# Impact of glucose tolerance on the severity of non-alcoholic steatohepatitis

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## ABSTRACT

**Aims/Introduction:** We investigated the relationship between non-alcoholic steatohepatitis (NASH) and different stages of fasting plasma glucose (FPG) concentrations, and the association between factors related to glucose tolerance and severity of NASH.

**Materials and Methods:** A total of 147 patients with non-alcoholic fatty liver disease (NAFLD) who had undergone a liver biopsy were divided into three groups: a normal glucose tolerance (NGT) group, an impaired fasting glucose (IFG) group and a diabetes (DM) group. In addition, to investigate progression factors of NASH in the DM group, we divided the diabetic patients into two groups: a group with NASH (NASH group) and a group without NASH, the simple steatosis (SS) group. The relationship between the patients' clinical parameters and the severity of NAFLD/NASH were analyzed.

**Results:** In the patients with liver biopsies, the IFG group had the highest percentage of NASH. There was no correlation between FPG and either total NAFLD activity scores (NAS) or staging of NASH, but the fasting serum insulin was correlated significantly with both, even after adjusting for age, sex and body mass index. Among the diabetic patients, the fasting insulin values in the NASH group were significantly higher than in the SS group, but there were no differences in FPG or A1c values between the two groups. The fasting serum insulin correlated significantly with total NAS, but the FPG and A1c values did not.

**Conclusions:** A high percentage of the IFG group developed NASH. Hyperinsulinemia, but not hyperglycemia, was associated with severity of NASH. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2011.00134.x, 2011)

**KEY WORDS: Hyperglycemia, Hyperinsulinemia, Non-alcoholic steatohepatitis**

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has now become a major liver disease in both Western countries and in Asia, including in Japan[1,2]. The spectrum of NAFLD ranges from simple steatosis (SS) to non-alcoholic steatohepatitis (NASH). NASH is the more aggressive form of fatty liver disease, which can progress to cirrhosis and complications of cirrhosis, including hepatic failure and hepatocellular carcinoma[3]. Furthermore, reduced survival and higher mortality from cardiovascular and liver-related causes have been reported among NASH patients in comparison with a reference population[4]. Several factors, including body mass index (BMI), age, hyperglycemia, dyslipidemia and hypertension, have been linked to the severity of NAFLD[5], and the presence of diabetes, in particular, has received a great deal of attention. One previous study found that type 2 diabetes was an independent predictor of progression to fibrosis in NASH patients[6], and the histological data of 458 Italian NAFLD patients showed that NASH was independently predicted by diabetes[7]. According to other papers[8,9], however, the stage of fibrosis is not significantly associated with diabetes. Thus, the association between glucose tolerance and the severity of NAFLD/NASH has not been fully elucidated, although the association of the prevalence of NAFLD with impaired glucose metabolism has been reported[10]. In the present study, we investigated the distribution of fasting glucose concentrations and the association between factors related to glucose tolerance and severity of NAFLD/NASH in patients who had had liver biopsies for suspected NAFLD. We also attempted to identify factors associated with the severity of NASH in Japanese diabetic patients, who tend to be leaner than Caucasian diabetic patients and are thus characterized to be more insulin deficient rather than insulin resistant[11].

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## MATERIALS AND METHODS

### Subjects

The subjects were 147 patients who were suspected of having NAFLD based on their clinical manifestations and recruited between 2004 and 2010 at Yokohama City University Hospital, Japan. A liver biopsy was carried out in all 147 NAFLD patients. The present study was approved by the institutional review board, and written informed consent was obtained from all patients. A detailed history was obtained from every patient, and a physical examination was carried out. The histological criterion for the diagnosis of NAFLD is the presence of macrovesicular fatty change in hepatocytes with displacement of the

nucleus to the edge of the cell[12]. The criteria for exclusion from participation in the present study were: history of hepatic disease, including chronic hepatitis C or concurrent active hepatitis B (serum positive for hepatitis B surface antigen), autoimmune hepatitis, primary biliary cirrhosis (PBC), sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's disease and current or past consumption of more than 20 g of alcohol daily. No subjects had taken any drugs that cause fatty liver, such as amiodarone, diltiazem, tamoxifen or steroids[12]. None of the subjects presented with clinical evidence of hepatic decompensation, such as hepatic encephalopathy, ascites, variceal bleeding or a serum bilirubin level more than twice the upper limit of normal.

