respectively. With regard to the detection of significant fibrosis (F2-F4), its sensitivity was 44.4% and its specificity was 77.0%. Although even the excellent marker APRI shows low sensitivity (25.9%) for distinguishing patients with or without significant fibrosis, the combination of the APRI and GA/HbA1c ratio increased the sensitivity up to 42.0%, with only a modest decrease in the specificity (from 90.2% to 83.6%).

**CONCLUSION:** The GA/HbA1c ratio increased in line with the histological severity of liver fibrosis, thus suggesting that this ratio is useful as a supportive index of liver fibrosis.

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**Key words:** Glycated albumin; Glycated hemoglobin; Liver fibrosis; Liver biopsy; Hepatitis C virus

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## INTRODUCTION

Glycated proteins are known to reflect the plasma glucose level and glycated hemoglobin (HbA1c) is used as a standard index of glycemic control in patients with diabetes mellitus[1,2]. Since the lifespan of erythrocytes is about 120 d, HbA1c reflects the glycemia for the recent few months[3]. Glycated albumin (GA) is another index of glycemic control which correlates with the plasma glucose levels during the past few weeks because the turnover of albumin is about 20 d[4,5]. Although the ratio of GA/HbA1c is usually close to 3, the value changes based on the patient's condition[6]. In patients with chronic liver disease (CLD), hypersplenism causes a shortened lifespan of erythrocytes, leading to lower HbA1c levels relative to the plasma glucose level. In contrast, the turnover periods of serum albumin in CLD patients is prolonged in order to compensate for the reduced production of albumin. Therefore, the GA levels in CLD patients are higher relative to the degree of glycemia[6].

Since HbA1c shows lower and GA shows higher values in CLD patients, the GA/HbA1c ratio is thought to be high in patients with liver cirrhosis. Indeed, the GA/HbA1c ratio in patients with CLD has been reported to show an inverse correlation with some indicators of hepatic function (including the hepaplastin test, cholinesterase and bilirubin) independent of the mean plasma glucose levels, thus suggesting that the GA/HbA1c ratio increases as the liver cirrhosis progresses[7]. However, it has not been examined whether the GA/HbA1c ratio correlates with the histological fibrotic stage in CLD patients.

Hepatitis C virus (HCV) is one of the main causes of liver cirrhosis and hepatocellular carcinoma and knowledge about the progression of liver fibrosis is important. In the present study, we analyzed the relationship between the histological grading of liver fibrosis and the GA/HbA1c ratio in 142 patients with HCV-related CLD. Our findings suggest that the GA/HbA1c ratio is associated with the progression of liver fibrosis and cirrhosis in HCV-positive patients.

## MATERIALS AND METHODS

### Patients

We retrospectively studied HCV-positive CLD patients ($n$ = 142) who had undergone percutaneous liver biopsy between January 2008 and March 2010 at our institution who met the following conditions: (1) HCV infection diagnosed by detectable HCV antibodies and HCV RNA in serum; and (2) blood samples were obtained on the same day of the liver biopsies. Patients with the following conditions were excluded from the study: the presence of other liver diseases, hepatocellular carcinoma, immunosuppressive therapy, hepatitis B virus co-infection and those with insufficient liver tissue for staging of fibrosis. The present study did not include patients whose GA/HbA1c ratios could have been influenced by poorly controlled diabetes.

The routine studies, including platelet counts, prothrombin time international normalized ratio (PT-INR), liver functional tests [alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase and total bilirubin] were performed. Since the index calculated by the combination of GA and HbA1c (CLD-HbA1c: defined as the average of the measured HbA1c and GA/3) was reported to be a good indicator for the evaluation of the mean plasma glucose level in patients with CLD[8], HbA1c and GA were also routinely measured in all patients. The values of GA and HbA1c were determined in the same sample and on the same day as the liver biopsies were performed. The AST-to-platelet ratio index (APRI), an excellent marker for the evaluation of liver fibrosis, was also calculated based on the formula proposed by Wai *et al*[9]: APRI = [(AST level/upper limit of normal)/platelet counts ($10^9$/L)] × 100. Written informed consent regarding the liver biopsy and retrospective use of clinical data was obtained from all patients on admission. This study was approved by the ethics committees of the institutional review board.

### Liver biopsy

Liver biopsy examinations were performed using the

**Table 1 Characteristics of the patients**

| | |
|---|---|
| Age (yr) | 60 (19-78) |
| Gender (male/female) | 60/82 |
| AST (IU/L) | 37.5 (14-328) |
| ALT (IU/L) | 36 (10-388) |
| γ-GTP (IU/L) | 29 ( 7-259) |
| ALP (IU/L) | 217 ( 97-556) |
| Total bilirubin (mg/dL) | 0.7 (0.1-2.1) |
| Albumin (g/dL) | 3.96 ± 0.36 |
| Hemoglobin (g/dL) | 13.4 ± 1.8 |
| Platelet (× $10^4$/$mm^3$) | 15.9 ± 5.5 |
| PT-INR | 1.04 ± 0.07 |

AST: Aspartate aminotransferase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; PT-INR: Prothrombin time international normalized ratio.

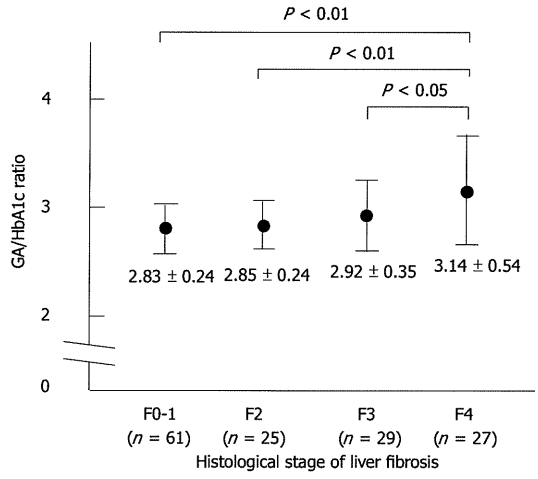


**Figure 1 The glycated albumin/glycated hemoglobin ratio in relation to the METAVIR fibrosis score in patients with hepatitis C virus-related chronic liver disease.** The glycated albumin (GA)/glycated hemoglobin (HbA1c) ratio increased as the fibrosis progressed. There was a significant difference between the F0-F1 *vs* F4, F2 *vs* F4, and F3 *vs* F4 groups.

standard procedures and all liver specimens were evaluated by well-trained pathologists at our institute, with evaluation of the fibrosis stage and activity grade according to the METAVIR scoring system[10]. Fibrosis was staged on a scale of 0-4 (F0: no fibrosis, F1: portal fibrosis without septa, F2: portal fibrosis with rare septa, F3: numerous septa without cirrhosis, F4: liver cirrhosis). The histological evaluation of the biopsy samples was also routinely performed in our department. All authors participated in the conference about the histological evaluation and the final results were confirmed by two authors (Enomoto H and Imanishi H) who received training for histological studies.

### Statistical analysis

In the present study, we attempted to clarify whether the GA/HbA1c ratio was associated with liver fibrosis and cirrhosis. The data for the comparisons among the groups "F0-1 *vs* F2 *vs* F3 *vs* F4" was analyzed by non-repeated measurements ANOVA and statistical significance was further examined by the Student-Newman-Keuls test. We compared the "F0-F3 (no cirrhosis) *vs* F4 (cirrhosis)", "F0-F2 (no - intermediate fibrosis) *vs* F3-F4 (severe fibrosis)" and "F0-F1 (no approximately minimal fibrosis) *vs* F2-F4 (significant fibrosis)" groups. The differences in the baseline characteristics and GA/HbA1c ratios of the groups were evaluated. Quantitative variables were expressed as the mean ± SD and those with an abnormal distribution were expressed as the median values (range). Statistical analysis was performed using Student's *t* test or the Mann-Whitney *U* test, as appropriate.

## RESULTS

### Characteristics of patients and clinical data

From January 2008 to March 2010, a total of 142 patients with HCV were consecutively included in the present study, based on the inclusion and exclusion criteria as described in the "Patients and Methods" section. The characteristics of the study population are summarized in Table 1. The population consisted of 60 (42%) males and 82 (58%) females, and the age of patients ranged from 19 to 78 years old (median 60). According to the METAVIR liver fibrosis staging[10], 56 (39%) patients had significant fibrosis (F3-F4) and 27 (19%) had cirrhosis (F4).

### The GA/HbA1c ratio in patients with HCV

The GA/HbA1c ratio in patients with CLD has been reported to show an inverse correlation with certain indicators of hepatic function. As shown in Figure 1, the mean values of the GA/HbA1c increased with the progression of the fibrosis stage, suggesting that the GA/HbA1c ratio was associated with the histological severity of liver fibrosis.

Comparing the F0-F3 (no cirrhosis) and F4 (cirrhosis) groups, we found that there was a significant difference in several parameters which correlated with hepatic function; that is, higher AST, ALT, γ-GTP alkaline phosphatase (ALP) and PT-INR levels and also a lower platelet count, and albumin values in the presence of cirrhosis (Table 2; left). However, no significant difference was observed in other parameters such as age and gender, which were not related to the hepatic function. Between the two groups, the GA/HbA1c ratio was significantly higher in patients with cirrhosis (Figure 2A), thus suggesting that the GA/HbA1c ratio is associated with the cirrhotic changes in the liver.

Next, we examined whether the GA/HbA1c ratio differed in patients with or without severe liver fibrosis. Comparing the F0-F2 (without severe fibrosis) and F3-F4 (with severe fibrosis) groups, we found significant differences, with higher AST, ALT, γ-GTP, ALP and PT-INR values and a lower platelet count, and albumin values in the presence of severe fibrosis (Table 2; middle). In patients with severe liver fibrosis, the GA/HbA1c ratio was significantly higher (Figure 2B) than that in patients without severe fibrosis, suggesting that the GA/HbA1c ratio

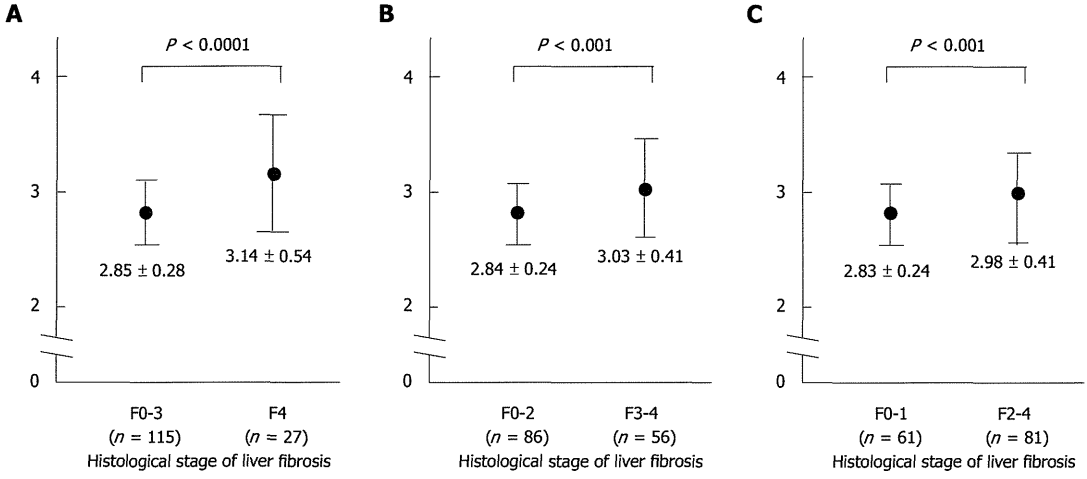


**Figure 2 The glycated albumin/glycated hemoglobin ratio in patients with hepatitis C virus-related chronic liver disease.** A: A comparison between the F0-F3 (no cirrhosis) group and F4 (cirrhosis) group. The glycated albumin (GA)/glycated hemoglobin (HbA1c) ratio was higher in patients with cirrhosis than that in non-cirrhotic patients; B: The comparison between the F0-F2 (no or intermediate fibrosis: without severe fibrosis) group and the F3-F4 (severe fibrosis) group. The GA/HbA1c ratio was higher in the patients with significant fibrosis than that in the patients with no or minimal fibrosis; C: A comparison between the F0-F1 (no or minimal fibrosis: without significant fibrosis) group and the F2-F4 (significant fibrosis) group. The GA/HbA1c ratio was higher in the patients with significant fibrosis than in those with either minimal fibrosis or none at all.

