MAJOR ARTICLE

Aberrant Serum Immunoglobulin G Glycosylation in Chronic Hepatitis B Is Associated With Histological Liver Damage and Reversible by Antiviral Therapy

Cheng-Hsun Ho,[1,a] Rong-Nan Chien,[6,a] Pin-Nan Cheng,[1] Jia-Huei Liu,[3] Cheng-Kun Liu,[6] Chih-Sheng Su,[6] I-Chin Wu,[1] I-Chen Li,[1] Hung-Wen Tsai,[2,4] Shiaw-Lin Wu,[7] Wen-Chun Liu,[1] Shu-Hui Chen,[5,b] and Ting-Tsung Chang[1,3,4,b]

[1]Department of Internal Medicine, [2]Department of Pathology, National Cheng Kung University Hospital, [3]Institute of Molecular Medicine, National Cheng Kung University Medical College, [4]Infectious Disease and Signaling Research Center, [5]Department of Chemistry, National Cheng Kung University, Tainan, and [6]Liver Research Unit, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Keelung, Taiwan; and [7]Barnett Institute and Department of Chemistry and Chemical Biology, Northeastern University, Boston, Massachusetts

Background. Aberrant serum immunoglobulin G (IgG) glycosylation and its immunomodulatory effect are rarely addressed in chronic hepatitis B virus (HBV) infection.

Methods. Serum IgG-Fc glycosylation profiles in 76 patients with HBV-related liver cirrhosis and 115 patients with chronic hepatitis B (CHB) before and after 48 weeks of anti-HBV nucleos(t)ide analogue treatment were analyzed using high-throughput liquid chromatography–mass spectrometry and were compared to profiles in 108 healthy controls.

Results. The level of aberrant serum IgG-Fc glycosylation, particularly galactose deficiency, was higher in patients with CHB and those with cirrhosis ($P < .001$ for both) than in healthy controls. IgG galactose deficiency was correlated with the severity of liver necroinflammation and fibrosis in CHB. Multivariate logistic regression analyses showed that the IgG-Fc glycoform with fucosylation and fully galactosylation was an independent factor for a total Knodell necroinflammation score of ≥7 (odds ratio, 0.74; 95% confidence interval, .56–.97) and an Ishak fibrosis score of ≥3 (odds ratio, 0.69; 95% confidence interval, .49–.97). Administration of antiviral therapy for 48 weeks reversed aberrant IgG-Fc glycosylation in patients with CHB from week 12 onward but did not reverse glycosylation in patients with cirrhosis. Attenuated IgG opsonization in patients with CHB, which was correlated with aberrant Fc-glycosylation, was reversed after treatment as well.

Conclusions. Aberrant serum IgG-Fc glycosylation in CHB, which is highly associated with histological liver damage, affects IgG opsonizing activity and can be reversed by antiviral therapy.

Keywords. chronic hepatitis B; IgG; glycosylation; liver histology; liquid chromatography–mass spectrometry.

Hepatitis B virus (HBV) is a global threat: >350 million people worldwide have chronic HBV infection [1]. Chronic hepatitis B (CHB) increases the risk of developing severe hepatic diseases, including liver cirrhosis and hepatocellular carcinoma, which cause >600 000 deaths annually. Because of systemic immune dysfunctions, patients with chronic liver diseases, particularly liver cirrhosis, are at a high risk of developing subclinical infections [2]. Under such circumstances, immunoglobulin G (IgG) against viral or bacterial antigen is abundantly synthesized, which leads to hypergammaglobulinemia [3]. Elevation of the serum IgG level is a clinical feature of chronic liver disease and is highly associated with disease progression [4].

N-linked glycosylation is the primary posttranslational modification of IgG. The glycosylation site of IgG is most often located in the CH2 domain of the

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[a]C.-H. H. and R.-N. C. contributed equally to this study.

[b]T.-T. C. and S.-H. C. contributed equally to this study.

Correspondence: Ting-Tsung Chang, MD, Department of Internal Medicine, National Cheng Kung University Hospital, 138 Sheng-Li Rd, Tainan 70403, Taiwan (ttchang@mail.ncku.edu.tw).

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Fc region at asparagine 297, and it is conserved in all subclasses of human IgG [5]. The structure of the *N*-glycosylation heptasaccharide core on IgG consists of 4 *N*-acetylglucosamine (GlcNAc) and 3 mannose residues. The diversity of the glycoform is derived from the addition of sugar residues, such as galactose, fucose, GlcNAc, and terminal sialic acid, on the core structure [6, 7]. Glycans on IgG are crucial for its biological and immune functions, including conformational integrity, subcellular transport, intracellular secretion, complement activation, and the binding affinity of Fcγ receptors (FcγRs) [8–10]. IgG with different glycan compositions may trigger variable downstream immune responses. For example, galactose-deficient IgG (IgG-G0) activates the complement cascade by interacting with mannan-binding lectin [11]. Moreover, a higher antiinflammatory activity was found in Fc-sialylated IgG [12]. Because modulation of the glycan on IgG considerably affects its effector functions, variations in glycosylation are physiologically significant.

Aberrant serum IgG glycosylation has been implicated in various autoimmune disorders, malignant diseases, and virus infections and it is a potential marker for monitoring the progression of diseases [13–17]. However, the clinical relevance and the effects of therapy on the IgG glycosylation pattern in HBV-related liver diseases remain largely unclear. Therefore, we conducted a glycoproteomics-based retrospective cohort study to investigate the association of aberrant serum IgG glycosylation to the severity of HBV-related chronic liver diseases and its possible reversal after anti-HBV nucleos(t)ide analogue (NA) therapy.