### Clinical and Laboratory Evaluation

The weight and height of the subjects were measured with a calibrated scale after they had removed their shoes and any heavy clothing. Venous blood samples were collected after an overnight fast (12 h). The serum insulin level was measured by radioimmunoassay, and the other biochemical parameters were measured in a conventional automated analyzer. The ratio of the computed tomography (CT) attenuation value of the liver to that of the spleen (L/S ratio) was used to quantitatively estimate the degree of liver steatosis[13].

Glucose tolerance was classified according to the 2006 World Health Organization (WHO) criteria[14]: normal glucose tolerance (NGT), fasting plasma glucose (FPG) < 110 mg/dL; impaired fasting glucose (IFG), FPG 110–125 mg/dL; and diabetes (DM), FPG ≥ 126 mg/dL. The A1c (%) value was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by using the formula: A1c (%) = A1c (Japan Diabetes Society [JDS]) (%) + 0.4%, considering the relational expression of A1c (JDS) (%) measured by the previous Japanese standard substance and measurement methods and $HbA_{1c}$ (NGSP)[15]. Subjects diagnosed with diabetes before the present study or whose A1c value was more than 6.5% were classified as having DM[16]. Of the 147 patients, 36 were divided into the DM group. Among these patients, 21 were treated without hypoglycemic medication; the remainder were treated with a sulfonylurea ($n$ = 2), a biguanide ($n$ = 2), a thiazolidinedione ($n$ = 1), an insulin ($n$ = 1), a combination of a sulfonylurea and a biguanide ($n$ = 5), a combination of a sulfonylurea and an α-glucosidase inhibitor ($n$ = 1), a combination of a biguanide and an α-glucosidase inhibitor ($n$ = 1), a combination of a biguanide and a thiazolidinedione ($n$ = 1), or a triple-drug combination of a sulfonylurea, a biguanide and a thiazolidinedione ($n$ = 1).

### Liver Histology

The liver biopsy specimens were stained with hematoxylin–eosin, reticulin and Masson trichrome stains, and all the specimens were examined by an experienced pathologist who was unaware of the clinical and biochemical data of the patients. All NASH cases were scored using the method of Brunt[17]. The degree of steatosis was assessed based on the percentage of hepatocytes containing macrovesicular fat droplets as follows: grade 0, no steatosis; grade 1, <33% hepatocytes containing macrovesicular fat droplets; grade 2, 33–66% hepatocytes containing macrovesicular fat droplets; and grade 3, >66% hepatocytes containing macrovesicular fat droplets. Necroinflammation was graded as follows: grade 0, absent; grade 1, occasional ballooned hepatocytes and no or very mild inflammation; grade 2, ballooning of hepatocytes and mild-to-moderate portal inflammation; and grade 3, intra-acinar inflammation and portal inflammation. Ballooning was graded as follows: grade 0, none; grade 1, few balloon cells; and grade 2, many cells/prominent ballooning. The severity of fibrosis, which represents staging of NASH, was expressed using a 4-point scale as follows: 0 = none, 1 = perivenular and/or perisinusoidal fibrosis in zone 3, 2 = combined pericellular portal fibrosis, 3 = septal/bridging fibrosis, 4 = cirrhosis. The NAFLD activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3) and ballooning (0–2), as reported by Kleiner *et al.*[18] A NAS of ≥5 correlated with a diagnosis of NASH. The cases were also classified as having steatosis or steatohepatitis on the basis of Matteoni's classification[19]. This classification was as follows: type 1, simple steatosis without inflammation or fibrosis; type 2, steatosis with lobular inflammation, but without fibrosis; type 3, additional presence of ballooned hepatocytes; and type 4, the presence of either Mallory's hyaline or fibrosis. If total NAS was >5 or the Matteoni classification was type 3 or 4, the patient was diagnosed as having NASH.