**Table 2 Characteristics of the patients (F0-F3 *vs* F4), (F0-F2 *vs* F3-F4) and (F0-F1 *vs* F2-F4)**

| | F0-F3 (*n* = 115) | F4 (*n* = 27) | *P* value | F0-F2 (*n* = 86) | F3-F4 (*n* = 56) | *P* value | F0-F1 (*n* = 61) | F2-F4 (*n* = 81) | *P* value |
|---|---|---|---|---|---|---|---|---|---|
| Age (yr) | 60 (19-78) | 62 (23-78) | NS | 60 (19-78) | 62 (23-78) | NS | 60 (19-78) | 62 (23-78) | NS |
| Gender (male/female) | 48/67 | 12/15 | NS | 31/55 | 29/37 | NS | 25/36 | 35/46 | NS |
| AST (IU/L) | 35 (14-195) | 50 (20-328) | < 0.001 | 32 (14-175) | 46 (20-328) | < 0.001 | 32 (14-104) | 42 (18-328) | < 0.001 |
| ALT (IU/L) | 38 (10-388) | 47 (10-310) | < 0.05 | 31.5 (10-388) | 48 (10-310) | < 0.01 | 31 (11-388) | 46 (10-310) | < 0.01 |
| γ-GTP (IU/L) | 25 ( 7-183) | 50 (12-259) | < 0.001 | 22 ( 7-183) | 42.5 (12-259) | < 0.0001 | 22 ( 8-183) | 36 ( 7-259) | < 0.01 |
| ALP (IU/L) | 207 ( 97-490) | 267 (133-556) | < 0.001 | 186 (97-465) | 275 (133-556) | < 0.0001 | 207 ( 97-465) | 258 (101-556) | < 0.001 |
| Total bilirubin (mg/dL) | 0.7 (0.1-1.6) | 0.7 (0.3-2.1) | NS | 0.7 (0.1-1.6) | 0.8 (0.3-2.1) | NS | 0.7 (0.1-1.6) | 0.7 (0.3-2.1) | NS |
| Albumin (g/dL) | 4.02 ± 0.31 | 3.70 ± 0.43 | < 0.001 | 4.03 ± 0.32 | 3.84 ± 0.37 | < 0.01 | 4.05 ± 0.31 | 3.89 ± 0.38 | < 0.01 |
| Hemoglobin (g/dL) | 13.5 ± 1.7 | 12.8 ± 2.0 | NS | 13.5 ± 1.8 | 13.3 ± 1.7 | NS | 13.7 ± 1.7 | 13.2 ± 1.8 | NS |
| Platelet ($\times 10^4$/mm$^3$) | 16.5 ± 5.3 | 13.2 ± 5.9 | < 0.001 | 17.2 ± 5.2 | 13.8 ± 5.5 | < 0.001 | 17.2 ± 4.8 | 14.9 ± 5.9 | < 0.05 |
| PT-INR | 1.03 ± 0.05 | 1.08 ± 0.06 | < 0.001 | 1.02 ± 0.05 | 1.07 ± 0.06 | < 0.001 | 1.02 ± 0.05 | 1.05 ± 0.08 | < 0.05 |

AST: Aspartate aminotransferase; ALT: Alanine transaminase; ALP: alkaline phosphatase; PT-INR: Prothrombin time international normalized ratio.

also correlates with the progression of liver fibrosis.

We also examined whether the GA/HbA1c ratio differed in patients with or without significant liver fibrosis. When we compared the F0-F1 (no or minimal fibrosis: without significant fibrosis) and F2-F4 (with significant fibrosis) groups, we also found significant differences, with higher AST, ALT, γ-GTP ALP and PT-INR values and a lower platelet count and albumin values in the presence of significant fibrosis (Table 2; right). In patients with significant liver fibrosis, the GA/HbA1c ratio was significantly higher than that in patients without significant fibrosis (Figure 2C).

Although the GA/HbA1c ratio is usually about 3, we found that the ratio increased in line with the progression of liver fibrosis (Figure 2). We therefore evaluated the diagnostic performance of the increased GA/HbA1c ratio (> 3.0) for the detection of patients with cirrhosis (F4), severe fibrosis (F3-F4) and significant fibrosis (F2-F4) (Table 3). Its sensitivity for the detection of liver cirrhosis was 16/27 (59.3%) and the specificity was 81/115 (70.4%). With regard to the detection of severe fibrosis, the sensitivity of the increased GA/HbA1c ratio (> 3.0) was 28/56 (50.0%) and its specificity was 64/86 (74.4%). With regard to the detection of significant fibrosis, the sensitivity of the increased GA/HbA1c ratio (> 3.0) was 36/81 (44.4%) and its specificity was 47/61 (77.0%).

### *Combination of the GA/HbA1c ratio and APRI for the detection of significant liver fibrosis*

As described above, the GA/HbA1c ratio in patients with significant liver fibrosis was higher than that in patients without significant fibrosis. However, the differences were small and the GA/HbA1c ratio had difficulty in distinguishing between F1 and F2.

Several biomarkers for the evaluation of fibrosis have

**Table 3 Glycated albumin/glycated hemoglobin ratio for the detection of cirrhosis (F4), severe fibrosis (F3-F4) and significant fibrosis (F2-F4) (%)**

| | F4 | F0-F3 | F3-F4 | F0-F2 | F2-F4 | F0-F1 |
|---|---|---|---|---|---|---|
| GA/HbA1c > 3.0 | 16/27 (59.3) | 34/115 (29.6) | 28/56 (50.0) | 22/86 (25.6) | 36/81 (44.4) | 14/61 (23.0) |
| GA/HbA1c ≤ 3.0 | 11/27 (40.7) | 81/115 (70.4) | 28/56 (50.0) | 64/86 (74.4) | 45/81 (55.6) | 47/61 (77.0) |

GA/HbA1c: Glycated albumin/glycated hemoglobin.

**Table 4 Aspartate aminotransferase-to-platelet ratio index for the detection of significant liver fibrosis (F2-F4)**

| | F2-F4 (%) | F0-F1 (%) | | F2-F4 (%) | F0-F1 (%) |
|---|---|---|---|---|---|
| APRI > 0.5 | 68/81 (84.0) | 32/61 (52.5) | APRI > 1.5 | 21/81 (25.9) | 6/61 (9.8) |
| APRI ≤ 0.5 | 13/81 (16.0) | 29/61 (47.5) | APRI ≤ 1.5 | 60/81 (74.1) | 55/61 (90.2) |

APRI: Aspartate aminotransferase-to-platelet ratio index.

**Table 5 Combination of aspartate aminotransferase-to-platelet ratio index and glycated albumin/glycated hemoglobin ratio for the detection of significant liver fibrosis (F2-F4)**

| | F2-F4 (%) | F0-F1 (%) | | F2-F4 (%) | F0-F1 (%) |
|---|---|---|---|---|---|
| APRI > 1.5 or GA/HbA1c > 3.0 | 43/81 (53.1) | 18/61 (29.5) | APRI > 1.5 or GA/HbA1c > 3.2 | 34/81 (42.0) | 10/61 (16.4) |
| Others | 38/81 (46.9) | 43/61 (70.5) | Others | 47/81 (58.0) | 51/61 (83.6) |

GA/HbA1c: Glycated albumin/glycated hemoglobin; APRI: Aspartate aminotransferase-to-platelet ratio index.

been reported previously and the APRI is a simple and useful marker for the prediction of significant fibrosis. We combined the GA/HbA1c ratio and the APRI in order to examine their utility for the detection of patients with significant liver fibrosis. At first, based on prior studies[9,11,12], we assessed two cut-off points (0.50 and 1.50) of the APRI to predict the absence or presence of significant fibrosis (Table 4). When we used the cut-off point as 0.5 (Table 4; left), the sensitivity was 68/81 (84.0%) and the specificity was 29/61 (47.5%). When we used the cut-off value of 1.5 (Table 4; right), the sensitivity was 21/81 (25.9%) and the specificity was 55/61 (90.2%). Therefore, as previously reported, the cut-off point of 1.50 had a high specificity but a low sensitivity to detect significant fibrosis.

We next asked whether a combination of the GA/HbA1c and the APRI could improve the sensitivity to detect the presence of significant fibrosis and help distinguish between the two groups (F0-F1 and F2-F4). When we examined the criteria "APRI >1.5 or GA/HbA1c ratio > 3.0", the sensitivity and the specificity for the detection of significant liver fibrosis was 43/81 (53.1%) and 43/61 (70.5%), respectively (Table 5; left). In addition, when we used the criteria "APRI >1.5 or GA/HbA1c ratio > 3.2", the sensitivity was 34/81 (42.0%) and the specificity was 51/61 (83.6%) (Table 5; right). Therefore, compared with the detection of significant liver fibrosis by using the APRI alone, the combination of GA/HbA1c and the APRI (APRI >1.5 or GA/HbA1c ratio > 3.2) improved the sensitivity from 25.9% to 42.0% without a major decrease in the specificity (only a modest reduction from 90.2% to 83.6% was observed).

## DISCUSSION

Liver biopsy is the gold standard method for histological evaluation of liver fibrosis[13]. Although a liver biopsy is generally a safe procedure, it is costly, invasive and has a small risk of complications. In addition, only 1/50 000 of the organ is removed and there can be sampling errors[13]. Furthermore, it has also been reported that there are inter- and intra-observer discrepancies of 10% to 20%[14,15]. Therefore, many noninvasive biomarkers readily available via laboratory tests have been proposed to predict the presence of significant fibrosis or cirrhosis in patients with HCV.

