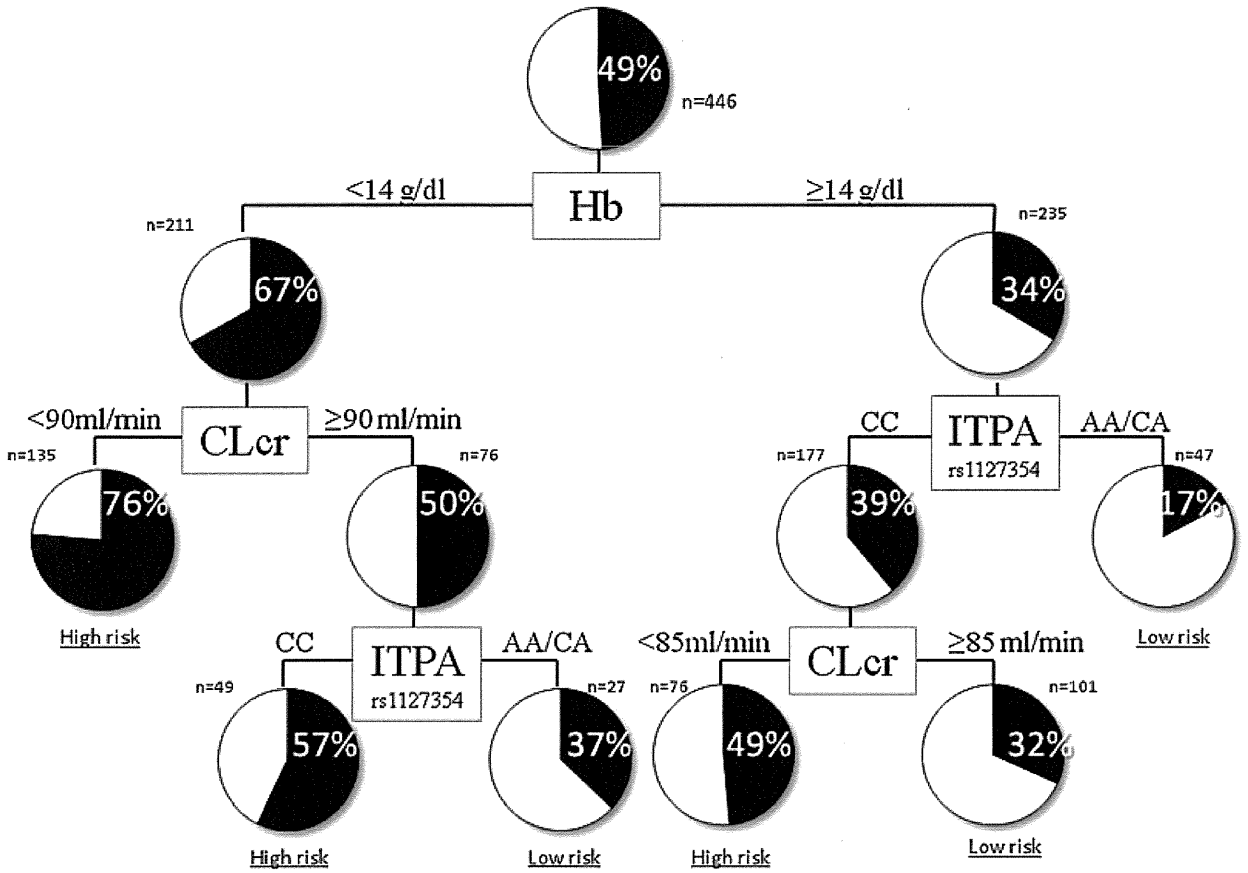

Fig. 1. The predictive model for severe anemia. The boxes indicate the factors used to differentiate patients and the cutoff values for the different groups. The pie charts indicate the rate of severe anemia (Hb <10.0 g/dl) for each group of patients, after differentiation. Terminal groups of patients differentiated by analysis are classified as at high risk if the rate is >40% and low risk if the rate is <40%. *ITPA*, inosine triphosphatase; CLcr, creatinine clearance; Hb, hemoglobin.

significantly lower rate of sustained virological response than the low-risk group (59% vs. 76%, $P = 0.013$) (Fig. 3D–F). In patients who did not achieve a complete early virological response, the *IL28B* genotype was a significant predictor of a sustained virological response (TT vs. TG/GG; 14% vs. 2%, $P < 0.0001$) but a high risk for anemia was not (high vs. low; 10% vs. 6%, $P = 0.361$).

From multivariate analysis (Table II), the *IL28B* genotype was the most important predictor of a sustained virological response at baseline [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$], along with female sex [0.42 (0.26–0.68), $P < 0.0001$], platelet count [1.09 (1.04–1.15), $P < 0.0001$], advanced fibrosis [0.49 (0.27–0.91), $P = 0.024$], and baseline HCV RNA load [4.14 (2.27–7.55), $P < 0.0001$]. At week 4, in patients without a rapid virological response, the *IL28B* genotype remained the most important predictor of a sustained virological response [7.16 (3.60–14.25), $P < 0.0001$], along with female sex and platelet count. At week 12, in patients with a complete early virological response, the risk of anemia was an independent and significant predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$], together with the platelet count and HCV RNA load, but the *IL28B* genotype was not associated with a sustained virological response. In patients without a complete early virological response, the *IL28B* genotype was a predictor of a sustained virological response [9.13 (2.02–41.3), $P = 0.004$] along with the platelet count. Thus, *IL28B* was a significant predictor of a sustained virological response at baseline and among virological non-responders at weeks 4 and 12. On the other hand, once a complete early virological response was achieved, the *IL28B* genotype was no longer associated with a sustained virological response but the risk of anemia was an independent predictor of a sustained virological response.

The Risk of Anemia, RBV Dose, and Treatment Outcome in Patients With a Complete Early Virological Response

Patients who achieved a complete early virological response were stratified according to adherence to

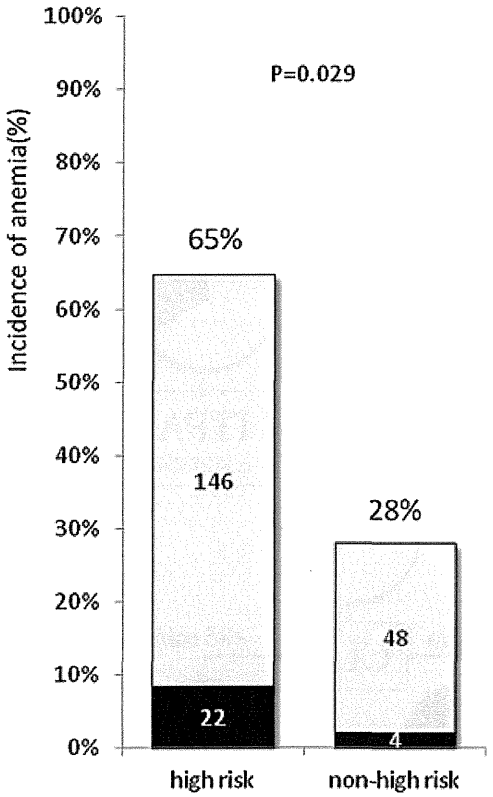

Fig. 2. The incidence of severe anemia stratified by risk of anemia. The incidence of anemia during therapy is shown for each group of patients at high and low risk of anemia. The black and white bars represent the percentages of patients with Hb concentrations below 8.5 g/dl and above 10 g/dl, respectively.