MATERIALS AND METHODS

Study Design and Patients

This retrospective cohort study was approved by the institutional review boards of National Cheng Kung University Hospital (NCKUH) and Keelung Chang Gung Memorial Hospital. Informed consent was obtained from each participant. Seventy-six patients with HBV-related liver cirrhosis and 115 with CHB were enrolled from outpatient clinics of both hospitals. They had detectable levels of HBV surface antigen (HBsAg) for >6 months. Baseline serum HBV DNA levels were >20 000 IU/mL in HBV e antigen (HBeAg)–positive patients and >2000 IU/mL in HBeAg-negative patients. Liver cirrhosis was diagnosed according to liver biopsy or classic ultrasound findings, combined with esophageal varices, gastric varices, or splenomegaly. Classic ultrasonography findings in liver cirrhosis include nodular contour and coarse echotexture of liver. All patients received anti-HBV NA treatment and attended regular follow-up visits for at least 48 weeks. A total of 108 healthy controls, who had normal serum alanine aminotransferase (ALT) levels but undetectable HBsAg, were enrolled from the Health Examination Center of NCKUH and were matched to patients with CHB on the basis of age and sex. All participants were negative for hepatitis C virus, human immunodeficiency virus, alcoholism- or autoimmune-induced liver diseases, biliary disorders, rheumatoid arthritis, juvenile-onset chronic arthritis, systemic lupus erythematosus, or Crohn disease. Serum samples from all patients were collected before and 48 weeks after initiation of treatment. Additional serum samples were obtained 4, 12, and 24 weeks after treatment initiation from 12 patients with CHB. All serum samples were stored at −80°C until use. In addition, baseline liver biopsy specimens obtained before treatment initiation from 66 patients with CHB were evaluated by a single experienced hepatopathologist. Liver necroinflammation and fibrosis stages were assessed according to the Knodell histology activity index and Ishak fibrosis scoring system, respectively.

HBV Virological and Serological Tests

Serum HBV DNA levels were assayed using the COBAS Amplicor/COBAS TaqMan HBV Test (Roche Diagnostics, Indianapolis, IN). Levels of serum ALT, aspartate aminotransferase (AST), albumin, and total bilirubin were determined using a modular analytics EVO analyzer (Modular DP; Roche Diagnostics). Serum IgG concentrations were detected using the Human IgG ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX). Blocking and sample dilution were performed using Superblock T20 (Thermo Fisher Scientific, Waltham, MA).

IgG Purification and In-Gel Trypsin Digestion

Serum IgG was purified by Protein G–Sepharose beads (GE Healthcare, Piscataway, NJ) and was then resolved using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein spot located between 50 and 55 kDa was in-gel digested by trypsin and stored at −80°C until use.

Liquid Chromatography–Mass Spectrometry (LC-MS) Analysis

A nano–high-performance liquid chromatography system (model 1200; Agilent, Santa Clara, CA) equipped with a C18 pre-column (300 μm × 5 mm, 5 μm) and a C18 nano-column (75-μm internal diameter × 15 cm, 3 μm; CVC Technologies, Fontana, CA) was coupled online to a mass spectrometer (LTQ-Orbitrap XL; Thermo Fisher Scientific). Mobile phase A was 0.1% fluoroacetic acid, and mobile phase B was 0.1% fluoroacetic acid in acetonitrile. The gradient consisted of (1) a linear increase from 2% to 40% B over 40 minutes, (2) a linear increase from 40% to 80% B over 10 minutes, and finally, (3) an isocratic elution at 80% B for 10 minutes at 300 nL/minute for separation. A survey MS spectrum (mass-to-charge ratio, 300–2000) with a mass resolution of 60 000 at a mass-to-charge ratio of 400 (with an ion target of 5×10^5 ions) was acquired, followed by 5 sequential collision-induced dissociation (CID)–MS^2 scans using the mass spectrometer in data-dependent mode. Selected ion chromatograms of different glycoforms

attached to the peptide backbone were extracted from the raw data. The peak height or peak area obtained from the extracted ion chromatogram of a particular glycoform of tryptic peptides was divided by the sum of all forms in the same LC-MS chromatogram. The percentage of each serum IgG glycoform from the average of 3 LC-MS runs was used for a relative comparison between healthy controls and patients. MS^2 spectra of each extracted glycopeptide were manually inspected to match all highly abundant product ions with a precursor ion mass accuracy of <5 ppm to confirm their assignments.

Opsonophagocytosis Assay

Human monocytic U937 cells (no. 60 435; Biological Resources Conservation and Research Center, Hsinchu, Taiwan, which originated from ATCC no. CRL-1593.2) were cultured in Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (Thermo Fisher Scientific), streptomycin (100 μg/mL), and penicillin (100 U/ml) at 37°C in 5% CO_2 for 1 week and were then maintained in serum-free medium (Macrophage-SFM; Invitrogen Life Technologies, Carlsbad, CA).

Cells were treated with 1 μg/mL of phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) for 72 hours to induce macrophage differentiation. Purified serum IgGs were incubated with fluorescent polystyrene latex beads (1 μm in diameter; Sigma-Aldrich) in phosphate-buffered saline (PBS) at 37°C for 1 hour. IgG-bead complexes were then incubated with adherent U937 macrophages (ratio of particles to cells, 5:1) at 37°C for 30 minutes. Bead internalization was synchronized using brief centrifugation. After the beads had been engulfed, the cells were trypsinized and washed 3 times with PBS to remove nonspecific bound beads. The geometric mean fluorescence intensity (GMFI) was measured using a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA). The opsonophagocytic activity was expressed as the GMFI difference (ΔGMFI) between the IgG-bead complexes of patient groups and the beads alone group, normalized to the ΔGMFI from the healthy control group.

Statistical Analysis

SPSS 17.0 for Windows was used for all statistical analyses. The χ^2 test was used for nominal variables. Continuous variables were compared using the Student *t* test or Mann–Whitney *U* test for 2 independent groups, a paired *t* test for 2 related groups, and one-way analysis of variance with Scheffe posterior comparison for 3 groups. Longitudinal data at different time points were analyzed using linear mixed-effects models. Multivariate logistic regression analysis was performed to evaluate possible factors involved in severe liver necroinflammation or fibrosis. The Pearson correlation coefficient (*r*) was used to evaluate the relationship between parameters. Significance was set at $P < .05$, and all reported *P* values are 2-tailed.