The Fibro-Test score is computed using the patient's age, sex and results of the analyses of serum haptoglobin, α2-macroglobulin, apolipoprotein A1, γ-GTP and bilirubin levels[16]. Forns *et al*[17] developed the Forns score, which is an algorithm including the platelet count, γ-GTP, age and cholesterol level. Wai *et al*[8] reported the APRI for fibrosis and cirrhosis prediction. In addition, some models such as the Hepascore[18], FibroMeter[19], FibroIndex[20] and FIB-4[21] have also been proposed for the evaluation of liver fibrosis. In addition, there are several noninvasive methods for the evaluation of liver fibrosis using ultrasound waves[22-26] such as Transient Elastography (FibroScan)[22,26]; SonoElastography (Real-Time Tissue Elastography)[23] and Acoustic Radiation Force Impulse[24-26]. Although each noninvasive tool has

an excellent positive predictive value for the diagnosis of moderate or significant fibrosis, none of the available methods completely meets the criteria of an ideal (simple, inexpensive and easily reproducible) method.

The Fibro-Test[16] is a combination of 6 markers and the Forns score[17] contains a complicated formula, indicating that while these markers are excellent, they lack simplicity. Recently introduced markers including APRI, FIB-4 and the FibroIndex are well-established, simple and inexpensive tools to assess liver fibrosis[9,20,21]. However, the values of these markers in one patient can vary within a short period, since the levels of AST or ALT or platelet count in the same patient often change daily. In addition, regarding APRI and FIB-4, the appropriate definition of the upper limit of normal (ULN) of the AST level remains uncertain, since each laboratory uses a different value for the ULN. With regard to the methods using special ultrasound tools, they are costly and cannot be routinely evaluated in all medical institutes.

In the present study, we have shown that the GA/HbA1c ratio of HCV-positive patients increases with the progression of liver fibrosis. Unlike the other previously established methods, the GA/HbA1c ratio is a simple and unique tool which is calculated based on the two glycated proteins and correlates with the degree of liver fibrosis. Since GA and HbA1c are stable over several weeks, the GA/HbA1c ratio does not change in a short period, resulting in a high reproducibility of its value. The stability of the two glycated proteins over weeks is a unique point, different from other biomarkers.

Bando *et al*[7] previously reported that the GA/HbA1c ratio in patients with CLD have an inverse correlation with the some indicators of hepatic function, regardless of the mean plasma glucose levels, thus suggesting that the increase of GA/HbA1c ratio indicates a reduction in the liver function caused by the progression of liver cirrhosis. Consistent with that report, our current histological evaluation revealed that the GA/HbA1c ratios of the cirrhotic patients were significantly higher than those of the patients without cirrhosis (Figure 2A). Furthermore, as shown in Figure 2B, the GA/HbA1c ratios increased in patients with severe fibrosis (F3-F4) compared to those in patients without severe fibrosis (F0-F2), thus suggesting that the GA/HbA1c ratio increased in correlation with the progression of fibrosis.

Since the GA/HbA1c ratio is usually about 3, we examined the diagnostic performance of the elevated GA/HbA1c ratio (GA/HbA1c > 3.0) and determined the sensitivity and specificity (Table 3). As described in the "Results" section, its solo diagnostic performance did not achieve satisfactory levels. However, when we combined the GA/HbA1c ratio with the APRI, the sensitivity to distinguish patients with significant fibrosis (F2-F4) from those without significant fibrosis was improved, with only a modest reduction in the specificity (Table 5). These findings suggest that the GA/HbA1c ratio can be used as a supportive index for the evaluation of liver fibrosis. Since only a small number of patients were investigated in the present study, we will therefore need to rigorously investigate the ratios in both larger and different populations.

In summary, we have shown that the GA/HbA1c ratio increases with the progression of the histological findings of liver fibrosis. However, its rate of change is relatively small. Although we have shown that the GA/HbA1c ratio improves the diagnostic performance of the APRI for the detection of significant fibrosis, it will be necessary to establish a new and better biomarker using a combination of the GA/HbA1c ratio and other parameter(s).

## COMMENTS

### Background

Hepatitis C virus (HCV) is one of the main causes of liver cirrhosis and hepatocellular carcinoma, and knowledge about the progression of liver fibrosis is important. Many noninvasive biomarkers readily available *via* laboratory tests have been proposed to predict the presence of significant fibrosis or cirrhosis in patients with HCV. The glycated albumin (GA)/glycated hemoglobin (HbA1c) ratio in patients with chronic liver disease (CLD) has been reported to show an inverse correlation with some indicators of hepatic function independent of the mean plasma glucose levels, thus suggesting that the GA/HbA1c ratio increases as the liver cirrhosis progresses. However, it has not been examined whether the GA/HbA1c ratio correlates with the histological fibrotic stage in CLD patients.

### Research frontiers

Liver biopsy is the gold standard method for histological evaluation of liver fibrosis. Although a liver biopsy is generally a safe procedure, it is costly, invasive and has a small risk of complications. It is very important to establish a simple, inexpensive and easily reproducible method for the evaluation of liver fibrosis.

### Innovations and breakthroughs

In the previous studies, many excellent noninvasive methods for the evaluation of liver fibrosis have been proposed. However, none of the available methods completely meets the criteria of an ideal (simple, inexpensive and easily reproducible) method. The present study has shown that the GA/HbA1c ratio of HCV-positive patients increases with the progression of liver fibrosis. Unlike the other previously established methods, the GA/HbA1c ratio is a simple and unique tool which is calculated based on the two glycated proteins and correlates with the degree of liver fibrosis.

### Applications

The study showed that the GA/HbA1c ratio increased in line with the histological severity of liver fibrosis, thus suggesting that this ratio is useful as a supportive index of liver fibrosis.

### Terminology

HbA1c is used as a standard index of glycemic control in patients with diabetes mellitus. Since the lifespan of erythrocytes is about 120 d, HbA1c reflects the glycemia for the recent few months; GA is another index of glycemic control which correlates with the plasma glucose levels during the past few weeks because the turnover of albumin is about 20 d.

### Peer review

The study focuses on the power of the GA/HbA1c ratio in estimation of liver fibrosis in people with HCV infection. Previously defined noninvasive fibrosis markers exist but none of them have proved to be equal to liver biopsy. Therefore, research on defining new but more effective fibrosis markers should be encouraged. People with HCV are always a good research base in this context. Therefore, the present study may be interesting for the readers.

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Original Paper

# Relationship between Elevation of Glycated Albumin to Glycated Hemoglobin Ratio in Patients with a High Bleeding Risk of Esophageal Varices

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**Key Words:** Liver cirrhosis; Portal hypertension; Esophageal varices; Glycated albumin; Glycated hemoglobin.

**Abbreviations:** Esophagogastroduodenoscopy (EGD); Glycated Albumin (GA); Chronic Liver Disease (CLD).

## ABSTRACT

**Background/Aims:** Variceal hemorrhaging due to portal hypertension is a severe complication of liver cirrhosis. Although several biomarkers have been reported as predictors of the presence of varices, it is still difficult to assess the risk of variceal bleeding without esophagogastroduodenoscopy (EGD). The ratio of glycated albumin (GA) to glycated hemoglobin (HbA1c) was reported to increase with the progression of liver fibrosis. The aim of the study was to investigate whether the GA/HbA1c ratio is related to the severity and bleeding-risk of the varices. **Methodology:** We measured the GA/HbA1c ratio of HCV-related cirrhotic patients with Child-Pugh class A status and analyzed its relationship with the presence and bleeding risk of varices. **Results:** The GA/HbA1c ratio was higher in the patients who had the varices with a high risk of hemorrhage than in the patients with a low risk of bleeding. In addition, the GA/HbA1c ratio was higher in patients with varices than that in patients without varices. Furthermore, the GA/HbA1c ratio was the most significantly different parameter of all the factors examined, including the platelet count, prothrombin activity and albumin level. **Conclusions:** The GA/HbA1c ratio is increased in patients with varices and with the bleeding risk of the varices.

## INTRODUCTION

Portal hypertension is a major complication of liver cirrhosis and can be a direct cause of variceal hemorrhage and of bleeding-related death. Therefore, esophagogastroduodenoscopy (EGD) is considered to be necessary for all cirrhotic patients to evaluate the risk of variceal bleeding (1-3). Three factors identify patients at a high risk of bleeding from varices: a large variceal size, red signs on the varices and advanced liver disease (Child-Pugh class B or C) (4). Several biochemical parameters have been reported as predictors of the presence of varices, such as a low platelet count, an advanced Child-Pugh class, hypoalbuminemia and low prothrombin activity (5-8). However, it is still difficult to predict the presence of varices without EGD. In addition, with regard to compensated cirrhotic patients with well-maintained liver function (Child-Pugh class A), the differences in the biochemical data between the patients with treatment-requiring high-risk varices and those with a low risk of hemorrhage have not yet been clarified.

Glycated proteins are known to reflect the plasma glucose level and glycated hemoglobin (HbA1c), is commonly used as an index of glycemic control in patients with diabetes mellitus (9,10). Since the lifespan of erythrocytes is about 4 months, the HbA1c level is correlated with the level of glycemia for the past few months (11). Another glycated protein, glycated albumin (GA), reflects the plasma glucose level during the past few weeks, because the turnover of albumin is about 3 weeks (12,13). Although the ratio of GA/HbA1c is usually close to 3, patients with chronic liver disease (CLD) have a shortened lifespan of erythrocytes due to the hypersplenism, thus resulting in lower HbA1c levels relative to the plasma glucose level. Conversely, the turn-over period of serum albumin in CLD patients is increased to compensate for the reduced albumin production. Therefore, the GA levels in CLD patients are higher relative to the degree of glycemia (14).

Since the HbA1c level is lower and the GA shows higher values, the GA/HbA1c ratio is assumed to be increased in CLD patients, especially in cirrhotic patients. In fact, the GA/HbA1c ratio in patients with CLD has been reported to show a reciprocal correlation with some indicators of hepatic function, irrespective of the mean plasma glucose levels (15). We previously examined the relationship between the histological grading of liver fibrosis and the GA/HbA1c ratio in patients with HCV-related

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CLD, and showed that the GA/HbA1c ratio was correlated with the progression of liver fibrosis (16).

In the present study, we analyzed the GA/HbA1c ratio of HCV-related compensated cirrhotic patients with Child-Pugh Class A disease. The GA/HbA1c ratio was higher in patients with varices than that in patients without varices. We also found the GA/HbA1c ratio to be higher in patients with high-risk varices than that in patients with a low risk of variceal bleeding.

## METHODOLOGY

EGD was routinely performed in CLD outpatients at our institution according to the standard procedures. If esophageal varices were detected, their size was graded as I-IV based on the Paquet grading system (17) and the presence of red signs on the varices was evaluated. Patients with large varices (grade III-IV) or small varices with red signs were categorized to be high-risk (treatment-requiring) varices. All HCV-related compensated (Child-Pugh class A) cirrhotic patients admitted to our department for the treatment of esophageal varices from June 2008 to June 2011 were included in the present study as the "high-risk varices group". Cirrhosis as the cause of portal hypertension was diagnosed by histological criteria and/or by the clinical (laboratory, endoscopic and/or ultrasonographic) findings.