### Statistical Analysis

The results are expressed as means ± SD. Differences between two groups were analyzed for statistical significance by Student's $t$-test. Individual comparisons among three groups were assessed with an analysis of variance (ANOVA), followed by a Fisher's PLSD test for comparisons between two groups or the Kruskal–Wallis test, as appropriate. The chi-square test was used to analyze the significance of differences in proportions among these groups. Calculations of correlation coefficients and linear regression analyses were carried out to test for associations between the variables. Multiple linear regression analyses were also carried out to calculate age-, sex- and BMI-adjusted coefficients between the variables. A $P$-value <0.05 was considered statistically significant.

## RESULTS

### Characteristics of the Subjects

The clinical and biochemical characteristics of the NGT, IFG and DM groups are shown in Table 1. There were no significant differences between the three groups in most of the parameters, including sex, age, BMI, L/S ratio, liver enzymes and lipid profiles, but, as expected, the FPG values in the IFG group and DM group were significantly higher than in the NGT group. The fasting insulin values in the IFG group were significantly higher

 

**Table 1** | Clinical and biochemical characteristics of the normal glucose tolerance, impaired glucose tolerance and diabetes groups

| | NGT | IFG | DM | *P*-value |
|---|---|---|---|---|
| *n* | 88 | 23 | 36 | |
| Sex (male/female) | 51/37 | 10/13 | 19/17 | 0.8700 |
| Age (years) | 47.5 ± 15.0 | 51.6 ± 14.5 | 51.4 ± 13.1 | 0.2585 |
| Body mass index (kg/m$^2$) | 27.0 ± 5.5 | 28.2 ± 3.9 | 28.6 ± 5.1 | 0.2511 |
| Liver/spleen ratio | 0.72 ± 0.25 | 0.70 ± 0.21 | 0.71 ± 0.21 | 0.9702 |
| Aspartate aminotransferase (IU/L) | 47.4 ± 32.1 | 50.3 ± 24.6 | 48.7 ± 25.9 | 0.9077 |
| Alanine aminotransferase (IU/L) | 76.3 ± 51.5 | 90.0 ± 63.0 | 76.6 ± 48.2 | 0.5224 |
| γ-Glutamyltranspeptidase (IU/L) | 83.3 ± 85.1 | 64.0 ± 35.0 | 83.1 ± 58.5 | 0.5180 |
| Cholinesterase (IU/L) | 378.3 ± 76.6 | 393.7 ± 64.0 | 382.3 ± 84.6 | 0.6936 |
| Albumin (g/dL) | 4.56 ± 0.31 | 4.57 ± 0.30 | 4.45 ± 0.40 | 0.2227 |
| Hemoglobin (g/dL) | 14.5 ± 1.4 | 14.7 ± 1.4 | 14.3 ± 1.8 | 0.6114 |
| Platelets (×10$^4$/μL) | 24.1 ± 6.4 | 23.5 ± 6.6 | 24.9 ± 6.6 | 0.7057 |
| Iron (μg/dL) | 116.2 ± 37.9 | 126.1 ± 46.6 | 111.9 ± 44.6 | 0.4279 |
| Ferritin (ng/mL) | 239.7 ± 214.5 | 297.0 ± 273.3 | 295.4 ± 228.5 | 0.3423 |
| Triglyceride (mg/dL) | 155.7 ± 65.8 | 202.3 ± 126.6 | 163.9 ± 89.9 | 0.0674 |
| HDL cholesterol (mg/dL) | 51.0 ± 12.1 | 46.0 ± 9.6 | 52.1 ± 16.2 | 0.1914 |
| LDL cholesterol (mg/dL) | 132.0 ± 32.7 | 134.0 ± 31.8 | 122.8 ± 39.0 | 0.3346 |
| Hyaluronic acid (ng/mL) | 34.4 ± 35.0 | 49.8 ± 43.6 | 45.6 ± 44.5 | 0.1503 |
| Type IV collagen 7S (ng/mL) | 4.40 ± 1.24 | 4.47 ± 1.11 | 4.79 ± 1.22 | 0.2679 |
| Fasting blood glucose (mg/dL) | 98.7 ± 6.0 | 115.5 ± 4.7* | 151.2 ± 43.6* | <0.0001 |
| Insulin (μU/mL) | 12.6 ± 8.2 | 20.7 ± 13.5*** | 11.6 ± 5.1 | 0.0001 |

Values are means ± SD.
*$P$ < 0.01 vs normal glucose tolerance (NGT); **$P$ < 0.01 vs diabetes (DM).
HDL, high-density lipoprotein; IFG, impaired fasting glucose; LDL, low-density lipoprotein.