RBV (≤40%, 41–60%, 61–80%, and >80%), which showed that patients with a high risk of anemia were predominantly in subgroups with a lower adherence to RBV (≤40%, 41–60%, and 61–80%), whereas patients with a low risk of anemia were predominantly in subgroups with a higher adherence to RBV (>80%) (Fig. 4, upper panel). The percentage of patients who received >80% of the planned dose of RBV was significantly higher in the low-risk group for anemia than in the high-risk group (74% vs. 55%, $P < 0.0001$).

Within the groups with high and low risks of anemia, there was a stepwise increase in the rate of sustained virological response according to the increase in adherence to RBV (Fig. 4, lower panel). The rate of sustained virological response was higher in patients who received >80% of the planned dose of RBV than those who received less, for both high-risk patients (71% vs. 47%, $P = 0.016$) and low-risk patients (81% vs. 60%, $P = 0.072$). Within the same subgroup of RBV adherence, however, the rate of sustained virological response did not differ between patients with a high risk and a low risk of anemia. Taken together, these results suggest that patients with a high risk of anemia have a disadvantage because they are likely to be intolerant to RBV, leading to reduced adherence to RBV throughout the 48 weeks of therapy and a reduced rate of sustained virological response. However, if >80% adherence to RBV could be obtained, the rate of sustained virological response would increase by 24%.

DISCUSSION

This study confirmed previous reports that the *IL28B* genotype is the most significant predictor of a sustained virological response to PEG-IFN plus RBV therapy in chronic hepatitis C patients at baseline [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010; Kurosaki et al., 2011c] and at week 4 [Thompson et al., 2010b], but it had no impact on the rate of sustained virological response among those patients who achieved a complete early virological response [Thompson et al., 2010b; Kurosaki et al., 2011c]. In contrast, the risk of anemia, assessed by the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr, was found to be associated with a sustained virological response in patients who achieved a complete early virological response. Generally, a complete early virological response is the hallmark of a high probability of a sustained virological response, but the rate of sustained virological responses in patients who achieved a complete early virological response and had a high risk of anemia was as low as 59%. This reduced rate of sustained virological response in these patients was attributable to poor adherence to RBV throughout the 48 weeks of therapy. Because administration of >80% of the planned RBV dose increased the rate of sustained virological response by 24%, it may be postulated that personalizing the treatment schedule to achieve a sufficient dose of RBV, such as extension of treatment duration, may improve sustained virological response rates in these patients. Clearly, this postulate needs to be confirmed in future study. Thus, the findings presented here may have the potential to support selection of the optimum, personalized treatment strategy for an individual patient, based on the risk of anemia.

The degree of hemolytic anemia caused by RBV varies among individuals. A reduction of the Hb concentration early during therapy predicts the likely development of severe anemia [Hiramatsu et al., 2008, 2011] but there are no reliable predictors at baseline. A breakthrough came from the results of a genome-wide association study that revealed that variants of the *ITPA* gene are protective against hemolytic anemia [Fellay et al., 2010]. The *ITPA* genotype has been shown repeatedly to be associated with the degree of hemolytic anemia and dose reduction of RBV [Fellay et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a; Seto et al., 2011; Tanaka et al., 2011; Kurosaki et al., 2011d]. However, factors other than the *ITPA* gene, such as baseline Hb concentrations [Ochi et al., 2010; Kurosaki et al., 2011d], platelet counts [Ochi

