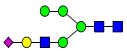
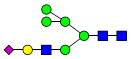
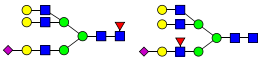
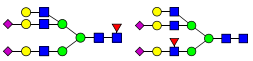
Clinical parameters	NAFLD (n=57)	SS (n=15)	NASH (n=42)	*P* value*
Age, yr	56 (17–78)	48 (27–74)	56 (17–78)	N.S.
Male/female, n	22/35	7/8	15/27	N.S.
Body mass index, kg/m^2	26.7 (17.9–38.4)	25.9 (17.9–36.1)	27.6 (19.3–38.4)	N.S.
Laboratory data				
Total bilirubin, mg/dL	0.6 (0.3–5.3)	0.6 (0.3–2.2)	0.6 (0.3–5.3)	N.S.
Albumin, g/dL	4.4 (3.1–5.0)	4.5 (3.2–5.0)	4.3 (3.1–4.9)	0.045
Prothrombin time, %	107 (52–135)	118 (69–135)	102 (52–135)	0.004
Platelet count, ×10^4/μL	22.9 (7.0–45.8)	25.2 (11.8–34.1)	20.0 (7.0–45.8)	0.028
Aspartate aminotransferase, IU/L	59 (14–548)	45 (14–548)	66 (28–201)	0.006
Alanine aminotransferase, IU/L	79 (16–452)	61 (16–275)	81 (17–452)	N.S.
Alkaline phosphatase, IU/L	304 (108–702)	251 (108–702)	308 (115–691)	N.S.
Gamma glutamyl transpeptidase, IU/L	78 (15–687)	78 (15–687)	78 (20–391)	N.S.
Total cholesterol, mg/dL	192 (102–354)	209 (134–308)	185 (102–354)	N.S.
LDL cholesterol, mg/dL	110 (35–240)	108 (59–229)	111 (35–240)	N.S.
HDL cholesterol, mg/dL	52 (33–118)	57 (33–93)	49 (34–118)	N.S.
Triglyceride, mg/dL	154 (32–687)	169 (65–687)	146 (32–392)	N.S.
Fasting plasma glucose, mg/dL	105 (85–269)	102 (88–269)	105 (85–181)	N.S.
HbA1c, %	5.5 (3.3–10.5)	5.3 (4.8–6.0)	5.5 (3.3–10.5)	N.S.
HOMA-IR	3.1 (0.3–47.6)	1.7 (0.3–47.6)	3.6 (1.3–12.8)	0.002
Ferritin, ng/mL	236 (15.6–6532)	201 (17–6532)	243 (15.6–1412)	N.S.
immunoglobulin G, mg/dl	1370 (901–2697)	1273 (939–1572)	1374 (901–2697)	N.S.
immunoglobulin A, mg/dl	259 (48–639)	211 (138–597)	284 (48–639)	0.049
immunoglobulin M, mg/dl	98 (28–220)	109 (53–178)	95 (28–220)	N.S.
Histological findings				
Steatosis 0/1/2/3, n	0/25/20/12	0/5/6/4	0/20/14/8	N.S.
Lobular inflammation 0/1/2/3, n	14/23/17/3	12/3/0/0	2/20/17/3	< 0.001
Ballooning 0/1/2, n	15/30/12	15/0/0	0/30/12	< 0.001
Fibrosis 0/1/2/3/4, n	15/8/10/15/9	15/0/0/0/0	0/8/10/15/9	< 0.001
Matteoni's classification 1/2/3/4, n	12/3/1/41	12/3/0/0	0/0/1/41	-

NOTE: *Differences between SS and NASH were calculated.

Values are the median (range) unless otherwise noted.

HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment-insulin resistance; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SS, simple steatosis; N.S., not significant .