**Table 2** | Percentages of subjects diagnosed with non-alcoholic steatohepatitis and scores for steatosis, inflammation, and ballooning, total non-alcoholic fatty liver disease activity scores and staging of non-alcoholic steatohepatitis

| | NGT | IFG | DM | *P*-value |
|---|---|---|---|---|
| Subjects diagnosed with NASH based on total NAS (%) | 21.6 | 65.2** | 41.7 | 0.0002 |
| Subjects diagnosed with NASH based on the Matteoni classification (%) | 40.9 | 65.2 | 58.3 | 0.0503 |
| Steatosis | 1.65 ± 0.63 | 2.00 ± 0.74* | 1.83 ± 0.66 | 0.0493 |
| Inflammation | 0.80 ± 0.79 | 1.17 ± 0.83 | 0.92 ± 0.73 | 0.1181 |
| Ballooning | 0.84 ± 0.74 | 1.22 ± 0.60* | 1.17 ± 0.81* | 0.0226 |
| Total NAS | 3.28 ± 1.44 | 4.39 ± 1.60** | 3.92 ± 1.63* | 0.0035 |
| Staging of NASH | 0.61 ± 0.90 | 1.09 ± 1.04* | 0.97 ± 1.03 | 0.0419 |

Values are means ± SD.
*$P$ < 0.05; **$P$ < 0.01 vs normal glucose tolerance.
DM, diabetes; IFG, impaired fasting glucose; NAS, non-alcoholic fatty liver disease activity scores; NASH, non-alcoholic steatohepatitis.

than in the NGT group and DM group. These results showed that the subjects in the IFG group were hyperinsulinemic.

### Percentages of Subjects Diagnosed with NASH in the NGT, IFG and DM groups

We investigated the percentages of subjects diagnosed with NASH in the three groups based on the histological findings in the liver (Table 2). Interestingly, the percentages of NASH judged on the basis of both the total NAS and Matteoni's classification were highest in the IFG group, and the percentages of NASH judged on the basis of the total NAS were significantly higher in the IFG group than in the NGT group. Analysis of the scores for each component of the total NAS showed a significantly higher percentage of steatosis in the IFG group than in the NGT group and a significantly higher percentage of ballooning in the IFG group and the DM group than in the NGT group. As a result, the total NAS was significantly higher in the IFG group and DM group than in the NGT group. Staging of NASH, which is associated with fibrosis progression, was significantly higher in the IFG group than in the NGT group.

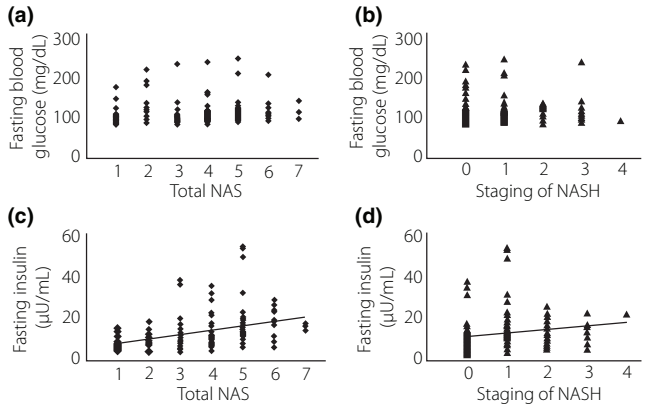


**Figure 1** | (a) Relationship between total non-alcoholic fatty liver disease activity scores (NAS) and fasting blood glucose values; no correlation exists ($r = 0.0824$, $P = 0.3208$). (b) Relationship between staging of non-alcoholic steatohepatitis (NASH) and fasting blood glucose values; no correlation exists ($r = 0.0811$, $P = 0.3290$). (c) Relationship between total NAS and fasting serum insulin values; a significant correlation exists ($r = 0.3642$, $P < 0.0001$). (d) Relationship between staging of NASH and fasting serum insulin values; a significant correlation exists ($r = 0.1777$, $P = 0.0313$). The values used to analyze these relationships were obtained from all 147 subjects.