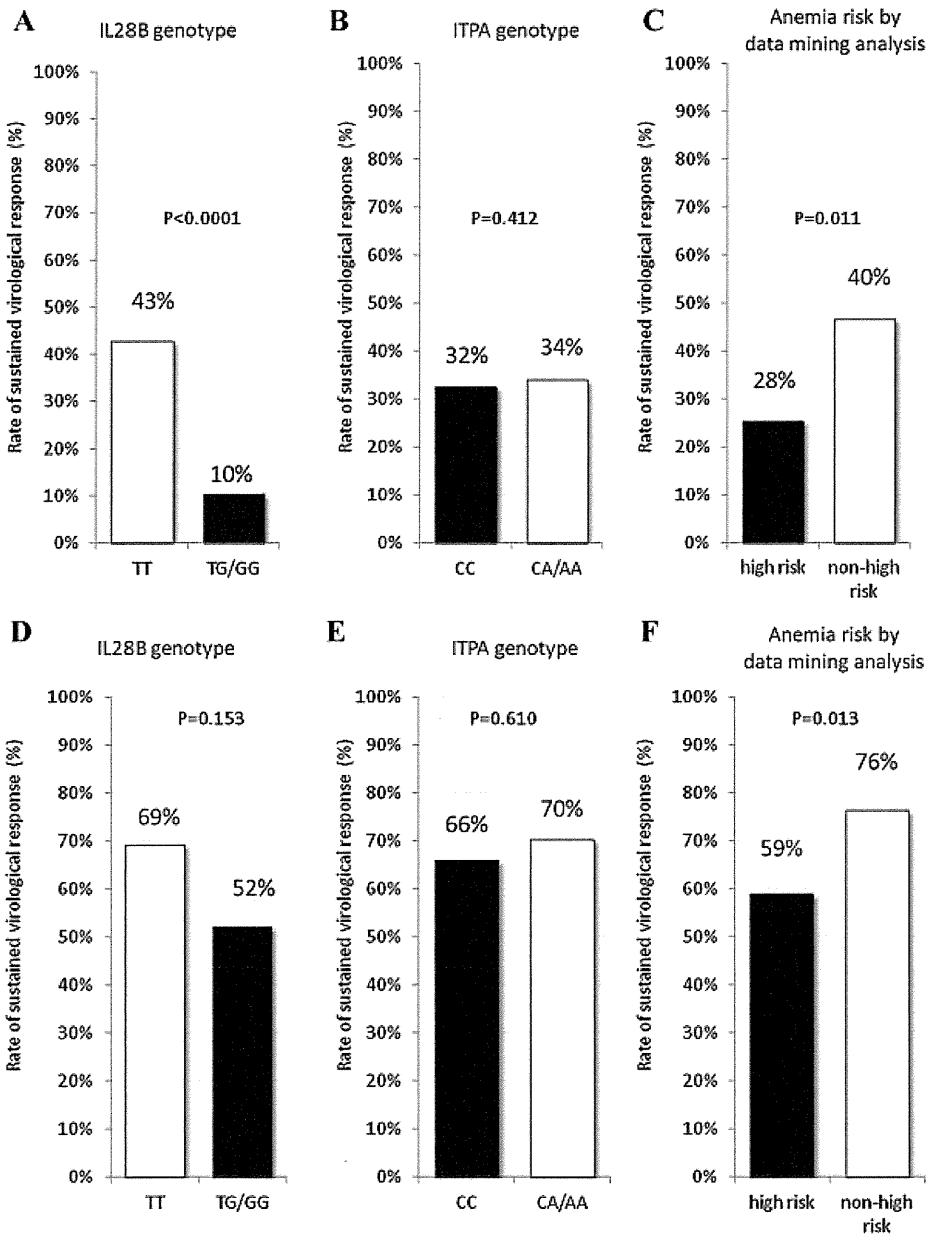

Fig. 3. Rates of sustained virological responses at baseline and among those with a virological response at week 12. The impacts of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response were studied at baseline (**A–C**) and among those with complete early virological responses (defined as undetectable HCV RNA at week 12) (**D–F**). At baseline, those with the TT allele of the *IL28B* gene had a significantly higher rate of sustained virological response than those with the TG or GG allele and the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group. Among patients with complete early virological responses, the *IL28B* genotype was not associated with a sustained virological response, while the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group.

et al., 2010], and CLcr [Kurosaki et al., 2011d], also contribute to the risk of severe anemia or RBV dose reduction. In the present study, the predictive model of anemia based on the data mining analysis selected the *ITPA* genotype, baseline Hb concentration, and baseline CLcr as predictive factors and identified six subgroups of patients with a variable rate of severe anemia, ranging from 17% to 76%. The specificity of the prediction of severe anemia was improved by 25.7% in the predictive model, compared to *ITPA*

TABLE II. Logistic Regression Analysis for Factors Associated With Sustained Virological Response at Baseline, Week 4 and Week 12

	Multi-variable		
	Odds	95% CI	*P*-value
Pre-treatment			
Sex: female	0.42	0.26–0.68	<0.0001
Platelet (10^9/L)	1.09	1.04–1.15	<0.0001
Fibrosis: F3-4	0.49	0.27–0.91	0.024
HCV RNA: <600,000 IU/L	4.14	2.27–7.55	<0.0001
IL28B rs8099917: TT	9.88	5.01–19.48	<0.0001
At week 4			
Non-RVR patients			
Sex: female	0.45	0.28–0.72	0.001
Platelet (10^9/L)	1.10	1.05–1.16	0.000
IL28B rs8099917: TT	7.16	3.60–14.25	<0.0001
At week 12			
cEVR patients			
Platelet (10^9/L)	1.09	1.02–1.17	0.015
HCV RNA: <600,000 IU/L	3.21	1.39–7.55	0.007
High-risk of anemia[a]	0.47	0.24–0.91	0.026
At week 12			
Non-cEVR patients			
Platelet (10^9/L)	1.11	1.02–1.21	0.017
IL28B rs8099917: TT	9.13	2.02–41.3	0.004

RVR: rapid virological response, defined as undetectable HCV RNA at week 4.
cEVR: complete early virological response, defined as undetectable HCV RNA at week 12.
[a]High-risk of anemia defined by decision tree analysis includes the following groups: (1) baseline hemoglobin <14.0 g/dl and creatinine clearance <90 ml/min, (2) baseline hemoglobin <14.0 g/dl, creatinine clearance ≥90 ml/min and ITPA rs1127354 genotype CC, and (3) baseline hemoglobin ≥14.0 g/dl, ITPA rs1127354 genotype CC, and creatinine clearance <85 ml/min.

genotyping alone. Because hemolytic anemia induced by RBV is one of the major adverse events leading to premature termination of therapy [Fried et al., 2002], a method to predict the risk of severe anemia before treatment is important clinically. A predictive model of anemia may have the potential to support individualized treatment strategies; patients at high risk of anemia may be tested intensively for anemia or may be candidates for erythropoietin therapy, whereas those with a low risk of anemia may be treated with a higher dose of RBV. Prediction of anemia will remain important in the era of direct antiviral agents for chronic hepatitis C, because these newer therapies still require RBV and PEG-IFN in combination, and the degree of anemia complicating these therapies may be even greater than with the current combination therapy [McHutchison et al., 2009; Kwo et al., 2010].

Studies of the impact of the *ITPA* genotype on treatment outcome have produced conflicting results. Previous studies of American [Thompson et al., 2010a] and Italian [Thompson et al., 2011] cohorts did not find any association between the *ITPA* genotype and treatment outcome, whereas a marginal difference was observed in a report from Japan [Ochi et al., 2010]. Moreover, with a subgroup analysis of Japanese patients, the variant of the *ITPA* gene was associated with a sustained virological response in patients with the *IL28B* major genotype [Kurosaki et al., 2011d], in patients infected with HCV other than genotype 1[Sakamoto et al., 2010], and in patients with pre-treatment Hb concentrations between 13.5 and 15 g/dl [Azakami et al., 2011]. These inconsistent results may be because the impact of anemia may be greater on a cohort of aged patients, such as in Japan. Another reason may be that the *ITPA* genotype is not the sole determinant of anemia; the *ITPA* genotype alone was not associated with treatment outcome in the present study but a high-risk of anemia, defined by the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr, was associated with sustained virological responses by patients with complete early virological responses, even after adjustment for the *IL28B* genotype and other relevant factors. This is in contrast to the finding that the *IL28B* genotype is an independent and significant predictor at baseline of a sustained virological response by patients without a rapid virological response and those without a complete early virological response, but not those with a complete early virological response. These results indicate that the *IL28B* genotype could be used to predict a sustained virological response at baseline or during therapy in patients in whom HCV RNA has not yet become undetectable, but it has no predictive value in patients in whom HCV RNA has become undetectable. The risk of anemia may be used to predict sustained virological responses in a selected subgroup of patients who achieve a complete early virological response.

Patients who received more than 80% of the planned dose of PEG-IFN or RBV had a higher rate of sustained virological responses than those who received a lower cumulative dose [McHutchison et al., 2002; Davis et al., 2003]. Patients who achieve a complete early virological response usually have a good chance of a sustained virological response and the treatment duration is not extended beyond 48 weeks. However, reduced adherence to drugs in these patients was related to relapse after the completion of 48 weeks of therapy [Hiramatsu et al., 2009; Kurosaki et al., 2012]. In the present study, the rate of sustained virological response was 59% in patients who achieved a complete early virological response but had a high risk of anemia, 17% lower than in patients with a low risk of anemia. However, there was a stepwise increase in the rate of sustained virological response according to the increase in adherence to RBV, and the rate of sustained virological response was higher in high-risk patients who received >80% of the planned dose of RBV (71% vs. 47%). This 24% increase in sustained virological response was observed among the patients in the present study who received 48 weeks of treatment. These findings suggest that receiving a sufficient RBV dose is essential for patients with a complete early virological response to attain a sustained virological response and that the treatment strategy should be personalized for patients with a