In order to investigate the GA/HbA1c ratio in the Child-Pugh class A cirrhotic patients without high-risk varices, we included consecutive HCV-positive CLD patients who were histologically diagnosed with cirrhosis by a liver biopsy at our institution, but who did not have (high-risk) varices which required treatment. The evaluation by EGD was performed within two months of the liver biopsy. Liver biopsy examinations were performed using the standard procedures and all liver specimens were evaluated by well-trained pathologists at our institute, with evaluation of the fibrosis stage and activity grade according to the METAVIR scoring system (18), and patients with the F4 stage were defined as having liver cirrhosis. The cirrhotic patients who did not have high-risk varices were divided into two groups; patients without detectable varices were categorized as the "no varices group" and patients with small varices without red signs were defined as the "low-risk varices group".

The HCV infection was diagnosed by detecting HCV antibodies and HCV-RNA in the serum. Blood samples were obtained to perform routine studies, including those of the platelet counts, prothrombin time and liver functional tests (ALT, AST, alkaline phosphatase and total bilirubin). In addition, the HbA1c and GA levels were also measured in all patients. All blood samples were obtained on the day of liver biopsy or endoscopic treatment for esophageal varices. Patients with the following conditions were excluded from the study: the presence of other liver diseases, immunosuppressive therapy, hepatitis B virus co-infection and insufficient liver tissue available for evaluation of liver fibrotic staging. The present study did not include patients whose GA/HbA1c ratios could have been influenced by poorly controlled diabetes. The study conformed to the ethical guidelines of the Helsinki declaration and written informed consent regarding the liver biopsy and the use of all clinical data was obtained from all patients on admission.

### Statistical analysis

In the present study, we investigated whether the GA/HbA1c ratios differed between the three groups ("no varices group", "low-risk varices group" and "high-risk varices group"). The data for the comparisons among the groups were analyzed by non-repeated measurements ANOVA and statistical significance was further evaluated with the Bonferroni correction. In addition, we examined whether the GA/HbA1c ratios differed between the groups with or without varices. The differences in the baseline characteristics of the two groups were also evaluated and we also evaluated whether the GA/HbA1c ratios could differentiate between the groups with or without high-risk varices. Quantitative variables were expressed as the mean values ±SD and those with an abnormal distribution were expressed as the median values (range). The statistical analysis was performed using Student's *t*-test or the Mann-Whitney U test, as appropriate.

## RESULTS

### Characteristics of patients and clinical data

Of the CLD outpatients in our department, 60 patients were found to have a high risk of variceal bleeding because they had large varices (grade III-IV) or small varices with red signs and 32 of these patients were HCV-positive. In the HCV-positive cirrhotic patients, the Child-Pugh classification was grade A in 12 patients, grade B in 19 patients and grade C in one patient. The 12 patients with Child-Pugh class A were enrolled as the "high-risk varices group" (HCV-related compensated cirrhotic patients with a high risk of variceal hemorrhage). In order to obtain the characteristics of compensated cirrhotic patients with a low risk of variceal bleeding, we examined the patients who underwent a liver biopsy as described in the methodology section. Out of a total of 184 HCV-positive patients who received a liver biopsy, 35 patients were categorized as compensated (Child-Pugh class A) cirrhotic patients with a low risk of variceal bleeding; 23 patients were diagnosed with liver cirrhosis (METAVIR score F4) without detectable varices ("no varices group") and the remaining 12 patients had small varices without red signs ("low-risk varices group"). The characteristics of all 47 patients evaluated in the present study are shown in **Table 1**. The population consisted of 29 (62%) males and 18 (38%) females and the age of patients ranged from 23 to 82 years old (median 64). No patients showed any clinical symptoms of hepatic encephalopathy. In the "high-risk varices group", two patients (2/12: 17%) had mild ascites, whereas neither the "no varices group" nor the "low-risk varices group" included patients with ascites.

**TABLE 1.** Patient characteristics.

| | |
|---|---|
| Age (years) | 64 (23-82) |
| Gender (Male / Female) | 29/18 |
| Ascites (Present/Absent) | 2/45 |
| AST (IU/L) | 47 (20-328) |
| ALT (IU/L) | 38 (10-310) |
| γ-GTP (IU/L) | 48 (12-259) |
| ALP (IU/L) | 279 (133-556) |
| Total bilirubin (mg/dL) | 0.8 (0.3-2.1) |
| Albumin (g/dL) | 3.68±0.37 |
| Hemoglobin(g/dL) | 12.5±1.8 |
| Platelet ($x10^4/mm^3$) | 10.8±3.3 |
| Prothrombin time (%) | 85.6±10.3 |

**The GA/HbA1c ratio and other parameters in patients with HCV-related cirrhosis**

The GA/HbA1c ratio in patients with CLD has been reported to show an inverse correlation with several parameters of hepatic function (15). In the compensated cirrhotic patients, the mean values of the GA/HbA1c ratio were elevated in patients with an increased bleeding risk, suggesting that the GA/HbA1c ratio reflects the severity of portal hypertension (**Figure 1**).

We also examined whether the GA/HbA1c ratio differed in patients with or without varices. Comparing the 25 patients with varices (the "high-risk varices group" and the "low-risk varices group") and 22 patients without varices (the "no varices group"), the GA/HbA1c ratio was significantly higher in patients with varices than that in patients without varices, suggesting that the increased GA/HbA1c ratio also correlates with the presence of varices (**Figure 2A**). We found significant differences between the groups, with a higher total bilirubin value and a lower platelet count, hemoglobin value and prothrombin time in the presence of varices. Interestingly, the GA/HbA1c ratio was the most significantly different parameter ($p$=0.0009, <0.001) out of all of the factors examined (**Table 2**).

When we compared the 12 patients in the "high-risk varices group" with the 35 patients in the "no varices group" or the "low-risk varices group", the GA/HbA1c ratio was significantly higher in patients in the "high-risk varices group" (**Figure 2B**), suggesting that the GA/HbA1c ratio is associated with an increased risk of variceal hemorrhage. We also found that there was a significant difference in the hemoglobin and albumin levels in the "high-risk varices group". However, no significant difference was observed in any other parameter, although there was a trend for there to be more advanced liver disease (lower platelet count and prothrombin time; higher total bilirubin level) in the "high-risk varices group". Also, in the comparison between the groups with or without varices (**Table 2**), it was observed that the GA/HbA1c ratio was the most significantly different factor ($p$=0.009, <0.01) of all of the parameters examined (**Table 3**).

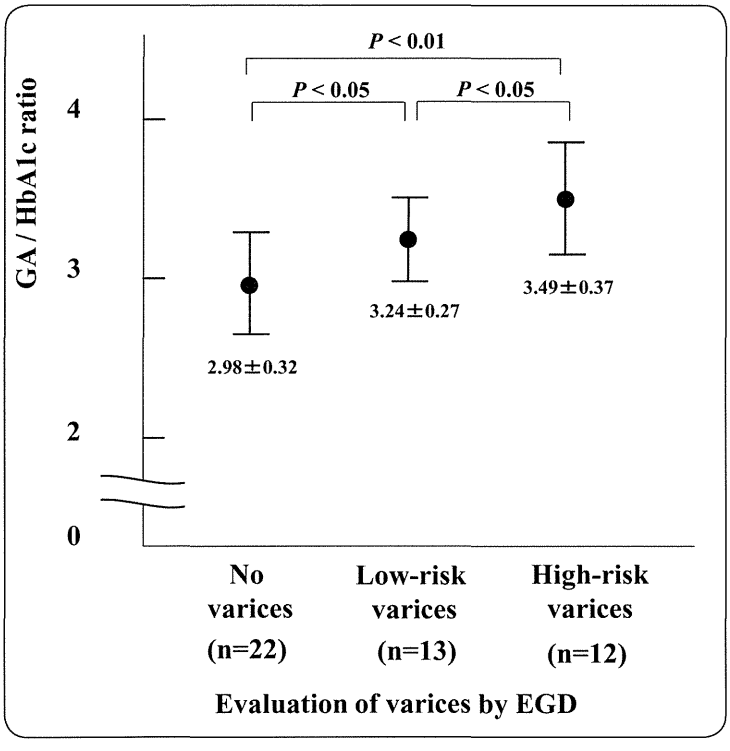


**FIGURE 1.** The GA/HbA1c ratio in patients with HCV-related compensated (Child-Pugh class A) cirrhosis. The GA/HbA1c ratio increased as the bleeding risk of esophageal varices increased. There was a significant difference between the "no varices group" *vs.* the "low-risk varices group", the "no varices group" *vs.* the "high-risk varices group" and the "low-risk varices group" *vs.* the "high-risk varices group.

**TABLE 2.** Characteristics of the patients with or without varices.

| | Varices detected by EGD | | |
|---|---|---|---|
| | **Absent (n=22)** | **Present (n=25)** | ***p* value** |
| Age (years) | 62.5 (23-78) | 67 (31-82) | NS |
| Gender (Male/ Female) | 11 / 11 | 18/ 7 | NS |
| AST (IU/L) | 47 (20-328) | 47 (27-251) | NS |
| ALT (IU/L) | 46.5 (10-310) | 37 (18-160) | NS |
| γ-GTP (IU/L) | 55.5 (12-259) | 41 (12-242) | NS |
| ALP (IU/L) | 268 (133-556) | 310 (181-556) | NS |
| Total bilirubin (mg/dL) | 0.65 (0.3-2.1) | 0.9 (0.3-2.1) | NS |
| Albumin (g/dL) | 3.81±0.32 | 3.57±0.39 | <0.05 |
| Hemoglobin (g/dL) | 13.1±1.8 | 11.9±1.5 | <0.05 |
| Platelet (x$10^4$/$mm^3$) | 12.4±3.3 | 9.4±2.8 | <0.01 |
| Prothrombin time (%) | 89.0±9.8 | 82.6±9.9 | <0.05 |
| GA/HbA1c ratio | 2.98±0.32 | 3.36±0.34 | <0.001 |

## DISCUSSION

EGD is the gold standard method for evaluating varices to determine whether a patient should be treated to prevent a first variceal hemorrhage (1-3,8). However, the need for repeating EGD is a burden for the patient with a high cost and a small, but significant, risk of complications. In addition, endoscopy has a relatively high interobserver variability for the diagnosis of small varices (2). Therefore, many non-invasive or minimally invasive methods have been proposed to evaluate the presence/size of such varices. For example, a low platelet count has been consistently reported to be associated with the presence of varices or of large varices (5-8,19-22). In addition, many other tools, such as the Fibro-Test score (23,24), transient elastography (FibroScan) (25-28), multi-detector CT (MDCT) (29-32) and capsule endoscopy (33-37), have been proposed to predict the presence or size of the varices. However, none of the available methods completely meets the criteria of an ideal (accurate, simple, inexpensive and easily reproducible) method (8,38).

Furthermore, previous reports regarding these non-invasive methods have not mentioned their predictive performance of the "red signs" on varices, although they are also important predictors of bleeding, as well as the size of the varices. Despite the fact that "small varices with severe red signs" have the same chance of bleeding as "large varices without red signs" (39), none of the non/minimally invasive methods, except capsule endoscopy, can evaluate patients for "red signs", thus indicating that they fail to detect small varices that are associated with a high risk of bleeding.