**Table 3** | Clinical and biochemical characteristics of the simple steatosis group and non-alcoholic steatohepatitis group of diabetic subjects

| | SS | NASH | *P*-value |
|---|---|---|---|
| *n* | 15 | 21 | |
| Sex (male/female) | 9/12 | 10/5 | 0.1583 |
| Age (years) | 50.9 ± 13.6 | 52.2 ± 12.6 | 0.7659 |
| Body mass index (kg/m$^2$) | 27.7 ± 4.6 | 29.7 ± 5.7 | 0.2597 |
| Liver/spleen ratio | 0.73 ± 0.20 | 0.67 ± 0.22 | 0.4069 |
| Aspartate aminotransferase (IU/L) | 37.9 ± 23.7 | 63.8 ± 21.4 | 0.0019 |
| Alanine aminotransferase (IU/L) | 58.0 ± 41.7 | 102.8 ± 45.3 | 0.0042 |
| γ-Glutamyltranspeptidase (IU/L) | 77.4 ± 64.2 | 91.1 ± 50.5 | 0.4971 |
| Cholinesterase (IU/L) | 380.1 ± 97.7 | 385.0 ± 67.7 | 0.8700 |
| Albumin (g/dL) | 4.42 ± 0.45 | 4.49 ± 0.32 | 0.6127 |
| Hemoglobin (g/dL) | 13.9 ± 1.2 | 15.0 ± 2.3 | 0.0980 |
| Platelets (×10$^4$/μL) | 24.3 ± 6.2 | 25.8 ± 7.4 | 0.4996 |
| Iron (μg/dL) | 108.4 ± 48.9 | 116.6 ± 39.2 | 0.5976 |
| Ferritin (ng/mL) | 193.1 ± 158.8 | 438.7 ± 238.3 | 0.0007 |
| Triglyceride (mg/dL) | 165.2 ± 86.0 | 162.1 ± 98.0 | 0.9199 |
| HDL cholesterol (mg/dL) | 52.3 ± 18.2 | 51.9 ± 13.4 | 0.9497 |
| LDL cholesterol (mg/dL) | 126.7 ± 40.6 | 117.3 ± 37.2 | 0.4811 |
| Hyaluronic acid (ng/mL) | 43.0 ± 42.8 | 49.3 ± 47.9 | 0.6797 |
| Type IV collagen 7S (ng/mL) | 4.39 ± 1.03 | 5.36 ± 1.25 | 0.0154 |
| Fasting blood glucose (mg/dL) | 153.8 ± 44.0 | 147.6 ± 44.5 | 0.6802 |
| Insulin (μU/mL) | 9.37 ± 3.31 | 14.7 ± 5.6 | 0.0036 |
| A1c (%) | 8.15 ± 1.99 | 7.67 ± 1.54 | 0.4355 |

Values are means ± SD.
HDL, high-density lipoprotein; LDL, low-density lipoprotein; NASH, non-alcoholic steatohepatitis; SS, simple steatosis.

### Relationship Between Glucose Tolerance and the Severity of NAFLD/NASH

Next, we evaluated the association between factors related to glucose tolerance and the severity of NAFLD/NASH. FPG was not correlated with the total NAS or staging of NASH, but the fasting insulin values were significantly correlated with both (Figure 1). Furthermore, multiple regression analysis showed that, even after adjustment for age, sex and BMI, fasting insulin was still correlated significantly with the total NAS (regression coefficient = 0.0652, standardized regression coefficient = 0.3812, $P < 0.0001$) or staging of NASH (regression coefficient = 0.0192, standardized regression coefficient = 0.1844, $P = 0.0300$). These results suggested that hyperinsulinemia, but not hyperglycemia, play a role in the severity of NASH.