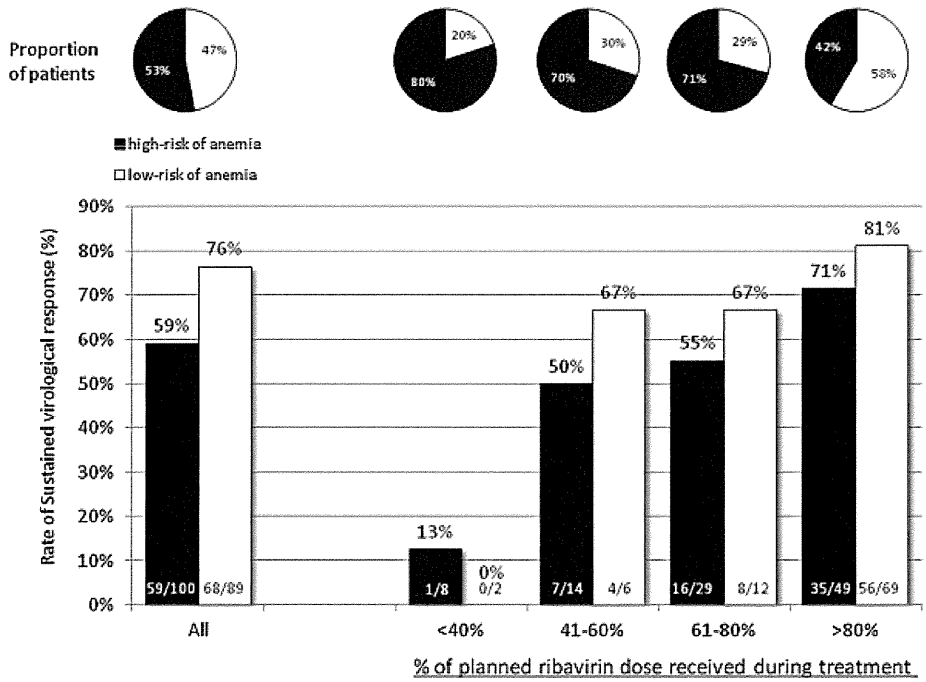

Fig. 4. The impact of risk of anemia and RBV dose on treatment outcome after a complete early virological response. Patients with complete early virological responses were divided into subgroups according to their adherence to RBV: ≤40%, 41–60%, 61–80%, and >80%. For each subgroup, the proportion of patients with a high risk and a low risk of anemia is shown in the upper panel by pie charts, and the rates of sustained virological responses, stratified by high risk and low risk of anemia, are shown in the lower panel by bar graphs. The black and white bars or charts represent patients with high and low risks of anemia, respectively.

high risk of anemia to extend the duration of treatment, even those patients with a complete early virological response, to obtain >80% adherence to RBV.

In conclusion, the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr could be used as a pre-treatment predictor of anemia. The risk of anemia thus identified is associated with adherence to RBV and impacts on the treatment outcome of patients who achieve a complete early virological response. This is in contrast to the major role of the *IL28B* genotype in the prediction of sustained virological responses at baseline and among non-responders at weeks 4 and 12. Patients who achieve a complete early virological response generally have a high probability of a sustained virological response but those who have a high risk of anemia have a high rate of relapse because of reduced adherence to RBV. To improve the rate of sustained virological responses in these patients, it may be postulated that the treatment schedule may be personalized to obtain >80% adherence to RBV. Clearly, this postulate needs to be confirmed in a future study.

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Original Article

Clinical usefulness of non-protein respiratory quotient measurement in non-alcoholic fatty liver disease

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Aim: Little is known about the effects of non-alcoholic fatty liver disease (NAFLD) on energy metabolism, although this disease is associated with metabolic syndrome. We measured non-protein respiratory quotient (npRQ) using indirect calorimetry, which reflects glucose oxidation, and compared this value with histological disease severity in NAFLD patients.

Methods: Subjects were 32 patients who were diagnosed with NAFLD histopathologically. Subjects underwent body composition analysis and indirect calorimetry, and npRQ was calculated. An oral glucose tolerance test was performed, and plasma glucose area under the curve (AUC glucose) was calculated.

Results: There were no differences in body mass index, body fat percentage or visceral fat area among fibrosis stage groups. As fibrosis progressed, npRQ significantly decreased (stage 0, 0.895 ± 0.068; stage 1, 0.869 ± 0.067; stage 2, 0.808 ± 0.046; stage 3, 0.798 ± 0.026; $P < 0.005$). Glucose intolerance worsened and insulin resistance increased with fibrosis stage. npRQ was negatively correlated with AUC glucose ($R = -0.6308$, $P < 0.001$), Homeostasis Model of Assessment – Insulin Resistance ($R = -0.5045$, $P < 0.005$), fasting glucose ($R = -0.4585$, $P < 0.01$) and insulin levels ($R = -0.4431$, $P < 0.05$), suggesting that decreased npRQ may reflect impaired glucose tolerance due to insulin resistance, which was associated with fibrosis progression. Estimation of fibrosis stage using npRQ was as accurate as several previously established scoring systems using receiver–operator curve analysis.

Conclusion: npRQ was significantly decreased in patients with advanced NAFLD. Our data suggest that measurement of npRQ is useful for the estimation of disease severity in NAFLD patients.

Key words: glucose intolerance, NAFLD, npRQ

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) is one of the most common chronic liver diseases. NAFLD is associated with metabolic syndrome and insulin resistance and often involves abnormal glucose and lipid metabolism.[1–6] Based on this, the NAFLD Asia–Pacific Working Party has recommended screening for metabolic syndrome and body composition in all NAFLD patients.[6] NAFLD treatment consists of diet and exercise interventions for weight loss,[4–6] and nutritional guidance and management are essential. As a part of a nutritional guidance and management program, our institution performs anthropometric measurement of NAFLD patients using a body composition analyzer, evaluation of glucose metabolism using a 75-g oral glucose tolerance test (OGTT), and evaluation of energy metabolism using indirect calorimetry. These basic tests are performed in routine practice.

Indirect calorimetry is a method used in physiological testing and enables easy and non-invasive evaluation of energy metabolism in real time.[7] The non-protein respiratory quotient (npRQ) calculated from indirect calorimetry data represents the ratio of carbohydrate to fat oxidation, and its value is said to be an indicator of prognosis in liver cirrhosis.[8] In addition, although it has been reported that NAFLD disease progression is

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associated with glucose intolerance[9–12] and visceral fat accumulation,[13] no previous study has examined the specific relationship between NAFLD pathology and these nutritional parameters.

One aim of the present study was to elucidate whether nutritional status, as estimated by indirect calorimetry, 75-g OGTT and body composition analysis, was related to NAFLD disease progression. The other aim was to elucidate whether these parameters were useful for prediction of the severity of disease.