RESULTS

Serum IgG-Fc Glycoforms Identified Using Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS)

MS data indicated 10 serum glycoforms on asparagine 297 of IgG (Figure 1*A*). These glycopeptides were eluted from the liquid chromatography column after 21–23 minutes, and their identities were detected using high-resolution, accurate mass measurements (5 ppm, using Orbitrap). Moreover, MS^2 spectra

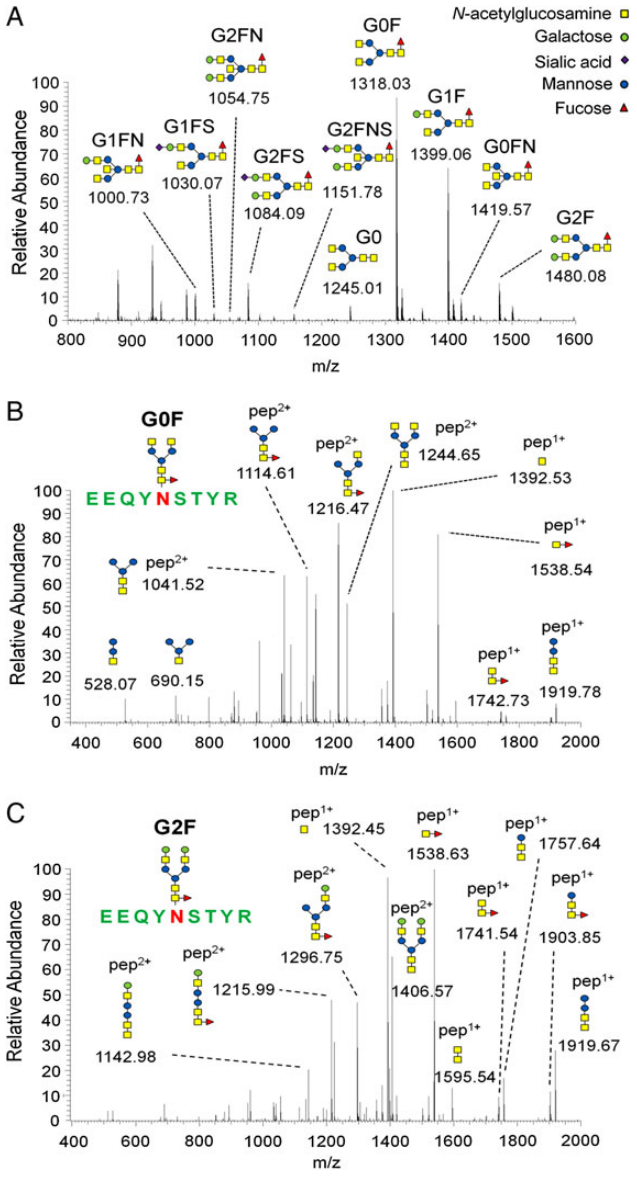

Figure 1. Identification by liquid chromatography–tandem mass spectrometry of 10 serum immunoglobulin G (IgG)–Fc glycoforms. *A*, The mass spectrum of trypsin-digested IgG showed ions with different glycoforms acquired during the liquid chromatography migration time (21–23 minutes). Moreover, each glycan structure was confirmed using the fragment ions generated by the collision-induced dissociation in fucosylated G0 (G0F; *B*) and fucosylated G2 (G2F; *C*).

Table 1. Baseline Clinical Data and Serum Immunoglobulin G_1 (IgG_1)–Fc Glycosylation Profiles of Patients With Hepatitis B Virus (HBV)–Related Liver Cirrhosis (LC), Patients With Chronic Hepatitis B (CHB), and Healthy Controls (HC)

Variable	LC (n = 76)	CHB (n = 115)	HC (n = 108)	*P* Value
Clinical data				
Sex, male:female	54:22	91:24	72:36	.107[b]
Age, y	55.6 ± 11.6	47.1 ± 11.1	45.4 ± 9.9	<.001
ALT level, U/L	106.8 ± 114.0	189.0 ± 167.8	21.0 ± 7.7	<.001
AST level, U/L	80.5 ± 71.6	93.0 ± 76.2	22.2 ± 4.3	<.001
Albumin level, g/dL	4.1 ± 0.4	4.4 ± 0.4	4.7 ± 0.3	<.001
Total bilirubin level, mg/dL	1.3 ± 0.9	1.0 ± 0.6	0.8 ± 0.4	<.001
Total IgG level, mg/mL	15.6 ± 6.0	12.5 ± 4.7	9.6 ± 3.3	<.001
HBV DNA level, log_{10} IU/mL	6.0 ± 1.4	7.3 ± 1.7	NA	<.05[a]
HBeAg, positive:negative	11:65	66:49	NA	<.001[b]
Child-Pugh score, A:B:C	72:2:2	NA	NA	NA
IgG glycoform, %				
Total G0	40.5 ± 9.0	39.5 ± 8.1	33.9 ± 7.0	<.001
G0F	34.4 ± 8.1	34.5 ± 7.6	27.9 ± 5.7	<.001
G0	2.4 ± 1.5	2.1 ± 1.6	2.9 ± 1.8	<.01
G0FN	3.7 ± 1.8	2.9 ± 1.7	3.2 ± 1.2	<.01
Total G1	38.2 ± 3.9	37.1 ± 3.4	40.4 ± 3.4	<.001
G1F	31.7 ± 3.8	31.6 ± 4.8	31.9 ± 4.2	.855
G1FN	5.5 ± 2.2	4.3 ± 2.6	7.0 ± 2.5	<.001
G1FS	1.0 ± 0.6	1.2 ± 1.0	1.5 ± 0.6	<.001
Total G2	21.3 ± 6.6	23.4 ± 6.5	25.6 ± 6.7	<.001
G2F	12.4 ± 4.4	12.5 ± 3.9	14.6 ± 3.3	<.001
G2FS	8.0 ± 2.9	9.5 ± 4.3	9.4 ± 5.3	<.05
G2FN	0.8 ± 0.6	1.2 ± 1.3	1.5 ± 0.7	<.001
G2FNS	0.1 ± 0.2	0.2 ± 0.3	0.2 ± 0.2	.527
Total N	10.1 ± 3.7	8.6 ± 4.6	11.8 ± 3.8	<.001
Total S	9.1 ± 3.1	10.8 ± 4.8	11.0 ± 5.6	<.05
G1 + G2/G0	1.6 ± 0.6	1.6 ± 0.6	2.1 ± 0.8	<.001

Data are mean ± standard deviation, unless otherwise indicated. One-way analysis of variance was used for comparisons among the HC, CHB, and LC groups, unless otherwise indicated.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; G, galactose; F, fucose; HBeAg, HBV e antigen; N, bisecting *N*-acetylglucosamine; NA, not available; S, sialic acid.