Table 2. Expression of serum glycans in patients with NASH

Glycans		Proposed structure[a]	Median glycan expression (pmol/μL)		*P* value[*]	AUROC[**]
Code	*m/z*		SS (n=15)	NASH (n=42)		
# 3500	1647.614		0.914	1.526	< 0.001	0.828
# 4301	1708.619		1.794	2.093	< 0.001	0.803
# 4500	1809.666		1.034	1.617	< 0.001	0.816
# 5301	1870.672		2.839	3.511	< 0.001	0.814
# 4510	1955.724		4.876	7.036	< 0.001	0.833
# 6301	2032.724		1.250	1.853	< 0.001	0.863
# 6511	2584.941		0.582	0.815	< 0.001	0.866
# 6512	2890.052		0.789	1.339	< 0.001	0.811

NOTE: *P* values are from Wilcoxon's rank sum test.

[a]Monosaccharide composition: rhombus, sialic acid; triangle, fucose; square, N-acetyl glucosamine; yellow circle, galactose; green circle, mannose.

[*]*P* values and [**]AUROC were calculated between SS and NASH

AUROC, area under the receiver operating characteristic curve; NASH, nonalcoholic steatohepatitis; SS, simple steatosis.

Table 3. Multivariate analyses of prognostic factors to discriminate NASH from SS

Variables	Multivariate analyses		
	OR	95% CI	*P* value
Clinical parameters			
Prothrombin time (> 107 %)	0.06	0.00–0.50	0.006
Aspartate aminotransferase (> 59 IU/L)	0.75	0.10–5.38	N.S.
HOMA-IR (> 3.1)	31.6	3.38–811	0.001
Glycan markers			
m/z 1955 (> median)	48.5	4.89–1440	< 0.001
m/z 2032 (> median)	6.46	1.15–53	0.032
m/z 2584 (> median)	11.8	2.02–106	0.005

NOTE: Only variables that demonstrated a *P* value less than 0.05 in univariate analysis were entered into the multiple logistic regression model. The odds ratio (OR) of glycan markers were examined separately in the multivariate analyses. The values shown in clinical parameters were those analyzed with *m/z* 1955.

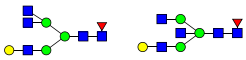
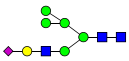
CI, confidence interval; HOMA-IR, homeostasis model assessment-insulin resistance; N.S., not significant.

Table 4. Correlation between histological characteristics and serum glycans

Variables	n	m/z 1955		m/z 2032		m/z 2584	
		Median glycan expression (pmol/μL)	*P* value	Median glycan expression (pmol/μL)	*P* value	Median glycan expression (pmol/μL)	*P* value
Steatosis			0.279		0.323		0.597
0	0	-		-		-	
1	25	6.840		1.579		0.782	
2	20	5.818		1.669		0.743	
3	12	6.047		2.004		0.824	
Lobular inflammation			0.001		0.016		0.018
0	14	4.804		1.273		0.600	
1	23	6.840		1.760		0.743	
2	17	6.638		1.835		0.828	
3	3	8.068		1.642		0.863	
Ballooning			< 0.001		< 0.001		< 0.001
0	15	4.876		1.250		0.582	
1	30	6.848		1.888		0.815	
2	12	7.090		1.698		0.817	
Fibrosis			< 0.001		< 0.001		< 0.001
0	15	4.876		1.250		0.582	
1	8	6.065		1.579		0.698	
2	10	6.738		1.797		0.815	
3	15	7.889		1.872		0.861	
4	9	7.059		1.951		1.085	

NOTE: *P* values are from Kruskal-Wallis test.

Supplementary Table. Expression of serum glycans in patients with HLT and NAFLD

Glycans		Proposed structure[a]	Median glycan expression (pmol/μL)		*P* value[*]	AUROC[**]
Code	*m/z*		HLT (n = 57)	NAFLD (n = 57)		
# 4510	1955.724		3.635	6.163	< 0.001	0.942
# 6301	2032.724		1.284	1.668	< 0.001	0.761
# 6511	2584.941		0.674	0.772	0.014	0.631

NOTE: p-values are from Wilcoxon's rank sum test.

[a]Monosaccharide composition: rhombus, sialic acid; triangle, fucose; square, N-acetyl glucosamine; yellow circle, galactose; green circle, mannose.

*P values and **AUROC were calculated between HLT and NAFLD

Abbreviations: AUROC, Area under Receiver Operating Characteristic curve; HLT, healthy controls; NAFLD, nonalcoholic fatty liver disease;