METHODS

Patients

SUBJECTS WERE 32 patients diagnosed with NAFLD/non-alcoholic steatohepatitis (NASH) by biopsy between April 2009 and March 2011 at our institution. All patients had untreated impaired glucose tolerance (no drug treatment) and present and past alcohol consumption of 20 g or less per week. No patient had been treated with drugs, such as tamoxifen, that can induce NAFLD/NASH. Patients were excluded if they had liver cirrhosis with decreased npRQ accompanied by protein energy malnutrition (PEM).[8] Other exclusion criteria included a history of liver diseases such as primary biliary cirrhosis, autoimmune hepatitis, hepatitis B infection or hepatitis C infection. Hepatocellular carcinoma (HCC) was not detected in any patient.

The study protocol was approved by the institutional review board. Written informed consent was obtained from all patients before trial registration. For all patients, tests were performed in the hospital under resting, fasted conditions in the early morning.

Study design

All subjects were hospitalized for at least 2 days to undergo a liver biopsy. Indirect calorimetry and 75-g OGTT were performed before liver biopsy, as described below. Anthropometric measurements and laboratory analysis were carried out before the indirect calorimetry study. All subjects received nutritional guidance from dieticians and were prescribed medical nutrition therapy (energy 25–30 kcal/kg ideal bodyweight).

Physical examination and serum biochemistry

Anthropometric measurements (body mass index [BMI], body fat percentage, and visceral fat area [VFA]) were performed using a body composition analyzer (InBody 720; BIOSPACE, Tokyo, Japan). We previously determined VFA values using a body composition analyzer and performed abdominal computed tomography using Fat Scan software (E2 system, Osaka, Japan) in 27 NAFLD patients. There was a strong correlation between these two modalities ($n = 27$, $R = 0.9319$, $P < 0.0001$; unpubl. data). Venous blood samples were collected in the early morning after patients had fasted for 12 h. These samples were used for several biochemical tests.

NAFIC score,[14] NAFLD fibrosis score[15] and FIB-4 index[16] were calculated using previously reported formulas.

75-g OGTT

A 75-G OGTT was performed, and plasma glucose and immunoreactive insulin (IRI) were measured at 0, 30, 60, 90 and 120 min after glucose loading. Based on the classification of the Expert Committee on the Diagnosis and Classification of DM,[17] individuals were diagnosed with impaired fasting glucose (IFG) if they had fasting plasma glucose levels of 110 mg/dL or more, but less than 126 mg/dL, and if they had a plasma glucose level less than 140 mg/dL at 120 min after glucose loading. Individuals were diagnosed with impaired glucose tolerance (IGT) if they had plasma glucose levels of less than 110 mg/dL at 0 min after loading and exceeding 140 mg/dL at 120 min after loading. Individuals were diagnosed with diabetes mellitus (DM) if they had plasma glucose levels exceeding 200 mg/dL at 120 min after loading. Homeostasis Model of Assessment – Insulin Resistance (HOMA-IR) was calculated using the following formula:[18] HOMA-IR = fasting insulin (mU/mL) × plasma glucose (mg/dL) / 405. Plasma glucose area under the curve (AUC glucose) and IRI area under the curve (AUC IRI) were calculated using methods reported previously.[19]

Indirect calorimetry

Energy metabolism was measured by indirect calorimetry (Aero Monitor AE-300s; Minato Medical Science, Osaka, Japan). A previously reported method[19] was used to measure oxygen uptake and carbon dioxide exhalation under resting, fasted conditions in the early morning. Twenty-four-hour urine nitrogen levels were also measured. The resulting values were used to calculate npRQ and resting energy expenditure (REE). The basal metabolic rate (BMR) was calculated using the Harris–Benedict formula.[20]

Pathology

All samples were diagnosed by a pathologist who was not notified of subjects' clinical data or course. The

classification of Brunt *et al.*[21] was used for fibrosis staging, and disease activity was assessed using the NAFLD activity score (NAS).[22]

Statistical analyses

Statistical analysis was performed using SPSS ver. 20.0 software (SPSS, Chicago, IL, USA). Results were expressed as mean ±standard deviation or standard error of the mean. A χ^2-test was used for categorical variables. A Student's *t*-test or Mann–Whitney *U*-test was used to compare two groups. One-way ANOVA or Kruskal–Wallis analysis followed by a post-hoc test was used to compare multiple independent groups. Correlation was assessed using Spearman's correlation coefficient. Receiver-operator curves (ROC) were used to assess discrimination ability. Statistical significance was defined as $P < 0.05$.

RESULTS

Patients

THE CLINICAL AND biochemical characteristics of patients enrolled in the study are summarized in Table 1. The 32 subjects (24 male, eight female) had a mean age of 45.4 years (range, 27–75). BMI ranged 22.0–38.8 kg/m^2 and averaged 27.2 kg/m^2. Serum alanine aminotransferase (ALT) levels ranged 22–200 IU/L and averaged 95.6 IU/L. Histological findings are shown also in Table 1. Fibrosis stages were determined according to Brunt *et al.*'s classification,[21] and there were eight patients at stage 0, 10 patients at stage 1, seven patients at stage 2 and seven patients at stage 3. For NAS, there were six patients with scores of less than 3, 20 patients with scores of 3 or 4, and six patients with scores of 5 or more.

Anthropometric measurements

Body mass index, body fat percentage and VFA tended to increase as fibrosis progressed. However, there was no significant difference in these parameters among groups with different Brunt stages (Table 2).

75-g OGTT

Four patients (12.5%) had HbA1c levels of at least 6.1% or fasting glucose of at least 110 mg/dL and in whom impaired glucose tolerance was suspected before the 75-g OGTT. The 75-g OGTT did not reveal a normal glucose tolerance pattern in any patient, and all patients had impaired glucose metabolism. One patient (3.1%) had IFG, 16 patients (50.0%) had IGT and 15 patients (46.9%) had DM.

Table 1 Characteristics of the patient population ($n = 32$)

Variable	
Sex (male/female)	24/8
Age (years)	45.4 ± 12.2
BMI (kg/m^2)	27.2 ± 4.0
AST (IU/L)	65.6 ± 73.4
ALT (IU/L)	95.6 ± 62.7
γ-GT (IU/L)	91.3 ± 76.8
Total cholesterol (mg/dL)	206.7 ± 44.7
Triglyceride (mg/dL)	155.4 ± 85.2
NEFA (μEq/L)	552.1 ± 212.0
Albumin (g/dL)	4.4 ± 0.5
Prothrombin time (%)	104.6 ± 11.2
Platelet count (X 10^4/μL)	23.7 ± 6.2
P-III-P (U/mL)	0.64 ± 0.28
Type IV collagen 7S (ng/mL)	4.4 ± 2.2
Fasting glucose (mg/dL)	94.4 ± 15.6
75-g oral glucose tolerance test	
Normal/IFG/ IGT/DM	0/1/16/15
Histological assessment	
Stage (0/1/2/3)†	8/10/7/7
Grade (0/1/2/3)†	8/6/14/4
NAS (<3/ 3,4/ ≥5)	6/20/6

Results are expressed as mean ± standard deviation.
†Stage and grade on histological assessment were determined using Brunt's classification.[21]
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DM, diabetes mellitus; γ-GT, γ-glutamyl transferase; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NEFA, non-esterified fatty acid.