[a] By a 2-tailed independent *t* test.

[b] By the χ^2 test.

acquired from electron transfer dissociation confirmed the glycosylation site on asparagine 297 (data not shown). The major glycan fragments detected using CID-MS^2 spectra confirmed the linkages of each glycoform, such as the glycan structure for fucosylated G0 (G0F; Figure 1*B*) and fucosylated G2 (G2F; Figure 1*C*) as shown in the sample spectra.

Aberrant IgG_1-Fc Glycosylation in Patients with CHB was Reversible After Anti-HBV Nucleos(t)ide Analogue Therapy

There were no differences in the proportions of males and females between patients with liver cirrhosis, patients with CHB, and healthy controls (Table 1). Patients with liver cirrhosis were significantly older because of the natural course of chronic HBV infection (Supplementary Table 1). Higher levels of ALT, AST, and total IgG and a lower level of albumin were found in patients with CHB and liver cirrhosis, compared with healthy controls. Serum IgG_1-Fc glycosylation profiles showed that both patient groups had higher galactose-deficient (total G0) IgG levels, lower partially galactosylated (total G1) IgG levels and fully galactosylated (total G2) IgG levels, and lower IgG galactosylation indexes (G1 + G2/G0) than healthy controls. HBeAg-positive patients with CHB seemed to have a higher baseline galactosylation index than HBeAg-negative patients with CHB ($P = .063$; Supplementary Figure 1*A*). The major contribution of total G0 and G2 changes were by G0F and G2F, respectively. A lower total G1 level was contributed by G1F with bisecting GlcNAc (G1FN) or by G1F with sialic acid (G1FS). The levels of major and total bisecting GlcNAc IgG_1-Fc (G1FN and total N) were also significantly lower in both patient groups, particularly in the CHB group. The level of total IgG_1-Fc

Table 2. Clinical Data and Serum Immunoglobulin G_1 (IgG_1)–Fc Glycosylation Profiles of Patients With Hepatitis B Virus (HBV)–Related Liver Cirrhosis (LC) and Chronic Hepatitis B (CHB) at Baseline and After 48 Weeks of Treatment (W48-PT)

	LC (n = 76)			CHB (n = 115)		
Variable	Baseline	W48-PT	*P* Value	Baseline	W48-PT	*P* Value
Clinical data						
ALT level, U/L	106.8 ± 114.0	38.5 ± 23.3	<.001	189.0 ± 167.8	43.3 ± 39.4	<.001
AST level, U/L	80.5 ± 71.6	37.7 ± 19.0	<.001	93.0 ± 76.2	31.0 ± 18.3	<.001
Albumin level, g/dL	4.1 ± 0.4	4.3 ± 0.4	<.001	4.4 ± 0.4	4.5 ± 0.4	<.01
Total bilirubin level, mg/dL	1.3 ± 0.9	1.2 ± 0.4	.251	1.0 ± 0.6	1.0 ± 1.1	.847
Total IgG level, mg/mL	15.6 ± 6.0	15.2 ± 7.3	.696	12.5 ± 4.7	10.5 ± 3.6	<.001
HBV DNA level, log_{10} IU/mL	6.0 ± 1.4	0.4 ± 2.0	<.001	7.3 ± 1.7	2.0 ± 2.3	<.001
HBeAg, positive:negative	11:65	5:71	.186[a]	66:49	49:66	<.05[a]
Child-Pugh score, A:B:C	72:2:2	73:2:1	.844[a]	NA	NA	NA
IgG glycoform, %						
Total G0	40.5 ± 9.0	39.0 ± 9.1	.129	39.5 ± 8.1	33.7 ± 8.1	<.001
G0F	34.4 ± 8.1	32.9 ± 7.6	.105	34.5 ± 7.6	28.6 ± 7.0	<.001
G0	2.4 ± 1.5	2.4 ± 1.7	.921	2.1 ± 1.6	2.4 ± 1.6	<.05
G0FN	3.7 ± 1.8	3.7 ± 1.9	.995	2.9 ± 1.7	2.7 ± 1.7	.180
Total G1	38.2 ± 3.9	38.3 ± 4.8	.829	37.1 ± 3.4	37.9 ± 3.1	<.05
G1F	31.7 ± 3.8	30.9 ± 4.3	.109	31.6 ± 4.8	30.3 ± 3.5	<.01
G1FN	5.5 ± 2.2	6.2 ± 4.3	.176	4.3 ± 2.6	6.0 ± 3.5	<.001
G1FS	1.0 ± 0.6	1.2 ± 0.9	<.05	1.2 ± 1.0	1.5 ± 0.8	<.001
Total G2	21.3 ± 6.6	22.7 ± 7.9	.125	23.4 ± 6.5	28.4 ± 7.7	<.001
G2F	12.4 ± 4.4	11.9 ± 3.7	.166	12.5 ± 3.9	13.4 ± 3.8	<.01
G2FS	8.0 ± 2.9	9.7 ± 6.7	<.05	9.5 ± 4.3	13.0 ± 5.4	<.001
G2FN	0.8 ± 0.6	1.0 ± 1.3	.114	1.2 ± 1.3	1.7 ± 1.2	<.001
G2FNS	0.1 ± 0.2	0.1 ± 0.1	.578	0.2 ± 0.3	0.2 ± 0.3	.074
Total N	10.1 ± 3.7	11.0 ± 4.9	.141	8.6 ± 4.6	10.6 ± 4.8	<.001
Total S	9.1 ± 3.1	11.0 ± 7.1	<.05	10.8 ± 4.8	14.8 ± 5.4	<.001
G1 + G2/G0	1.6 ± 0.6	1.7 ± 0.6	.110	1.6 ± 0.6	2.2 ± 0.9	<.001

Data are mean ± standard deviation, unless otherwise indicated. *P* values for comparisons between baseline and W48-PT are from 2-tailed paired *t* tests.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; G, galactose; F, fucose; HBeAg, HBV e antigen; N, bisecting *N*-acetylglucosamine; NA, not available; S, sialic acid.