### NASH Severity Factors in Japanese Diabetic Patients

To investigate the NASH severity factors in the DM group, the members of the DM group were divided into a group with NASH (NASH group) and a group without NASH; that is, a simple steatosis (SS) group. The clinical and biochemical characteristics of the SS group and NASH group are shown in Table 3. There were no significant differences between these two groups in most of the parameters, including sex, age, BMI, L/S ratio and lipid profiles, but the serum aspartate aminotransferase, alanine aminotransferase, ferritin and type IV collagen 7S values were significantly higher in the NASH group than in the SS group. The fasting serum insulin values were also significantly higher in the NASH group than in SS group, but there were no differences between their FPG or A1c values.

Next, we analyzed the relationship between clinical parameters related to glucose tolerance and NAFLD/NASH severity in the DM group (Figure 2a–f). There were no correlations between the FPG or A1c values and the total NAS or staging of NASH, but the fasting insulin values were significantly correlated with both. Furthermore, multiple regression analysis showed that, even after adjustment for age, sex and BMI, fasting insulin was still correlated significantly with the total NAS (regression coefficient = 0.1583, standardized regression coefficient = 0.4734, $P = 0.0122$), which was consistent with the results for the subjects as a whole, including the subjects in the NGT group and IFG group. In addition, the fasting serum insulin values were significantly correlated with both the aspartate aminotransferase and type IV collagen 7S values, and there were weak associations between the fasting insulin values and the alanine aminotransferase and ferritin values, but they were not statistically significant (Figure 2g–j).

Insulin values might be influenced by hypoglycemic medications. We therefore carried out a subanalysis in diabetic patients without hypoglycemic medication. In this subgroup, the fasting insulin values in the NASH group ($n = 10$) were significantly higher than in the SS group ($n = 11$), but there were no differences in FPG or A1c values between the two groups (data not shown). The fasting serum insulin correlated significantly with total NAS ($r = 0.4456$, $P = 0.0429$), but the FPG and A1c

 