Fasting glucose, HbA1c and AUC glucose increased as fibrosis progressed, and glucose metabolism was significantly worsened with fibrosis progression (Table 2). The 75-g OGTT revealed a correlation between postprandial hyperglycemia and Brunt stage (Fig. 1a). There were significant differences among fibrosis stages in plasma glucose levels at 0, 30 and 120 min after loading ($P < 0.05$ using Kruskal–Wallis analysis).

In patients with fibrosis stages 1–3, fasting insulin levels were increased and HOMA-IR was elevated, indicating the presence of insulin resistance. This tendency was significantly more pronounced in more advanced fibrosis stages (Table 2). In the 75-g OGTT (Fig. 1b), insulin secretion was delayed and postprandial hyperinsulinemia was observed for all stages compared with healthy controls, as previously reported.[23] In particular, stage 3 patients had significantly greater hyperinsulinemia than patients at the other stages at 0, 90 and 120 min after loading ($P < 0.05$ using Kruskal–Wallis analysis).

Table 2 Clinical features and laboratory data of NAFLD/NASH patients determined using Brunt *et al.*'s classification[21]

Variable	Stage 0 ($n = 8$)	Stage 1 ($n = 10$)	Stage 2 ($n = 7$)	Stage 3 ($n = 7$)	*P*-value†
Sex (male/female)	5/3	8/2	5/2	6/1	0.7348
Age (years)	46.1 ± 11.4	40.3 ± 13.0	46.3 ± 11.9	50.8 ± 11.8	0.3713
BMI (kg/m^2)	25.1 ± 1.9	27.2 ± 5.9	27.6 ± 2.2	29.1 ± 3.1	0.2714
Percent body fat (%)	30.3 ± 6.8	28.7 ± 8.4	33.6 ± 6.7	33.2 ± 7.1	0.5082
VFA (cm^2)	116.7 ± 22.3	122.3 ± 47.5	141.4 ± 26.7	163.5 ± 46.8	0.1029
AST (IU/L)	29.8 ± 5.4	48.6 ± 22.6	78.7 ± 35.5	117.7 ± 142.3	<0.01
ALT (IU/L)	43.3 ± 26.7	91.5 ± 51.8	131.0 ± 43.2	125.7 ± 86.0	<0.05
γ-GT (IU/L)	74.4 ± 29.1	85.1 ± 73.9	75.7 ± 45.1	134.9 ± 127.6	0.5789
Total cholesterol (mg/dL)	200.6 ± 23.0	214.8 ± 59.5	200.9 ± 30.0	188.0 ± 52.1	0.5131
Triglyceride (mg/dL)	116.8 ± 6.5	181.9 ± 109.3	170.8 ± 87.2	145.1 ± 75.6	0.5364
NEFA (μEq/L)	494.0 ± 231.9	571.8 ± 285.0	619.2 ± 31.0	532.9 ± 171.4	0.8629
Type IV collagen 7S (ng/mL)	3.51 ± 0.71	3.46 ± 0.98	3.82 ± 0.54	6.94 ± 3.12	<0.05
Ferritin (ng/mL)	157.0 ± 45.9	225.1 ± 208.9	178.4 ± 67.9	474.4 ± 578.5	<0.05
Fasting glucose (mg/dL)	85.5 ± 8.69	88.6 ± 7.71	109.7 ± 21.5	110.4 ± 34.7	<0.05
Fasting insulin (μU/mL)	8.00 ± 3.62	11.3 ± 4.81	15.2 ± 10.8	26.1 ± 15.3	<0.005
HOMA-IR	1.70 ± 0.80	2.46 ± 1.19	4.11 ± 3.03	6.56 ± 2.78	<0.005
Hemoglobin A1c (%)	5.61 ± 0.55	5.41 ± 0.38	5.97 ± 0.89	6.74 ± 1.75	<0.05
75-g OGTT					
Normal/IFG/IGT/DM	0/0/6/2	0/1/6/3	0/0/4/3	0/0/0/7	
AUC glucose (mg·h/dL)	363.8 ± 64.9	406.4 ± 87.2	450.6 ± 39.0	497.1 ± 45.8	<0.005
AUC IRI (μU·h/mL)	247.4 ± 130.6	247.7 ± 99.1	238.54 ± 96.2	536.2 ± 305.1	<0.05
Indirect calorimetry					
npRQ	0.895 ± 0.068	0.869 ± 0.067	0.808 ± 0.046	0.798 ± 0.026	<0.005
REE/BMR	0.922 ± 0.091	0.922 ± 0.160	1.034 ± 0.069	1.021 ± 0.073	0.2495
NAFIC score[14]	0.125 ± 0.354	0.600 ± 0.699	0.857 ± 0.378	2.143 ± 0.900	<0.0005
NAFLD fibrosis score[15]	−2.77 ± 1.13	−2.55 ± 1.08	−2.06 ± 0.69	−0.73 ± 1.92	<0.05
FIB-4 index[16]	0.880 ± 0.331	0.952 ± 0.472	1.326 ± 0.674	2.564 ± 2.391	<0.05

Results are expressed as mean ± standard deviation.

†*P*-value for four-group comparisons.

Differences and correlations among the four groups were determined using one-way ANOVA or Kruskal–Wallis analysis followed by a post-hoc test.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC glucose, plasma glucose area under the curve; AUC IRI, immunoreactive insulin area under the curve; BMI, body mass index; BMR, basal metabolic rate; DM, diabetes mellitus; γ-GT, γ-glutamyl transferase; HOMA-IR, homeostasis model assessment of insulin resistance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NEFA, non-esterified fatty acid; npRQ, non protein respiratory quotient; 75-g OGTT, 75-g oral glucose tolerance test; REE, resting energy expenditure; VFA, visceral fat area.

Indirect calorimetry

Non-protein respiratory quotient values determined using indirect calorimetry data significantly decreased as fibrosis progressed (Table 2, Fig. 2a). In addition, npRQ values significantly decreased as NAS increased (Fig. 2b). There was no relationship between npRQ and disease activity (Fig. 2c).

Resting energy expenditure and BMR predicted using the Harris–Benedict formula[20] did not differ among Brunt stages or NAS classifications (data not shown). The ratio of REE to BMR (REE/BMR) also did not differ among Brunt stages (Table 2) or NAS classifications (data not shown). In addition, this ratio was within the normal range ($0.9 < \text{REE/BMR} < 1.1$),[8] indicating that most subjects were in normal metabolic states, and not hyper- or hypometabolic states.