[a] By the χ^2 test.

sialylation (total S) was lower only in patients with liver cirrhosis, which primarily resulted from the decrease in the G2FS glycoform (Supplementary Table 1). IgG glycoforms between patients with compensated and those with decompensated cirrhosis were not compared because of the low number of patients with a Child-Pugh score of B (n = 2) or C (n = 2).

After 48 weeks of anti-HBV NA therapy, HBV DNA, ALT, and AST levels decreased and the albumin level increased in both patient groups (Table 2). In patients with CHB, the total G0 level of IgG-Fc reversed after treatment and resembled that in healthy controls (*P* = .842). Moreover, the total posttreatment G1, G2, S, and N levels of these patients increased. A 17.1% decrease in total G0 and a 19.5% increase in total G2, relative to their baseline level, were found in patients with CHB who were receiving antiviral treatment. A lower week-48 galactosylation index was found in HBeAg-negative patients than in HBeAg-positive patients, but it was not associated with HBeAg seroconversion (Supplementary Figure 1*A* and 1*B*). The magnitude of the change in IgG-Fc sialylation (total S, 37.0%) was even higher than that of galactosylation. In patients with liver cirrhosis, even with remarkably reduced levels of serum HBV DNA, ALT, and AST after treatment, only sialylated IgG glycoforms (G1FS, G2FS, and total S) were reversed (Table 2). These results showed that after 48 weeks of anti-HBV NA therapy, IgG-Fc glycosylation was robustly restored in patients with CHB but not in patients with liver cirrhosis.

We next analyzed the efficacies of various anti-HBV NAs on the restoration of IgG-Fc glycosylation in patients with CHB. Significant decreases in ALT, AST, IgG, HBV DNA, and IgG-Fc total G0 levels were found in both the entecavir monotherapy group and the lamivudine or adefovir dipivoxil monotherapy group (Table 3). In comparison to lamivudine or adefovir dipivoxil, entecavir had stronger efficacies on HBV suppression and the increase in IgG-Fc bisecting GlcNAc (G1FN, G2FN, and total N) but not on the restoration of IgG-Fc galactosylation.

Table 3. Effects of 48-Week Treatment With Nucleos(t)ide Analogues on Different Clinical Characteristics and Serum Immunoglobulin G_1 (IgG_1)–Fc Glycosylation Pattern in Patients With Chronic Hepatitis B

	Entecavir (n = 64)				Lamivudine or Adefovir Dipivoxil[b] (n = 51)				
Variable	Baseline	W48-PT	*P* Value[a]	Change	Baseline	W48-PT	*P* Value[a]	Change	*P* Value[c]
Clinical data									
Age, y	46.3				48.1				
ALT level, U/L	194.4	33.5	<.001	−160.9	182.1	55.5	<.001	−126.6	.277
AST level, U/L	98.6	28.7	<.001	−69.9	85.9	33.9	<.001	−52.0	.201
Total IgG level, mg/mL	12.5	11.1	<.05	−1.4	12.5	9.7	<.001	−2.8	.120
HBV DNA level, $\log_{10}$ IU/mL	7.8	1.9	<.001	−5.9	7.6	3.2	<.001	−4.4	<.001
Glycosyl patterns, %									
Total G0	40.4	33.9	<.001	−6.5	38.4	33.5	<.001	−4.9	.276
G0F	35.2	28.5	<.001	−6.7	33.6	28.7	<.001	−4.9	.209
G0	2.1	2.4	.134	0.2	2.1	2.5	<.05	0.4	.488
G0FN	3.1	3.1	.950	0	2.6	2.2	<.05	−0.4	.095
Total G1	37.4	38.5	<.01	1.2	36.8	37.1	.516	0.3	.183
G1F	32.0	30.1	<.01	−2.0	31.0	30.7	.481	−0.3	.079
G1FN	4.3	6.8	<.001	2.5	4.3	5.1	<.05	0.8	<.01
G1FS	1.0	1.7	<.001	0.7	1.4	1.4	.938	0	<.001
Total G2	22.3	27.5	<.001	5.3	24.8	29.4	<.001	4.6	.592
G2F	12.2	12.8	.204	0.5	12.9	14.3	<.01	1.4	.185
G2FS	8.5	12.4	<.001	3.9	10.8	13.9	<.01	3.1	.429
G2FN	1.4	2.1	<.001	0.7	1.0	1.2	.125	0.2	<.001
G2FNS	0.2	0.3	.077	0.1	0.1	0.1	.803	0	.059
Total N	9.0	12.3	<.001	3.4	8.1	8.5	.314	0.4	<.001
Total S	9.7	14.3	<.001	4.7	12.3	15.4	<.01	3.1	.165
G1 + G2/G0	1.6	2.2	<.001	0.6	1.7	2.1	<.001	0.4	.255

All data are mean values.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; G, galactose; F, fucose; HBV, hepatitis B virus; N, bisecting *N*-acetylglucosamine; S, sialic acid; W48-PT, week 48 after treatment initiation.

[a] Differences between baseline and W48-PT are from 2-tailed paired *t* tests.

[b] A total of 28 received lamivudine and 23 received adefovir dipivoxil.

[c] Differences between changes among entecavir recipients and changes among lamivudine or adefovir dipivoxil recipients are from 2-tailed independent *t* tests.