In the present study, we have shown that the GA/HbA1c ratio in HCV-positive cirrhotic patients (Child-Pugh class A) increases with the severity of the esophageal varices. The GA/HbA1c ratio is a simple and unique tool which is calculated based on the levels of the two glycated proteins and is associated with the endoscopic findings of esophageal varices. In particular, the GA/HbA1c ratio was found to be significantly increased in the pa-

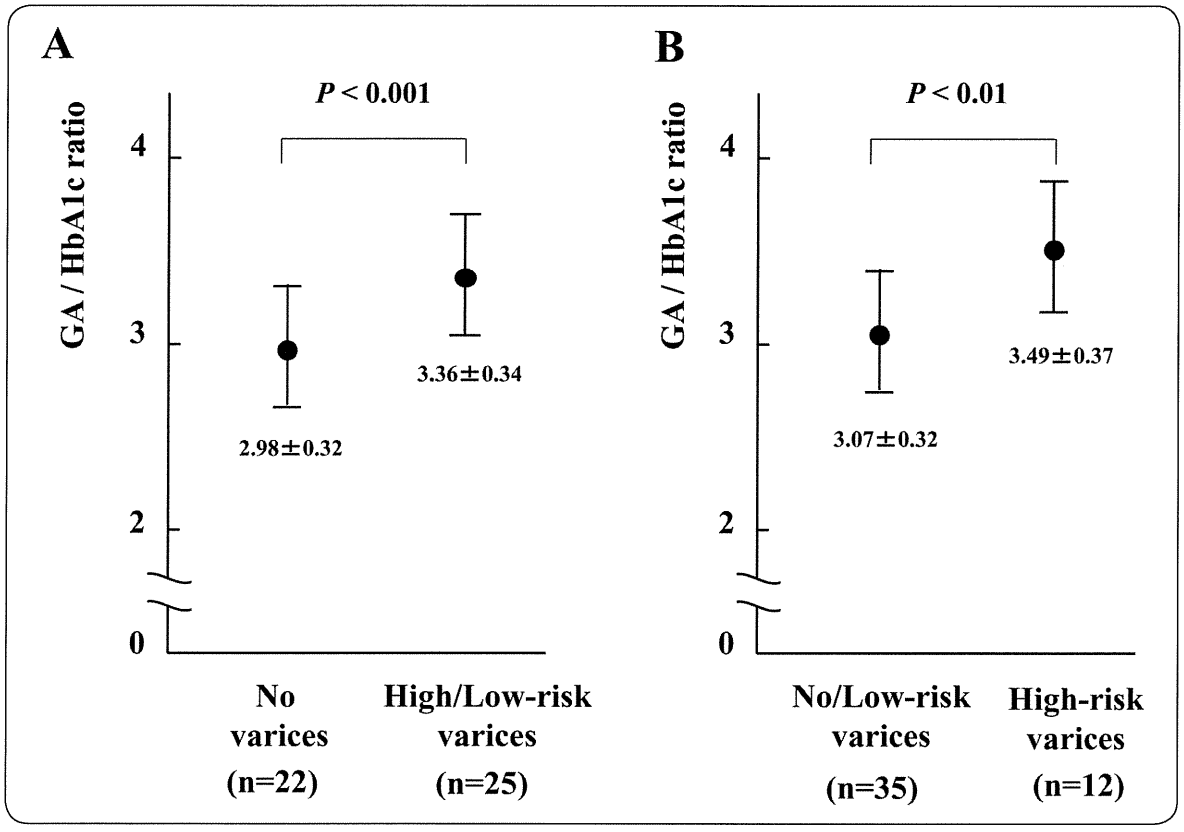


**FIGURE 2.** (**A**) The GA/HbA1c ratio in patients with HCV-related compensated (Child-Pugh class A) cirrhosis with or without varices. A comparison between the 25 patients in the "high-risk varices group" or the "low-risk varices group" and the 22 patients in the "no varices group". The GA/HbA1c ratio was higher in the patients with varices than that in the patients without varices. (**B**) The GA/HbA1c ratio in patients with HCV-related compensated (Child-Pugh class A) cirrhosis with or without high-risk varices. A comparison between the 12 patients in the "high-risk varices group" and the 35 patients in the "no varices group" or the "low-risk varices group". The GA/HbA1c ratio was higher in patients with high-risk varices than that in patients without high-risk varices.

tients with high-risk varices. Our findings should be of interest in that the ratio is elevated in the patients with a high risk of bleeding, including patients who have small varices with "red signs". Although the rate of change was relatively small, using the ratio may help predict the presence of any varices and discrimination of low-risk from high-risk of varices, because the GA/HbA1c ratio was the most significantly different among all of the parameters tested (**Tables 2** and **3**).

Bando *et al.* (15) have previously reported that the GA/HbA1c ratio in patients with CLD has an inverse correlation with some indicators of hepatic function, regardless of the mean plasma glucose level, suggesting that the increase in the GA/HbA1c ratio reflects the reduction of liver function caused by the progression of liver cirrhosis. In addition, we found that the GA/HbA1c ratio was elevated with the histological grade of liver fibrosis in patients with HCV-related CLD (16).

In the present study, all patients were classified as cirrhotic patients with a well-maintained liver function (Child-Pugh class A). Therefore, the elevation of the GA/HbA1c ratio may reflect the severity of the portal hypertension, rather than the progression of liver fibrosis. However, the degree of the portal hypertension does not always correlate with the size of varices and the risk of bleeding and future studies will be needed to clarify the reason for this discrepancy.

In summary, we have shown that the GA/HbA1c ratio increases with the progression of the severity and bleeding risk of the varices. Since only a small number of patients were enrolled in the present study, it will be necessary to investigate the GA/HbA1c ratio in both larger and different populations.

**TABLE 3.** Characteristics of the patients with or without high-risk varices.

| | High risk varices detected by EGD | | |
|---|---|---|---|
| | **Absent (n=35)** | **Present (n=12)** | ***p* value** |
| Age (years) | 64 (23-78) | 71.5 (31-82) | NS |
| Gender (Male/ Female) | 19/ 16 | 10 / 2 | NS |
| AST (IU/L) | 44 (20-328) | 48.5 (27-68) | NS |
| ALT (IU/L) | 42 (10-310) | 35.5 (18-86) | NS |
| γ-GTP (IU/L) | 54 (12-259) | 34 (12-159) | NS |
| ALP (IU/L) | 269 (133-556) | 304 (191-462) | NS |
| Total bilirubin (mg/dL) | 0.7 (0.3-2.1) | 1.0 (0.6-1.4) | NS |
| Albumin (g/dL) | 3.74±0.39 | 3.53±0.28 | <0.05 |
| Hemoglobin(g/dL) | 12.8±1.7 | 11.4±1.4 | <0.05 |
| Platelet (x$10^4$/$mm^3$) | 11.2±3.4 | 9.5±3.1 | NS |
| Prothrombin time (%) | 86.4±10.6 | 83.2±9.3 | NS |
| GA/HbA1c ratio | 3.07±0.32 | 3.49±0.37 | <0.01 |

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# Original Article

# The glycated albumin to glycated haemoglobin ratio increases along with the fibrosis stage in non-alcoholic steatohepatitis

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## Abstract

**Background:** We previously reported that the indicator of glycaemic control, glycated albumin (GA) levels, are low in relation to glycaemia in patients with high alanine aminotransferase (ALT) levels in non-alcoholic fatty liver disease because of chronic inflammation, and that the GA/glycated haemoglobin ratio (G/H ratio) is inversely correlated with hepatic function in patients with chronic liver disease. The severity of liver fibrosis is known to be a good indicator for surveillance, and for determining the prognosis and optimal treatment of non-alcoholic steatohepatitis (NASH). In this study, we aimed to investigate the clinical usefulness of measuring the G/H ratio for predicting the severity of liver fibrosis in patients with NASH.
**Methods:** The study subjects were 36 patients with histologically diagnosed NASH (19 men, 17 women; mean age 54.8 $\pm$ 12.2 years, body mass index 28.3 $\pm$ 5.0 kg/m$^2$). The relationships of the G/H ratio to hepatic function tests and fibrosis stage in the liver were investigated.
**Results:** The G/H ratio in patients with NASH was inversely correlated with ALT ($P < 0.001$) and platelet count ($P < 0.0001$). Furthermore, the G/H ratio was positively correlated with the fibrosis stage in liver ($P = 0.003$).
**Conclusions:** These results suggest that the G/H ratio increases along with the fibrosis stage in patients with NASH.

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## Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease, affecting up to 20% of the population in Western countries, and 70–80% of obese individuals.[1,2] The incidence of NAFLD is increasing due to the ongoing epidemic of its two major risk factors, obesity and type 2 diabetes mellitus, related to a sedentary lifestyle and inadequate dietary choices. It encompasses a spectrum of distinct histological entities with different natural histories and outcomes, ranging from simple fat accumulation in hepatocytes to liver steatosis accompanied by a microinflammatory component that may have associated fibrosis. In contrast to simple steatosis, which is defined as a benign form of NAFLD with minimal risk of progression, non-alcoholic steatohepatitis (NASH) can progress to liver cirrhosis in up to 20% of patients and subsequently lead to hepatic failure or hepatocellular carcinoma.[3,4]

Diabetic patients are known to show greater glycation of various proteins than non-diabetic subjects and it has been suggested that some of these glycated proteins are involved in the onset and development of the chronic complications of diabetes.[5] One of these glycated proteins, glycated haemoglobin ($HbA_{1c}$), is often used in clinical settings as an indicator of glycaemic control.[6,7] Since the lifespan of erythrocytes is approximately 120 days, $HbA_{1c}$ reflects the glycaemic control status of the previous 2–3 months. However, measurement of $HbA_{1c}$ is affected in diseases in which the erythrocyte lifespan is shortened, such as haemolytic anaemia and renal anaemia, and with variant haemoglobins, and thus $HbA_{1c}$ does not accurately reflect the glycaemic control status in such cases.[8,9]

Glycated albumin (GA) is used as another glycaemic control indicator.[6] Since the half-life of serum albumin is shorter than that of erythrocytes, at 14 days, GA reflects plasma glucose concentrations over a shorter period (approximately two weeks). Moreover, GA has a benefit in that its measurements are not affected by haemoglobin disorders.[10] However, measurements of GA are affected in patients with disorders

of albumin metabolism, such as in nephrotic syndrome or thyroid dysfunction, and do not correctly reflect the glycaemic control status in such cases.[11]

It has been recently demonstrated that GA is low relative to plasma glucose concentrations in obese patients with diabetes.[12] We observed a significant negative correlation between body mass index and GA even in non-diabetic subjects.[13] We also demonstrated a significant negative correlation between highly sensitive C-reactive protein (hs-CRP) and GA, and that hs-CRP was a significant negative explanatory variable for GA in a multivariate analysis.[13] From the finding that hs-CRP is elevated as a result of accelerated release of various cytokines in obesity, we hypothesized that the negative regulatory mechanism of GA resulting from obesity is based on accelerated albumin catabolism accompanied by chronic microinflammation.[13] We have also reported low levels of GA by similar mechanisms in smoking,[14] hyperuricaemia[15] and hypertriglyceridaemia,[16] which are representative conditions causing elevated hs-CRP. High levels of hs-CRP have also been reported in NAFLD patients with high alanine aminotransferase (ALT).[17,18] We reported GA concentrations to be low in relation to glycaemia in patients with high ALT without a drinking habit.[19]

It has been demonstrated that patients with chronic liver disease (CLD) have apparently low $HbA_{1c}$[20,21] due to a shortened lifespan of erythrocytes that is caused by hypersplenism. Furthermore, since the ability of albumin synthesis is impaired and the half-life of serum albumin is prolonged in patients with CLD,[22] their GA concentrations appear to be high relative to glycaemia.[23] Therefore, the GA/$HbA_{1c}$ ratio (G/H ratio) in patients with CLD was shown to be correlated with hepatic function irrespective to glycaemia.[24]

The severity of liver fibrosis is known to be a good indicator for surveillance, and for determining the prognosis and optimal treatment of NASH. In this study, we aimed to investigate the clinical usefulness of measuring the G/H ratio for predicting the severity of liver fibrosis in patients with NASH.