Blood biochemistry

There were significant differences among stages in AST, ALT, type IV collagen 7S and ferritin levels (Table 2). However, there were no significant differences among stages with respect to other parameters, including γ-glutamyl transferase (γ-GT), total cholesterol, triglyceride and non-esterified fatty acid (NEFA) (Table 2),

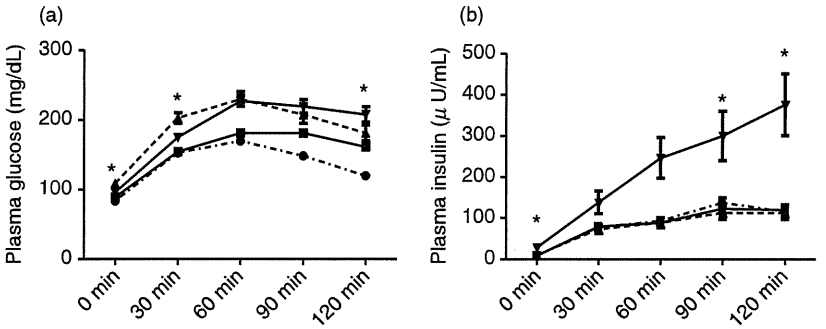

Figure 1 (a) Serum glucose levels during a 75-g oral glucose tolerance test in non-alcoholic fatty liver disease (NAFLD) patients. *There were significant differences among stages in plasma glucose levels at 0, 30 and 120 min ($P < 0.05$) after loading as determined by Kruskal–Wallis analysis. (b) Serum insulin levels during a 75-g oral glucose tolerance test in NAFLD patients. *There were significant differences among stages in plasma glucose levels at 0, 90 and 120 min ($P < 0.05$) after loading as determined by Kruskal–Wallis analysis. Results are expressed as mean ± standard error of the mean. ·-●-, Stage 0; —■—, stage 1; -▲-, stage 2; —▼—, stage 3.

AST/ALT ratio, hyaluronic acid, platelet count and prothrombin time (data not shown).

Comparison of patients with mild fibrosis (stages 0–1) and patients with more advanced fibrosis (stages 2–3)

To identify factors correlated with fibrosis in NAFLD patients, we divided subjects into two groups – those with mild fibrosis (stages 0–1) and those with more advanced fibrosis (stages 2–3) – and compared clinical features.

Patients with more advanced fibrosis had significantly higher values than patients with mild fibrosis for the following parameters: VFA, serum AST, ALT, P-III-P, type IV collagen 7S, fasting glucose, fasting insulin, HOMA-IR, HbA1c and AUC glucose (Table 3). Patients having more advanced fibrosis had significantly lower npRQ values than patients with mild fibrosis (Table 3). There were no significant differences between the two groups with respect to other parameters, including γ-GT, total cholesterol, triglyceride and NEFA (data not shown).

Correlation of npRQ with parameters of glucose and fat metabolism and body composition

We next compared npRQ to parameters of glucose and fat metabolism and body composition. There was a negative correlation between npRQ and AUC glucose ($R = -0.6308$, $P < 0.001$ using Spearman's correlation coefficient) (Fig. 3a). There was also a negative correla-

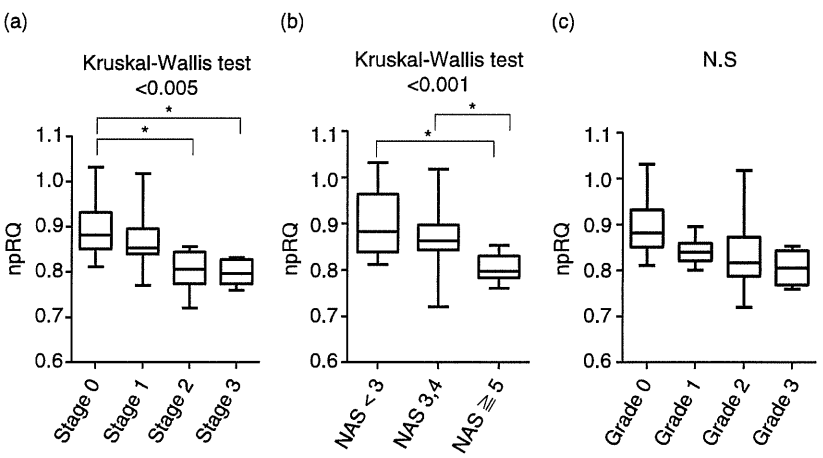

Figure 2 Non-protein respiratory quotient (npRQ) in non-alcoholic fatty liver disease (NAFLD) patients. (a) By stage. (b) By NAFLD activity score (NAS). (c) By grade. There was a significant difference in npRQ among (a) stages and (b) NAS. *Post-hoc test showed a significant difference ($P < 0.05$). N.S., not significant.

Table 3 Comparison of clinical features and laboratory data between stages 0–1 versus stages 2–3 in NAFLD/NASH patients

	Stages 0–1 ($n = 18$)	Stages 2–3 ($n = 14$)	*P*-value†
Sex (male/female)	13/5	11/3	0.6807
Age (years)	42.9 ± 12.35	48.6 ± 11.6	0.1947
BMI (kg/m^2)	26.3 ± 4.6	28.4 ± 2.8	0.1419
Percent body fat (%)	29.4 ± 7.6	33.4 ± 6.7	0.1398
VFA (cm^2)	119.7 ± 36.8	153.3 ± 39.0	<0.05
AST (IU/L)	40.2 ± 19.4	98.2 ± 101.7	<0.05
ALT (IU/L)	70.1 ± 48.2	128.4 ± 65.5	<0.01
P-III-P (U/mL)	0.53 ± 0.10	0.81 ± 0.38	<0.01
Type IV collagen 7S (ng/mL)	3.48 ± 0.84	5.64 ± 2.83	<0.005
Fasting glucose (mg/dL)	87.2 ± 8.1	104.3 ± 18.2	<0.005
Fasting insulin (μU/mL)	9.9 ± 4.5	21.3 ± 14.2	<0.005
HOMA-IR	2.1 ± 1.1	5.2 ± 3.1	<0.0005
Hemoglobin A1c (%)	5.5 ± 0.5	6.0 ± 0.7	<0.05
75-g OGTT			
Normal/IFG/IGT/DM	0/1/12/5	0/0/4/10	
AUC glucose (mg·h/dL)	379.4 ± 64.2	473.4 ± 49.4	<0.0005
AUC IRI (μU·h/mL)	254.2 ± 100.6	371.8 ± 261.6	0.1448
Indirect calorimetry			
npRQ	0.881 ± 0.067	0.803 ± 0.036	<0.0005

Results are expressed as mean ± SD.
†*P*-value for two comparisons.
Differences between two groups were determined using Student's *t*-test, Mann-Whitney's *U*-test, or chi-square test.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC glucose, blood glucose area under the curve; AUC IRI, immunoreactive insulin area under the curve; BMI, body mass index; DM, diabetes mellitus; γ-GT, γ-glutamyl transferase; HOMA-IR, Homeostasis Model of Assessment – Insulin Resistance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; npRQ, non-protein respiratory quotient; 75-g OGTT, 75-g oral glucose tolerance test; VFA, visceral fat area.

tion between npRQ and HOMA-IR ($R = -0.5045$, $P < 0.005$) (Fig. 3b). A weak negative correlation was found between npRQ and fasting glucose ($R = -0.4585$, $P < 0.01$) (Fig. 3c), fasting insulin ($R = -0.4431$, $P < 0.05$) (Fig. 3d), γ-GT ($R = -0.4428$, $P < 0.05$), plasma glucose levels 120 min after loading ($R = -0.3684$, $P < 0.05$) and IRI levels 120 min after loading in the OGTT ($R = -0.3772$, $P < 0.05$). No significant correlation was found between npRQ and AUC IRI ($R = -0.2992$, $P = 0.1021$), total cholesterol ($R = -0.2499$, $P = 0.1678$), triglyceride ($R = -0.0617$, $P = 0.7599$), NEFA ($R = -0.0629$ $P = 0.7367$), BMI ($R = -0.3165$, $P = 0.0776$), body fat percentage ($R = -0.1233$, $P = 0.5088$) or VFA ($R = -0.2308$, $P = 0.2199$). Based on these results, we speculated that low npRQ in NAFLD is associated with impaired glucose tolerance due to insulin resistance.