Table 4. Multivariate Logistic Regression Analyses of Serum Immunoglobulin G_1 (IgG_1)–Fc Glycoform for Liver Histological Damage in Patients With Chronic Hepatitis B

	Knodell Necroinflammation Score ≥7		Ishak Fibrosis Score ≥3	
Variable	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
Age, y	1.00 (.92–1.08)	.985	1.01 (.92–1.11)	.851
ALT level, U/L	1.00 (.97–1.03)	.974	0.98 (.95–1.01)	.151
AST level, U/L	1.03 (.97–1.09)	.390	1.06 (.99–1.13)	.071
Albumin level, g/dL	0.00 (.00–.09)	<.01	0.01 (.00–.56)	<.05
Total bilirubin level, mg/dL	3.03 (.51–17.81)	.221	1.78 (.31–10.18)	.518
HBV DNA level, $\log_{10}$ IU/mL	0.67 (.32–1.41)	.292	0.97 (.46–2.09)	.947
HBsAg level, IU/mL	0.66 (.18–2.37)	.525	0.42 (.09–2.06)	.285
HBeAg, positive:negative	2.49 (.25–24.99)	.437	1.53 (.10–23.06)	.758
G1F, %	0.81 (.63–1.04)	.101	1.22 (.92–1.61)	.170
G1FS, %	2.00 (.55–7.25)	.292	3.88 (.77–19.54)	.101
G2F, %	0.74 (.56–.97)	<.05	0.69 (.49–.97)	<.05

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; G, galactose; F, fucose; HBeAg, hepatitis B virus e antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; OR, odds ratio; S, sialic acid.

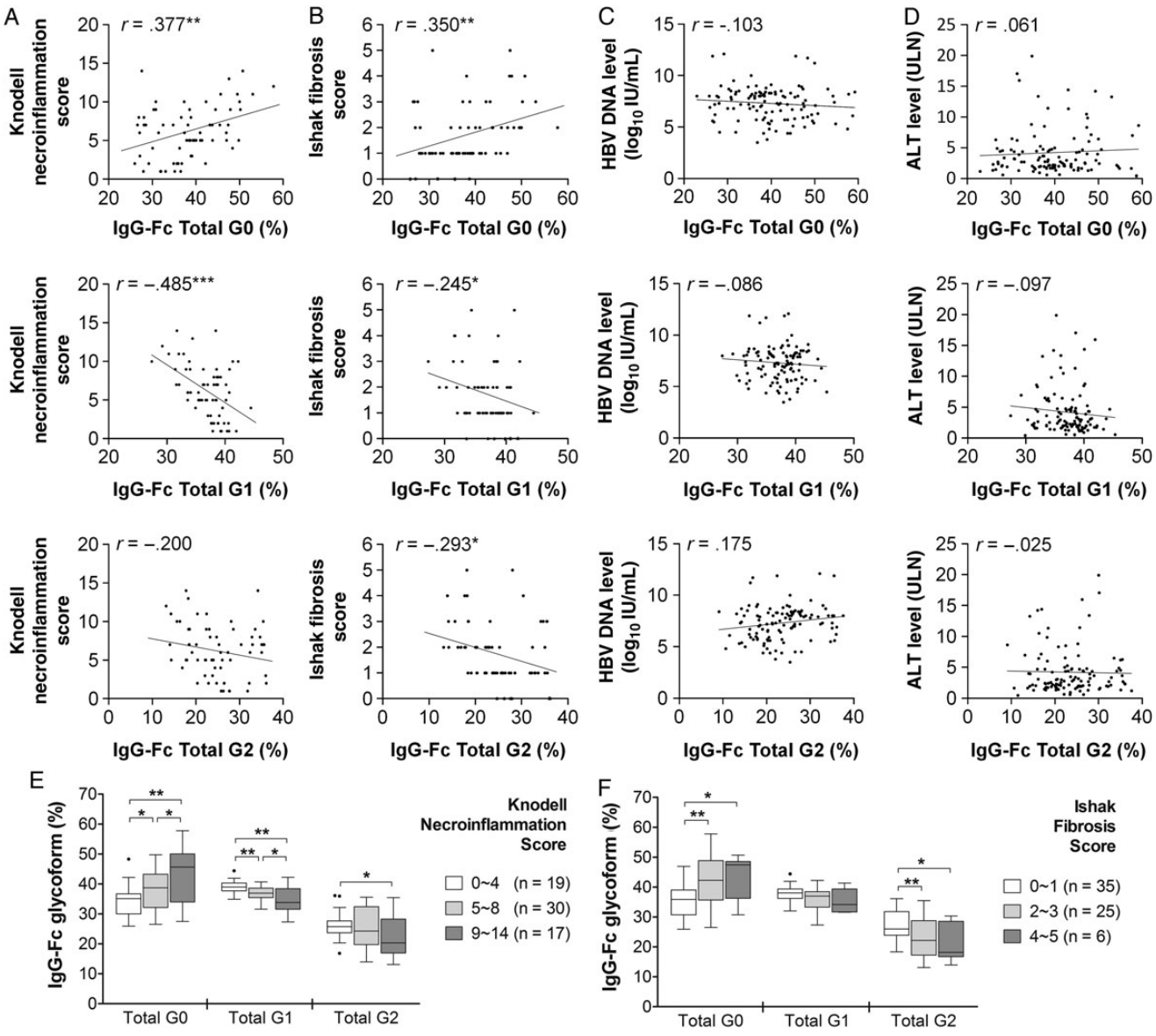

Figure 2. Associations of immunoglobulin G (IgG)–Fc glycosylation patterns and liver necroinflammation and fibrosis in patients with chronic hepatitis B. Correlations (coefficient *r* in scatterplots and regression lines) of IgG-Fc glycoforms to total Knodell necroinflammation scores (*A*), Ishak fibrosis scores (*B*), hepatitis B virus (HBV) DNA levels (*C*), and alanine aminotransferase (ALT) levels (*D*) were shown. Levels of serum IgG-Fc glycoforms in different total Knodell necroinflammation scores (*E*) and Ishak fibrosis scores (*F*) were shown as Tukey box plots. *P* values in panels *A*–*D* and those in panels *E* and *F* are from Pearson correlation tests and Mann–Whitney *U* tests, respectively. **P* < .05, ***P* < .01, and ****P* < .001. ULN, upper limit of normal.