## Methods

### Study patients

GA and $HbA_{1c}$ were simultaneously measured in 36 patients with NASH (Table 1) undergoing treatment at Fukui-ken Saiseikai Hospital or Hyogo College of Medicine. Among the 36 patients, 26 patients (72%) displayed complications of type 2 diabetes mellitus. Patients with unstable glycaemic control were excluded. The diagnosis of NASH was made histologically, and the fibrosis stage was classified as S1–S4 according to the method of Brunt *et al.*[25] This study was approved by the ethics committee at Fukui-ken Saiseikai Hospital and Hyogo College of Medicine. The purpose of the study was explained to all patients, and all patients provided their written informed consent.

### Laboratory measurements

$HbA_{1c}$ was measured by high-performance liquid chromatography, with calibration using Japan Diabetes Society (JDS) Lot 2.[26] The value for $HbA_{1c}$ (%) was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated using the formula $HbA_{1c}$ (%) = $HbA_{1c}$ (Japan Diabetes Society: JDS) (%) + 0.4%, considering the relational expression of $HbA_{1c}$ (JDS) (%) measured using previous Japanese standard substances and measurement methods and $HbA_{1c}$ (NGSP).[27] Serum GA was determined by enzymatic methods using albumin-specific protease, ketoamine oxidase and albumin assay reagent (Lucica GA-L; Asahi Kasei Pharma, Tokyo, Japan).[28] GA was hydrolysed to amino acids by an albumin-specific proteinase and then oxidized by ketoamine oxidase to produce hydrogen peroxide, which was measured quantitatively.

### Statistical analysis

All data are shown as mean ± SD. The significance of the differences between the two groups was calculated using Student's *t*-test. To evaluate the relationship between the G/H ratio and other variables, Spearman's correlation coefficient was performed. The relationship between the stages of fibrosis on histology and the G/H ratio was tested by analysis of variance with StatView (Version 5.0 for Windows; Abacus Concepts, Berkeley, CA, USA) software. A $P$ value $<0.05$ was considered significant.

## Results

The clinical characteristics of the study patients classified by the stage of fibrosis are shown in Table 1. Serum ALT,

**Table 1** Clinical characteristics of the study patients classified by the stage of fibrosis*

| | S1 | S2 | S3 | S4 | *P* |
|---|---|---|---|---|---|
| *n* (M/F) | 7 (5/2) | 17 (8/9) | 6 (3/3) | 6 (3/3) | 0.546 |
| Age (y) | 53.0 ± 10.3 | 50.2 ± 3.1 | 63.5 ± 4.0 | 60.8 ± 3.6 | 0.051 |
| BMI (kg/m$^2$) | 28.7 ± 3.6 | 28.9 ± 5.7 | 26.9 ± 4.8 | 27.5 ± 5.3 | 0.099 |
| DM (%) | 3 (43) | 12 (71) | 5 (83) | 5 (83) | 0.252 |
| Serum ALT (U/L) | 80.9 ± 40.0 | 58.1 ± 30.3 | 51.2 ± 25.0 | 42.2 ± 16.9 | 0.026 |
| Platelet ($\times 10^3/\mu$L) | 20.9 ± 3.1 | 25.5 ± 5.1 | 18.6 ± 5.7 | 11.8 ± 1.4 | 0.001 |
| Hb (g/dL) | 15.2 ± 1.7 | 14.5 ± 1.0 | 13.5 ± 1.6 | 12.6 ± 1.6 | 0.001 |
| $HbA_{1c}$ (%) | 6.4 ± 0.7 | 6.3 ± 0.7 | 7.6 ± 1.3 | 7.0 ± 1.6 | 0.077 |
| GA (%) | 15.5 ± 2.5 | 14.8 ± 2.4 | 20.0 ± 3.4 | 19.5 ± 5.4 | 0.004 |
| G/H ratio | 2.43 ± 0.25 | 2.37 ± 0.28 | 2.62 ± 0.13 | 2.79 ± 0.27 | 0.003 |

BMI, body mass index; DM, diabetes mellitus; ALT, alanine aminotransferase; Hb, haemoglobin; $HbA_{1c}$, glycated haemoglobin; GA, glycated albumin; G/H, GA/$HbA_{1c}$

*The fibrosis stage was classified as S1–S4 according to the method of Brunt *et al.*[25]

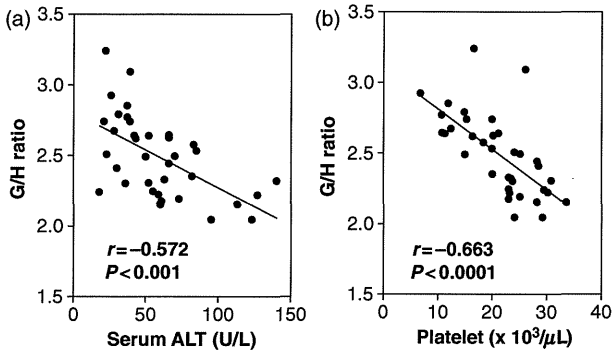


**Figure 1** Correlation between the GA/$HbA_{1c}$ (G/H) ratio and serum ALT (a) and platelet count (b) in patients with NASH. $HbA_{1c}$, glycated haemoglobin; GA, glycated albumin; NASH, non-alcoholic steatohepatitis; ALT, alanine aminotransferase

platelet count and haemoglobin decreased with the progression of fibrosis. On the other hand, higher GA, but not $HbA_{1c}$, was seen with the progression of fibrosis. Higher G/H ratios were also seen with the progression of fibrosis (Table 1). A significant inverse correlation was seen between the G/H ratio and serum ALT in patients with NASH ($R = -0.572$, $P < 0.001$) (Figure 1a). The G/H ratio was also inversely correlated with platelet count ($R = -0.663$, $P < 0.0001$) (Figure 1b).

## Discussion

A significant inverse correlation was seen between the G/H ratio and serum ALT in patients with NASH. We previously reported that serum ALT showed significant inverse correlations with both GA and the G/H ratio in patients with NAFLD.[19] In those patients, hs-CRP had a significant positive correlation with serum ALT and a significant inverse correlation with GA,[19] indicating the possibility that GA values declined as a result of accelerated albumin metabolism due to chronic inflammation[29] in patients with NAFLD with high serum ALT. This suggests that GA concentrations are set lower relative to plasma glucose concentrations because of hepatic inflammation in the early stages of NASH, resulting in lower G/H ratios.

The G/H ratio in patients with NASH showed a significant inverse correlation with platelet count, which reflects hepatic function. A significant increase in the G/H ratio was also seen with progressing fibrosis stage histologically. While there was an apparent decrease in $HbA_{1c}$ and an apparent increase in GA in patients with CLD, we previously showed that these indicators diverged markedly with the state of glycaemic control as hepatic function decreased.[23] We also showed that the G/H ratio was related to hepatic function irrespective to glycaemia in patients with CLD.[24] Therefore, it is thought that in patients with NASH, hepatic function declines with the progression of histological findings including fibrosis, finally reaching a state of cirrhosis, while the G/H ratio becomes higher.

The above results are thought to indicate that the G/H ratio in patients with NASH declines in the early stage of the disease, but increases with the progression of the disease, and becomes high in the final stage. These results suggest that the G/H ratio is associated with the progression of fibrosis in patients with NASH. Since other fibrosis markers (hyaluronic acid and type IV collagen) were measured only in a small number of the patients in this study, statistical analysis was difficult to compare the G/H ratio with these markers. The relation between the G/H ratio and such fibrosis markers will need to be studied in future.

### DECLARATIONS

**Competing interests:** None.
**Funding:** No funding was required in this research.
**Ethical approval:** The ethics committee of Fukui-ken Saiseikai Hospital and Hyogo College of Medicine approved this study.
**Guarantors:** KaN and S-hN.
**Contributions:** MK wrote the first draft of the manuscript. YB, DT, KaN, NT, S-hN, KeN, HN and SK edited and contributed to the final manuscript. HK, KA, NT and HE looked after the patients. All authors reviewed and edited the manuscript and approved its final version.
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# Interferon-Gamma–Mediated Tissue Factor Expression Contributes to T-Cell-Mediated Hepatitis Through Induction of Hypercoagulation in Mice

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**Concanavalin A (Con A) treatment induces severe hepatitis in mice in a manner dependent on T cells, interferon (IFN)-gamma, and tumor necrosis factor (TNF). Treatment with the anticoagulant heparin protects against hepatitis, despite healthy production of IFN-γ and TNF. Here, we investigated molecular and cellular mechanisms for hypercoagulation-mediated hepatitis. After Con A challenge, liver of wild-type (WT) mice showed prompt induction of *Ifnγ* and *Tnf*, followed by messenger RNA expression of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1), which initiate blood coagulation and inhibit clot lysis, respectively. Mice developed dense intrahepatic fibrin deposition and massive liver necrosis. In contrast, *Ifnγ*$^{-/-}$ mice and *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice neither induced *Pai1* or *Tf* nor developed hepatitis. In WT mice TF blockade with an anti-TF monoclonal antibody protected against Con A–induced hepatitis, whereas *Pai1*$^{-/-}$ mice were not protected. Both hepatic macrophages and sinusoidal endothelial cells (ECs) expressed *Tf* after Con A challenge. Macrophage-depleted WT mice reconstituted with hematopoietic cells, including macrophages deficient in signal transducer and activator of transcription-1 (STAT1) essential for IFN-γ signaling, exhibited substantial reduction of hepatic *Tf* and of liver injuries. This was also true for macrophage-depleted *Stat1*$^{-/-}$ mice reconstituted with WT macrophages. Exogenous IFN-γ and TNF rendered T-cell-null, Con A–resistant mice deficient in recombination-activating gene 2, highly susceptible to Con A–induced liver injury involving TF. *Conclusions*: Collectively, these results strongly suggest that proinflammatory signals elicited by IFN-γ, TNF, and Con A in both hepatic macrophages and sinusoidal ECs are necessary and sufficient for the development of hypercoagulation-mediated hepatitis.** (HEPATOLOGY 2013;57:362-372)