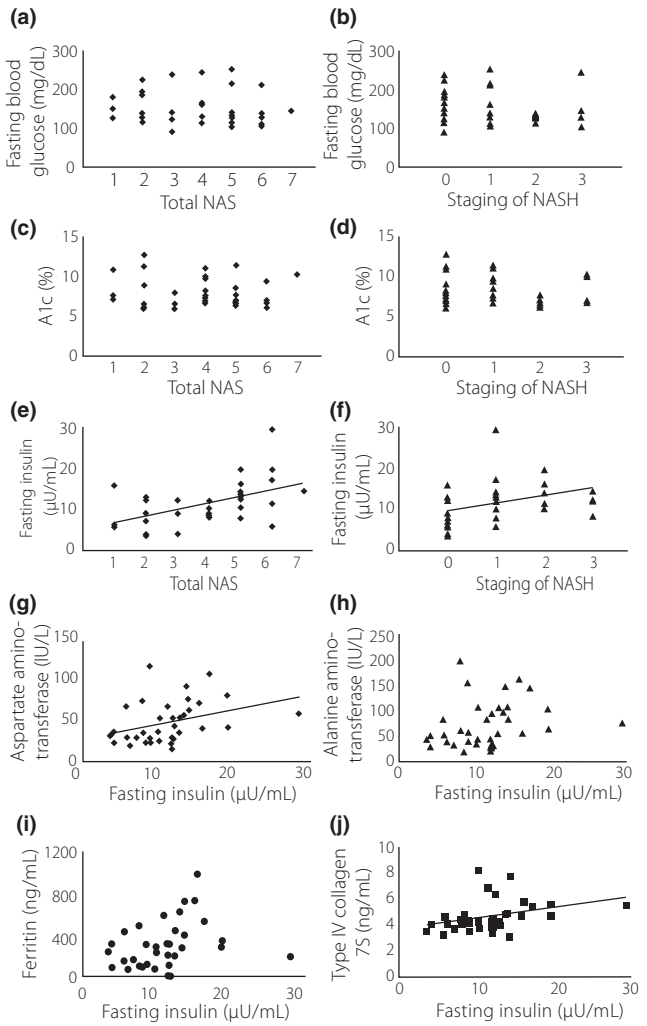


**Figure 2** | (a) Relationship between total non-alcoholic fatty liver disease activity scores (NAS) and fasting blood glucose values in the diabetes (DM) group; no correlation exists ($r = 0.1236$, $P = 0.4727$). (b) Relationship between staging of non-alcoholic steatohepatitis (NASH) and fasting blood glucose values in the DM group; no correlation exists ($r = 0.0957$, $P = 0.5749$). (c) Relationship between total NAS and blood A1c values; no correlation exists ($r = 0.1081$, $P = 0.5304$). (d) Relationship between staging of NASH and blood A1c values; no correlation exists ($r = 0.0376$, $P = 0.8276$). (e) Relationship between total NAS and fasting serum insulin values; a significant correlation exists ($r = 0.5103$, $P = 0.0015$). (f) Relationship between staging of NASH and fasting serum insulin values; a significant correlation exists ($r = 0.3599$, $P = 0.0311$). (g) Relationship between fasting serum insulin values and aspartate aminotransferase values in the DM group; a significant correlation exists ($r = 0.3560$, $P = 0.0331$). (h) Relationship between fasting serum insulin values and alanine aminotransferase values in the DM group; a weak correlation exists, but it is not significant ($r = 0.2540$, $P = 0.1349$). (i) Relationship between fasting serum insulin values and ferritin values; a weak correlation exists, but it is not significant ($r = 0.2940$, $P = 0.0818$). (j) Relationship between fasting serum insulin values and type IV collagen 7S values; a significant correlation exists ($r = 0.3559$, $P = 0.0332$). These relationships were analyzed based on data obtained from 36 diabetic subjects.

values did not (FPG: $r = 0.2605$, $P = 0.2540$; A1c: $r = 0.2653$, $P = 0.2451$). These results were consistent with the results obtained from all diabetic patients. Therefore, there might be an association between hyperinsulinemia and the severity of NASH, even in diabetic patients.

## DISCUSSION

The starting point is that we had a group of 147 patients who had liver biopsies, and in the 23 of these who had IFG, we made important observations about the severity of their liver disease (Table 2). IFG, in which glucose metabolism abnormalities are present, but the glucose level is still below the cut-off point for a diagnosis of diabetes, is referred to as prediabetes. β-Cells secrete additional insulin in response to insulin resistance, and the insulin levels initially increase in the prediabetic state[20]. Clinical data have shown that subjects with prediabetes have higher fasting insulin concentrations than subjects with NGT[21]. The results of the comparison with the NGT group in the present study showed that the IFG group was hyperinsulinemic (Table 1), which is in agreement with these reports. Then, why did the IFG group have the highest percentage of NASH? We assume that hyperinsulinemia, but not hyperglycemia, plays an important role in the severity of NASH, because the FPG values were not correlated with the total NAS or staging of NASH, but the fasting insulin values were significantly correlated with both (Figure 1).

The pathogenesis of NASH occurs in two steps. In the first step, the healthy liver becomes steatotic, mainly as a consequence of peripheral resistance to insulin, which results in an increase in fatty acid transport from adipose tissue to the liver. Then, the second step is elicited by oxidative stress and cytokines occurs, resulting in an inflammatory process, hepatocellular degeneration and fibrosis. Hyperinsulinemia resulting from insulin resistance is very much involved in these first and second hits[22]. In the second hit especially, a direct link between insulin, inflammation and oxidative stress has been suggested by the observation that chronic activation of IKK-β in *ob*/*ob* mice triggered by cytokines involved in oxidant and inflammatory stresses is associated with insulin resistance[23]. Our finding that fasting serum insulin values were significantly correlated with staging of NASH supports these notions. We recently reported the effect of long-term, high-fat diet loading on the development of NASH and hepatocellular carcinoma in C57bl/6J male mice and in mice with β-cell specific haploinsufficiency of the glucokinase gene ($Gck^{+/-}$) having the same genetic background, an animal model for type 2 diabetes with an insulin secretory defect[24]. The same degrees of liver steatosis, inflammation, fibrosis and nodular lesions were observed in the $Gck^{+/-}$ mice as in the wild-type mice on the high-fat diet, a finding that is consistent with our clinical findings in the present study, showing that hyperglycemia *per se* did not cause such pathological alterations.