Association Between Serum IgG-Fc Glycoform and Liver Necroinflammation and Fibrosis in Patients With CHB

Aberrant IgG-Fc glycosylation in patients with CHB was correlated with the severity of liver necroinflammation and fibrosis but not with the levels HBV DNA and ALT (Figure 2*A*–*D*). Patients with CHB who had more severe liver necroinflammation (total Knodell necroinflammation score, ≥9) showed a significantly higher IgG-G0 level than those with moderate (total score, 5–8) or mild (total score, ≤4) liver necroinflammation (Figure 2*E*). IgG total G1 and total G2 levels decreased as the severity of liver necroinflammation in these patients. Furthermore, patients with an Ishak fibrosis score of ≥2 had a significantly higher IgG-G0 level and a lower IgG-G2 level than those with a fibrosis score of ≤1 (Figure 2*F*). Multivariate logistic regression analyses, with factors that affect liver histology and were significant in a univariate logistic regression, were conducted to investigate factors involved in severe liver necroinflammation and fibrosis in 66 patients with CHB (Table 4). In addition to the albumin level, IgG-Fc G2F glycoform was associated with a total Knodell necroinflammation score of ≥7 (odds ratio, 0.74; 95% confidence interval, .56–.97) and Ishak fibrosis score of ≥3 (odds ratio, 0.69; 95% confidence interval, .49–.97). This result

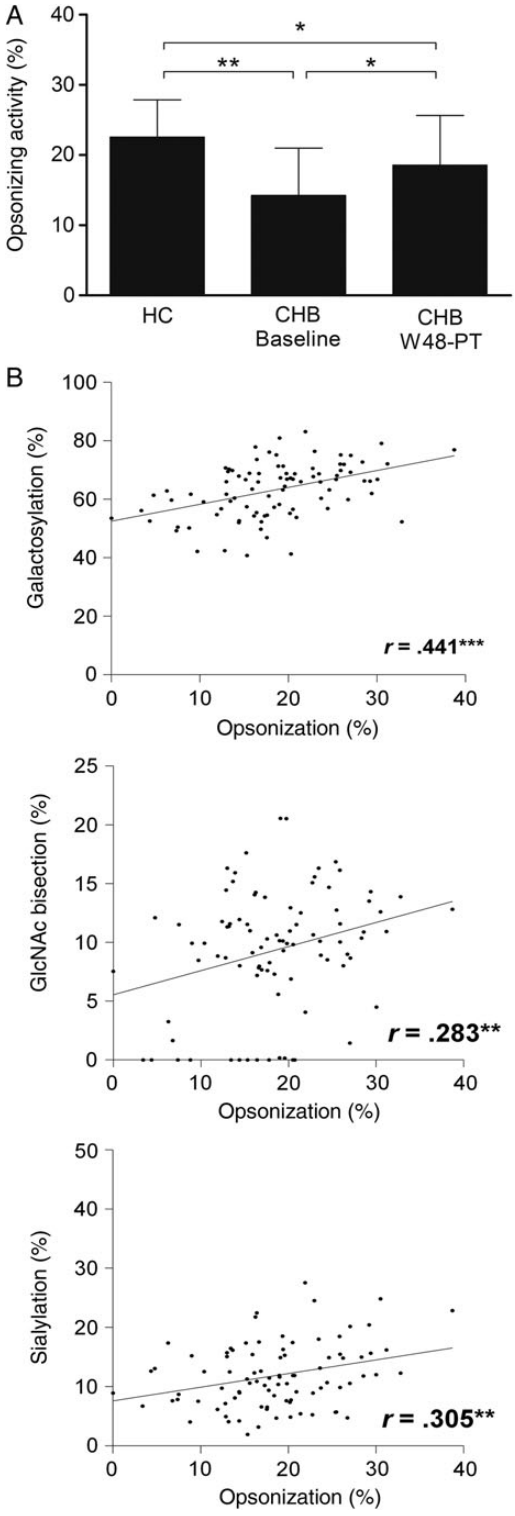

Figure 3. Reduced opsonophagocytic activity of serum immunoglobulin G (IgG) from patients with chronic hepatitis B. *A*, IgG opsonizing activities in 30 healthy controls (HC) and 30 patients with chronic hepatitis B (CHB) before and 48 weeks after treatment initiation (W48-PT) are shown. *B*, Correlations of serum IgG opsonizing activity and its glycan structures are shown. Values in all bar graphs are means ± SD. All *P* values are from 2-tailed independent or paired *t* tests (**P* < .05 and ***P* < .01). The coefficient *r* is from the Pearson correlation test (***P* < .01 and ****P* < .001).

revealed that a lower level of albumin or IgG-Fc G2F glycoform was related to high-grade liver necroinflammation and fibrosis during CHB.

IgG-Fc Glycosylation Dynamics in Patients With CHB During the Course of Anti-HBV NA Treatment

To comprehend the dynamics of the change in IgG_1-Fc glycosylation during antiviral treatment, we examined IgG_1-Fc glycosylation patterns at different time points in 12 randomly selected patients with CHB. Serum ALT, HBV DNA, and IgG levels in these patients were lower after 4 weeks of treatment (Supplementary Figure 2*A*). Interestingly, an alteration of IgG-Fc glycosylation was found at week 12 (Supplementary Figure 2*B*). The change in IgG_1-G1 levels was smaller and slower than that in IgG_1-G2 levels. The galactosylation index of IgG_1-Fc began to increase at week 12 (from 1.41 to 1.68; $P < .001$) and continued until week 48 (2.08; $P < .001$). The magnitude of the change in Fc galactosylation in IgG_2 during treatment was smaller overall than that of IgG_1 (Supplementary Figure 2*C*).

Attenuated IgG Opsonizing Activity Was Highly Associated With Aberrant Fc Glycosylation

To determine the immunomodulatory effect of aberrant IgG glycan composition during CHB, we performed an opsonophagocytic assay on purified serum IgG molecules from 30 healthy controls and 30 patients with CHB before and after 48 weeks of anti-HBV NA treatment. The serum IgG opsonizing activity in patients at baseline was significantly lower (at 63.1% of the value for controls; $P < .01$) than that in healthy controls, but it significantly rebounded (to 82.3% of the value for controls; $P < .05$) after 48 weeks of anti-HBV NA treatment (Figure 3*A*). The opsonophagocytic activity of IgG-Fc was significantly correlated with its level of galactosylation, GlcNAc bisection, and sialylation (Figure 3*B*).