Concanavalin A (Con A)-induced hepatitis is a well-characterized, representative mouse model of T-cell-mediated acute liver failure.[1] After Con A challenge, mice show elevation of circulating proinflammatory cytokine levels, subsequently resulting in massive liver necrosis with dense infiltration of leukocytes. Because interferon (IFN)-γ or tumor necrosis factor (TNF) blockade and gene depletion of *Ifnγ* or

*Abbreviations: Abs, antibodies; ALI, acute liver injury; ALT, alanine aminotransferase; B6, C57BL/6; BM, bone marrow;* Ccl2, *CC chemokine ligand 2 gene clodronate liposome, liposome-encapsulated dichloromethylene bis-phosphonate; Con A, concanavalin A; ECs, endothelial cells; H&E, hematoxylin and eosin; IFN, interferon; IFNAR1, IFN-α receptor 1; IgG, immunoglobulin G; IHC, immunohistochemistry;* Il6, *interleukin-6 gene;* Il1β, *interleukin-1β gene; IP, intraperitoneal; IV, intravenously; KO, knockout; Mø, macrophages; mAb, monoclonal antibody; mRNA, messenger RNA; PAI-1, plasminogen activator inhibitor-1; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time reverse-transcriptase polymerase chain reaction; RAG2, recombination-activating gene 2; rIFN-γ, recombinant IFN-γ; rRNA, ribosomal RNA; rTNF, recombinant TNF; SC, subcutaneously; SEC, sinusoidal endothelial cells; STAT1, signal transducer and activator of transcription 1; TAT, thrombin antithrombin III complex; TF, tissue factor; TNF, tumor necrosis factor; tPA, tissue plasminogen activator; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphage nick-end labeling; WT, wild type.*

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**These authors equally contributed to the present study.*

*Tnf* rescues mice from Con A–induced hepatitis,[2,3] IFN-$\gamma$ and TNF are convincingly regarded as the cytokines necessary for the development of this type of liver injury. Thus, one may assume that endogenous IFN-$\gamma$ and TNF initiate both the hepatic inflammatory responses and the liver parenchymal cell death.[4] However, as previously reported, massive liver necrosis is accompanied by severe thrombocytopenia and intrahepatic hemostasis, and heparin pretreatment substantially protects against liver injury without down-regulating the production of IFN-$\gamma$ and TNF.[2] This may imply that microcirculatory disturbances resulting from hepatic thrombosis contribute to liver injury independent of IFN-$\gamma$/TNF-mediated hepatic inflammation and hepatocytotoxicity. Alternatively, IFN-$\gamma$ and/or TNF might be causative for hepatic thrombosis, perhaps by inducing procoagulant activity within the liver. Thus, it is important to elucidate whether and how IFN-$\gamma$ and/or TNF contribute to the hepatic hypercoagulation and whether IFN-$\gamma$ and/or TNF are sufficient to trigger these pathological changes.

Tissue factor (TF) is a transmembrane cofactor for the coagulation factor, VIIa, and is constitutively expressed in the blood vessel wall and its expression is induced by various mediators in several cell types including macrophage (Mø) and endothelial cells (ECs).[5-7] Endothelial damage or TF expression on circulating monocytes/macrophages brings TF in contact with circulating factor VIIa to initiate the blood coagulation cascade, which eventually results in the activation of prothrombin, leading to fibrin formation and platelet activation. The coagulation system is tightly regulated by the fibrinolytic system, which comprises plasminogen, the tissue-type plasminogen activator (tPA), and its inhibitor, plasminogen activator inhibitor-1 (PAI-1).[8,9] *Pai1*$^{-/-}$ mice have been reported to be resistant to alcohol-induced or cholestatic liver injuries.[10-12] Therefore, it is possible that PAI-1 as well as TF may play a role in coagulation-mediated liver injuries.

In this study, we investigated the mechanisms by which Con A treatment induces the prothrombotic state. We found strong induction of hepatic *Tf* and *Pai1* expressions, dense hepatic fibrin deposits, and massive liver necrosis in Con A–treated wild-type (WT) mice, but not in *Ifn*$\gamma^{-/-}$ *Tnf*$^{-/-}$ mice. TF blockade protected WT mice from the intrahepatic fibrin deposition and resultant hepatitis. Both hepatic macrophages (Mø) and sinusoidal ECs (SECs) expressed *Tf* in Con A–challenged WT mice. IFN-$\gamma$ signaling was crucial for *Tf* induction in both these cell types. Con A–resistant mice that have Mø and SECs, but not T cells, became highly susceptible to Con A when treated simultaneously with IFN-$\gamma$ and TNF. Collectively, these results indicate that IFN-$\gamma$-, TNF-, and Con A–activated signaling pathways in hepatic Mø and SECs are necessary and sufficient for the development of intrahepatic hemostasis-mediated massive liver injuries.

## Materials and Methods

***Reagents.*** Con A was purchased from J-Oil Mills (Tokyo, Japan). Neutralizing rat antimouse TF monoclonal antibody (mAb) (1H1) was described elsewhere.[13] Purified rat immunoglobulin G (IgG) was purchased from Beckman Coulter (Fullerton, CA). Recombinant murine IFN-$\gamma$ and TNF were from PeproTech (Rocky Hill, NJ). Liposome-encapsulated dichloromethylene bis-phosphonate (clodronate liposome) and phosphate-buffered saline (PBS) liposomes were prepared as described previously.[14,15]

***Induction of Acute Hepatitis.*** Con A was administered to mice (20 mg/kg) through a tail vein.[2] In some experiments, mice received Con A intravenously (IV), promptly followed by intraperitoneal (IP) treatment with recombinant IFN-$\gamma$ (rIFN-$\gamma$; 500 ng) and recombinant TNF (rTNF; 500 ng). In some experiments, mice were treated IP with neutralizing anti-TF mAb, 1H1, or subcutaneously (SC) with heparin (5,000 U/kg) 30 minutes before Con A challenge. At various time points after challenge, plasma and liver specimens were sampled.[16] Plasma alanine aminotransferase (ALT) and aspartate aminotransferase levels were measured (SRL, Osaka, Japan).

***Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction.*** We performed quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR), as shown in the Supporting Materials. RNA content was normalized based on amplification of 18S ribosomal RNA (rRNA) (18S).[17] Change folds = normalized data of experimental sample/normalized data of control.

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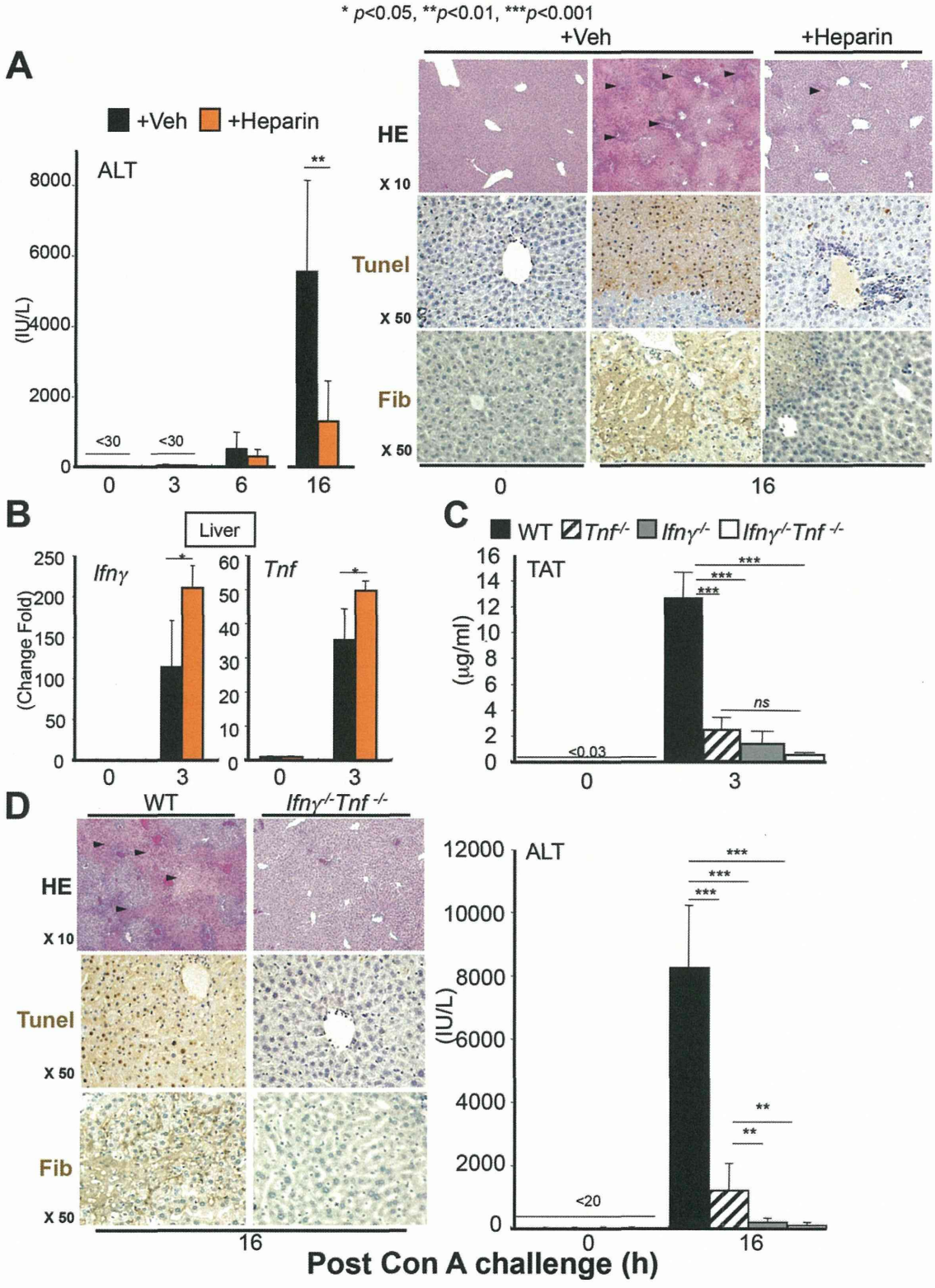


Fig. 1. Both IFN-$\gamma$ and TNF are necessary for the development of thrombus-associated ALI. (A and B) WT mice were treated SC with heparin (red columns) or vehicle (Veh, closed columns), then with Con A. At the indicated time point, plasma and liver specimens were sampled for measurement of ALT (A) and hepatic *Ifnγ* (B) and *Tnf* (B), respectively. Fold increase of mRNA expression was calculated after normalization to 18S (B). Histological (H&E) and immunohistological study for TUNEL and fibrin deposition (A) were also performed. (C and D) WT (closed bars), $Tnf^{-/-}$ (hatched bars), $Ifn\gamma^{-/-}$ (gray bars), and $Ifn\gamma^{-/-}Tnf^{-/-}$ mice (open bars) were treated with Con A. At the indicated time point, plasma and liver specimens were sampled for measurement of TAT (C) and histological/immunohistological study, as shown in (B), respectively. Representative data are shown (A and D left panels). Original magnification, ×10 (A and D, left upper panels) and ×50 (A and D, left lower panels). Arrowheads indicated necrotic area.