The serum adiponectin concentrations of the patients with hyperinsulinemia might have been low, but, unfortunately, we did not measure them. Because adiponectin has been found to have an anti-inflammatory effect and an antifibrogenic effect in

a mouse model[25], and a stepwise decrease in the serum adiponectin in parallel to the severity of hepatic fibrosis has been reported in diabetic subjects[26], hypoadiponectinemia might be involved in the pathogenesis and progression of NASH. Thus, the effect of hyperinsulinemia on the severity of NASH might be at least partly adiponectin-mediated.

It has recently been reported that decreases in A1c and the use of insulin to treat diabetes were independently associated with improvement of liver fibrosis in Japanese NAFLD patients[27], and many of the diabetics in the improved group had started insulin treatment. Based on the results of our observation that hyperinsulinemia, but not hyperglycemia, was associated with the severity of NASH, we speculate that insulin therapy suppressed endogenous insulin secretion by β-cells and led to a decreased insulin influx into the liver. Thus, our result would not constitute a contradiction with the concept of that report, although it is not clear how much insulin treatment could be directly involved in acting on hepatic insulin signaling. We are currently investigating the effect of hepatic insulin signaling on the development of NASH and HCC in insulin receptor substrate-1 knockout mice[28] on a high-fat diet, which represents impaired insulin action in the liver and severe hyperinsulinemia, and are dramatically spared from liver steatosis (Nakamura A, Tajima K, Khadbaatar Z, Terauchi Y, unpublished observation, 2011). This mouse model should provide a clue to the associations between hyperinsulinemia, hepatic insulin actions and the development of NASH.

Epidemiological studies have shown that diabetes might increase the risk of developing cancer, especially liver cancer[29,30]. Although several mechanisms might be involved in the molecular link between glucose intolerance and the risk of developing cancer, Johnson and Pollak[31] recently commented that the accumulation of experimental and epidemiological evidence was more consistent with the hyperinsulinemia hypothesis and less so with the hyperglycemia hypothesis. It should be noted that our results also suggest that hyperinsulinemia, but not hyperglycemia, might promote the severity of NASH. Because NASH has been considered to be the cause of hepatocellular carcinoma[3], correction of hyperinsulinemia would be important in the prevention of not only NASH, but also hepatocellular carcinoma.

One limitation of the present study was related to the small sample. Liver biopsy is recommended as the gold standard method for the diagnosis of NASH. This procedure, however, is invasive and associated with a relatively high risk of complications. Thus, the number of the NASH patients in the present study was small. Because we started with a group of 147 subjects suspected of having NAFLD, who had all had a liver biopsy, and found that 23 of these had IFG, the IFG group could be highly selective and not necessarily representative of the wider IFG population. Another limitation is that the subjects did not have a 75-g oral glucose tolerance test to determine if they had impaired glucose tolerance (IGT). Although IGT represents risk for cardiovascular diseases[32], it remains unclear whether IFG is a risk category for atherosclerosis. Because it has been reported that there was a difference of β-cell dysfunction and insulin resistance to the pathogenesis between IFG and IGT[21], results could be different by classification using the 75-g oral glucose tolerance test. Because NASH patients tend to die as a result of cardiovascular events[4], it will be interesting to evaluate the association between IGT and NASH. Further study is needed to test this issue. However, our data deliver a key message that people with prediabetes might be at high risk of severity of NASH.

In conclusion, a high percentage of the subjects of the present study in the IFG group developed NASH. Hyperinsulinemia, but not hyperglycemia, was associated with severity of NASH. These findings should lead to better prevention and treatment for NASH, such as weight loss and exercise, which could reduce hyperinsulinemia resulting from insulin resistance.

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