DISCUSSION

In the present study, we found that aberrant serum IgG-Fc glycosylation during CHB was correlated with the severity of liver necroinflammation and fibrosis. Aberrant glycosylation of IgG was associated with its attenuated opsonizing activity and could be reversed after antiviral treatment in patients with CHB. The dynamic change in IgG-Fc glycosylation during the treatment course was illustrated. This is the first report to show that antiviral treatment reverses the aberrant glycosylation of IgG and increases its opsonizing activity during infection.

The Fc-glycan structure is pivotal for determining the binding tropism of IgG to FcγRs and the subsequent downstream immune activities [18]. The results of the opsonization assay suggest that even when the differences in IgG-Fc glycoforms during CHB are not large, their immunomodulatory effects are vast. HBeAg-negative patients with CHB seemed to have a

lower level of serum IgG-Fc galactosylation than HBeAg-positive patients with CHB because of a more progressive disease course. IgG-Fc galactosylation deficiency was significantly correlated with histological liver necroinflammation and fibrosis but not with HBV DNA or ALT levels. A limitation of this study was that histological data could not be obtained for each patient because of the invasiveness and possible complications of liver biopsy. Analysis of histological data from 66 patients implied that the change in IgG glycan composition represents a long-term deterioration of liver histology and immune dysfunction, rather than a current hepatocellular injury. As the HBV and ALT levels declined at week 4 of antiviral treatment, the reversal of aberrant IgG-Fc glycosylation was delayed until week 12. This probably occurred because of the long half-life of serum IgG (>20 days in most subclasses) or a gradual histological improvement after HBV has been suppressed. One year of antiviral therapy efficaciously reduced liver necroinflammation, but it reduced fibrosis only to a limited extent [19]; therefore, the reversal of aberrant IgG glycosylation after 48 weeks of treatment could be detected in patients with CHB but not in patients with cirrhosis. It takes a few years to reverse severe liver fibrosis and to improve the immune function of liver cirrhosis [20]. Hence, restored IgG-Fc glycosylation in patients with liver cirrhosis might be seen after years of treatment. Reports from clinical trials revealed that 48 weeks of entecavir therapy was more efficacious than lamivudine and adefovir dipivoxil for suppressing HBV and improving liver histology [19, 21, 22]. In the present study, entecavir more potently suppressed HBV but had a similar effect on the restoration of IgG-Fc galactose contents, compared to lamivudine and adefovir dipivoxil. More cases are needed to accurately evaluate the degree of improvement in liver histology after IgG-Fc galactosylation is restored.

An increase of serum β-galactosidase activity [23], alteration of β-1,4-galactosylransferase-1 activity in B cells [24], and overexpression of interleukin 6 [25] have been linked to an increase in IgG-G0. These mechanisms might regulate IgG galactose biosynthesis during sustained liver damage. Interestingly, Rademacher et al [26] reported that IgG-G0 is proinflammatory. IgG-G0 preferentially interacts with activating FcγRs and subsequently triggers downstream immune responses [18, 27]. Thus, CHB-induced IgG-G0 might form a vicious cycle and lead to progressive liver necroinflammation or fibrosis. In contrast to IgG-G0, fully galactosylated IgG (IgG-G2) is antiinflammatory because of its high affinity for FcγRIIB, the only known inhibitory FcγR in humans [28]. Because G2F is the major component of IgG-G2, its deficiency might lead to an impairment of the negative regulatory activity of the immune responses during CHB. The dysregulation of immune activity induced by aberrant IgG-Fc glycosylation, particularly galactose deficiency, might be crucial for the exacerbation of liver necroinflammation and fibrosis that arise from CHB or other etiologies. We were unable to draw any conclusion about a causal relation between serum IgG-Fc glycosyl modifications and liver histological damage, but we believe that they affect each other. Multivariate logistic regression analyses showed that a low G2F level was associated with high-grade liver necroinflammation and fibrosis, which suggested that IgG-Fc glycoform might be a potential noninvasive indicator of severe liver necroinflammation and fibrosis during CHB.

The etiology of attenuated opsonization during CHB [29] is unclear. Kumpel et al [30] showed that the opsonizing efficiency of anti-RhD monoclonal antibody decreased slightly after β-galactosidase treatment, which suggested the importance of galactosylation on IgG opsonizing properties. We found that IgG opsonizing activity was correlated not only with its Fc-galactosylation but also with GlcNAc bisection and sialylation, which suggested a complex glycan-dependent modulation of IgG on opsonization or other immune activities. Aberrant serum IgG-Fc glycosylation might be essential in the systemic immune dysfunction of patients with HBV-related liver diseases.

In conclusion, aberrant IgG-Fc glycosylation indicates histological liver damage and immune dysfunction in patients with CHB. Anti-HBV NA therapy reverses aberrant serum IgG-Fc glycosylation and attenuated IgG opsonizing activity.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Cheng-Hsun Ho was responsible for experimental design, experiment performance, clinical and IgG-Fc glycan data analyses, and manuscript writing; Cheng-Kun Liu and Chih-Sheng Su analyzed clinical data from patients with liver cirrhosis; Pin-Nan Cheng provided samples from healthy controls; Hung-Wen Tsai was responsible for liver histological data from patients with CHB; Jia-Huei Liu and I-Chen Li assisted in IgG sample preparation and clinical data arrangement; I-Chin Wu and Wen-Chun Liu assisted in statistical analysis; Shiaw-Lin Wu assisted in IgG-Fc glycan analysis; Shu-Hui Chen supervised the LC-MS/MS instrumentation and analyzed IgG-Fc glycan data; Rong-Nan Chien supervised the treatment and collected clinical data and serum samples from patients with liver cirrhosis; and Ting-Tsung Chang coordinated the study, supervised the treatment, collected clinical data and serum samples from patients with CHB, and composed the manuscript.

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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