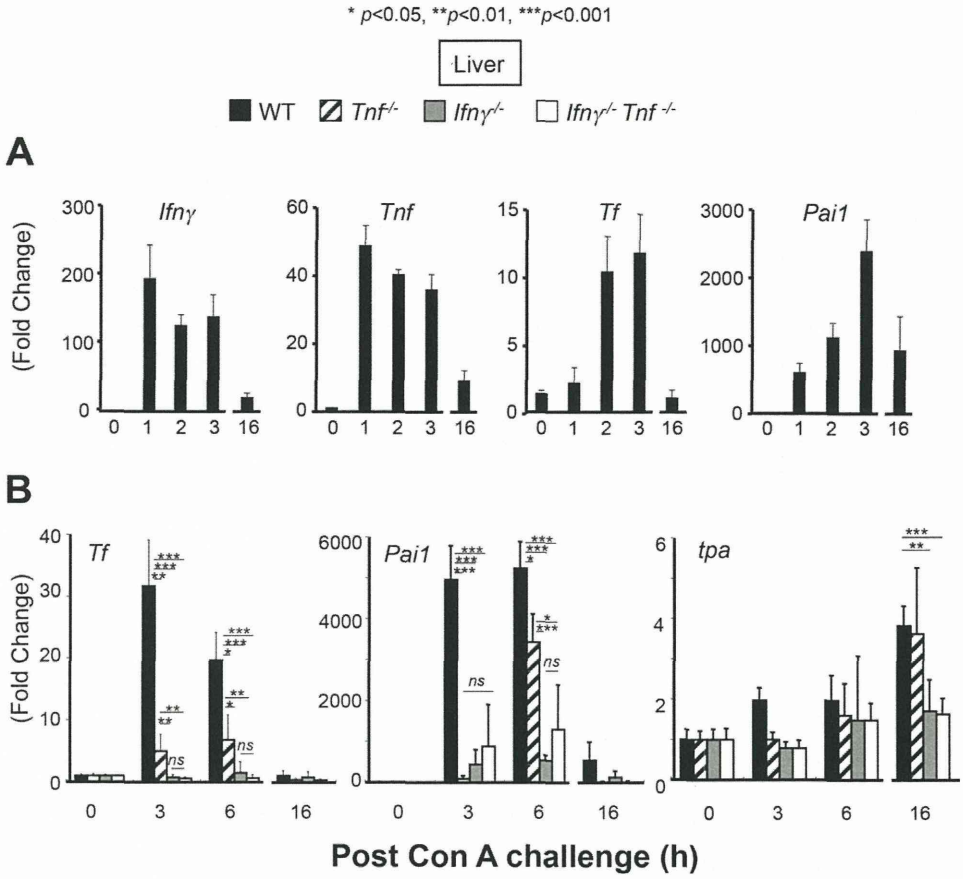


Fig. 2. Requirement of IFN-$\gamma$ and TNF for the induction of hepatic *Tf* and *Pai1*. WT (closed bars), *Tnf*$^{-/-}$ (hatched bars), *Ifn*$\gamma^{-/-}$ (gray bars), or *Ifn*$\gamma^{-/-}$*Tnf*$^{-/-}$ mice (open bars) were challenged IV with Con A, and their liver specimens were sampled for measurement of mRNA expression levels of IFN-$\gamma$ (A), TNF (B), TF (A and B), PAI-1 (A and B), and tPA (B) by real-time qRT-PCR. Fold increase of mRNA expression was calculated after normalization to 18S.

***Assay for Thrombin Antithrombin III Complex.*** Plasma levels of thrombin antithrombin III complex (TAT) were measured by commercially available kits for TAT (Enzyme Research Laboratories, South Bend, IN).[16]

***Preparation of Liver Cells.*** Hepatic nonparenchymal cells from 3 mice were pooled.[15] CD11b$^{+}$ hepatic Mø and CD146$^{+}$ SECs were then enriched by magnetic-activated cell sorting (Miltenyi Biotec GmbH, Cologne, Germany) using anti-CD11b and anti-CD146 microbeads (Miltenyi Biotec), according to the manufacture's instruction, respectively. Stellate cells and liver parenchymal cells were prepared as described.[18,19]

***Histological and Immunochemical Analyses.*** Formalin-fixed tissue sections were stained with hematoxylin and eosin (H&E).[2]

For detection of fibrin deposition, livers were perfused through a portal vein with PBS,[2] and liver specimens were rapidly sampled, fixed in 10% zinc fixative (Becton Dickinson, San Diego CA), and embedded in paraffin. Tissue sections were incubated overnight with rabbit antimouse fibrinogen antiserum (1:5,000) (Molecular Innovations, Inc., Novi, MI), followed by treatment with the rabbit Vectastatin Elite ABC kit (Vector Laboratories, Burlingame, CA). Antigen-antibody (Ab) complexes were detected by using a DAB Substrate Kit (Vector Laboratories). Formalin-fixed liver sections were analyzed for apoptosis by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphage nick-end labeling (TUNEL) assay.[20]

**In Vivo *Depletion of Mø.*** Mø were depleted by the IV injection of clodronate liposome, as described previously.[14]

***Mouse Reconstitution.*** To abolish irradiation-resistant Mø, we injected IV clodronate liposome into host mice and, 2 days later, irradiated them, followed by transfer of donor bone marrow (BM) cells.[21,22] CD45.1 WT mice were transferred with CD45.2 WT or CD45.2 *Stat1*$^{-/-}$ BM cells, and CD45.2 *Stat1*$^{-/-}$ mice were transferred with CD45.1 WT BM cells.[22] Two months later, the reconstituted mice were used.

***Statistical Analyses.*** All data are shown as the mean $\pm$ standard deviation of samples in each experimental group. Five to seven mice were used for each experimental group. Significance between the experimental and control groups was examined by the unpaired Student *t* test. *P* values less than 0.05 were considered significant. Two to three experiments were

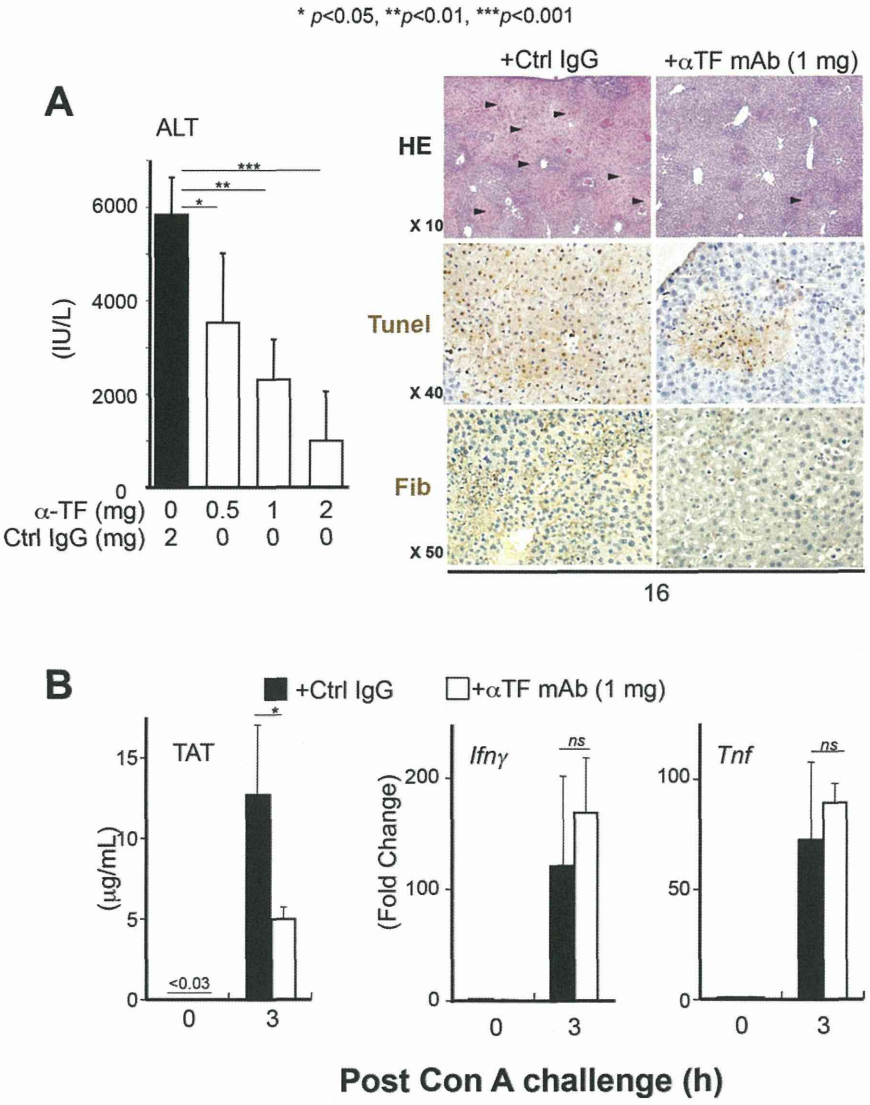


Fig. 3. TF is necessary for the development of Con A hepatitis. We administered various doses of neutralizing anti-TF mAb (open bars) or control rat IgG (Ctrl IgG) (closed bars) into WT mice 30 minutes before Con A challenge. At the indicated time points after Con A challenge, plasma and liver specimens were sampled for measurement of ALT (A) and TAT (B) as well as measurement of *Ifnγ* and *Tnf* expressions (B) and histological/immunohistological studies (A), respectively. Arrowheads indicated necrotic area.

separately performed, and representative data were shown.

## Results

***IFN-γ- and TNF-Dependent Hepatic Hypercoagulation Underlies Con A–Induced Hepatitis.*** We previously reported that by use of electron microscopy, many microthrombi, consisting of platelets, red blood cells, and fibrin deposits were observed in hepatic sinusoids of Con A–treated mice.[2] Immunohistochemistry (IHC) with antifibrinogen Abs further substantiated the dense fibrin deposition in the hepatic sinusoids (Fig. 1A, right middle lower panel). Pretreatment with the anticoagulant, heparin, protected against Con A–induced liver injuries with abundant TUNEL-positive hepatocytes and resulted in greatly reduced fibrin deposition (Fig. 1A). Consistent with our previous report,[2] heparin pretreatment did not down-regulate hepatic *Ifnγ* or *Tnf* (Fig. 1B). This is also true for interleukin-1β (*Il1β*), interleukin-6 (*Il6*), and CC chemokine ligand 2 (*Ccl2*) genes (Supporting Fig. 1). These results clearly indicated the importance of intrahepatic fibrin deposition for liver injury, and suggested that induction of these proinflammatory cytokines/chemokine was insufficient for the development of liver injuries in the absence of hepatic thrombosis.

Because plasma TAT is an excellent indicator of thrombin formation in the circulation,[16] we measured plasma TAT levels of Con A–challenged mice. Concomitant with dense fibrin deposition in the liver (Fig. 1A), plasma TAT levels were strongly elevated after challenge of WT mice with Con A, indicating that Con A treatment induced a systemic coagulation response along with hepatic hypercoagulation. Our previous report revealed that blockade of IFN-γ and