Comparison of npRQ to several parameters and previously established scoring systems

We calculated area under the ROC (AUROC) for npRQ and for several of the parameters shown in Table 2. We compared these AUROC to see if they could differentiate stage 3 from stages 0–2, stages 2–3 from stages 0–1, and stage 0 from stages 1–3. Table 4 summarizes these results. For differentiation of stages 3 from stages 0–2, the calculated AUROC was greatest for NAFIC score (0.9200), followed by HOMA-IR (0.9100), type IV collagen 7S (0.8820), AUC glucose and fasting insulin (0.8743), ferritin (0.8690) and npRQ (0.8343). For differentiation of stages 2–3 from stages 0–1, the AUROC for npRQ was greatest (0.8849), followed by HOMA-IR (0.8846), AUC glucose (0.8690), fasting glucose (0.8651), NAFIC score (0.8373) and fasting insulin (0.8234). For differentiation of stage 0 from stages 1–3, the AUROC for ALT was greatest (0.8568), followed by AST (0.8542), fasting insulin (0.8490), HOMA-IR and AUC glucose (both 0.8478), NAFIC score (0.8281) and npRQ (0.8203).

In each of the three comparisons of stages, AUROC for npRQ, HOMA-IR, AUC glucose, fasting insulin and NAFIC score were all over 0.8000 and showed relatively good results. To differentiate stage 3 from stages 0–2, the AUROC for type IV collagen 7S and ferritin were

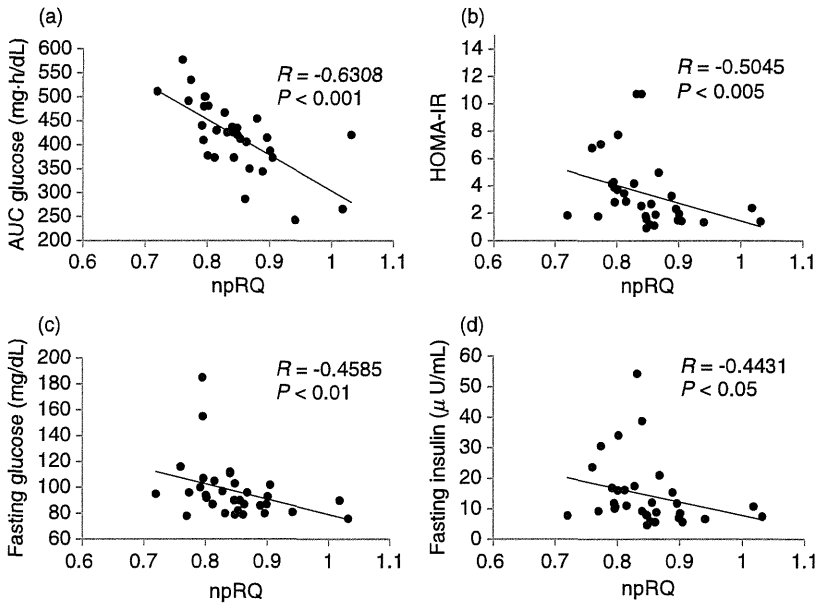

Figure 3 Correlation between non-protein respiratory quotient (npRQ) and other parameters ($n = 32$). (a) With area under the curve (AUC) glucose. (b) With Homeostasis Model of Assessment - Insulin Resistance. (c) With fasting glucose. (d) With fasting insulin.

Table 4 AUROC for npRQ, other biochemical parameters and scoring systems for NAFLD/NASH patients

Variable	AUROC Stage 0 vs. Stages 1–3	AUROC Stages 0–1 vs. Stages 2–3	AUROC Stages 0–2 vs. Stage 3
npRQ	0.8203	0.8849	0.8343
HOMA-IR	0.8478	0.8846	0.9100
AUC glucose	0.8478	0.8690	0.8743
AUC IRI	0.5924	0.6154	0.8133
Fasting glucose	0.7813	0.8651	0.7143
Fasting insulin	0.8490	0.8234	0.8743
Hemoglobin A1c	0.5617	0.7668	0.8080
Type IV collagen 7S	0.5893	0.7870	0.8820
NAFIC score[14]	0.8281	0.8373	0.9200
NAFLD fibrosis score[15]	0.6615	0.7460	0.8057
FIB-4 index[16]	0.6250	0.7222	0.7143
AST	0.8542	0.7976	0.6514
ALT	0.8568	0.7778	0.6343
Ferritin	0.5893	0.6912	0.8690

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC glucose, plasma glucose area under the curve; AUC IRI, immunoreactive insulin area under the curve; AUROC, area under the receiver-operator curve; HOMA-IR, homeostasis model assessment of insulin resistance; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; npRQ, non-protein respiratory quotient.

high; however, AUROC for these parameters were not able to differentiate stage 0 from stages 1–3. This is due to the fact that these two parameters had elevated values in stage 3 and there was no significant difference from stage 0 to stage 2. The AUROC for NAFLD fibrosis score and FIB-4 index were lowest for differentiation of stage 0 from stages 1–3, and increased for differentiation of stages 2–3 from stages 0–1 and differentiation of stage 3 from stages 0–2. This result suggests that these two methods of scoring fibrosis had a relatively high degree of accuracy in distinguishing severe from mild or no fibrosis. AUROC for AST and ALT could be used to differentiate stage 0 from stages 1–3, but were not as accurate in differentiating stage 3 from stages 0–2.

DISCUSSION

NON-ALCOHOLIC FATTY LIVER disease comprises a wide spectrum of conditions ranging from simple steatosis to NASH, which can progress to cirrhosis and HCC. Patients with advanced liver fibrosis are considered to be at high risk for liver failure and HCC.[1–6] Thus, it is important to efficiently identify patients at risk for advanced fibrosis among a large number of NAFLD patients. In addition, differentiation of early-stage NASH allows for early intervention, which can improve patients' outcomes. Liver biopsy is the most reliable method for the diagnosis and determination of fibrosis stage in patients with NASH. However, it is

widely acknowledged that biopsy is costly and runs the risk of sampling error and procedure-related morbidity and mortality. Some guidelines recommend that liver biopsy should be considered in patients who are at risk for NASH with advanced fibrosis,[4-6] but this recommendation is not universally accepted. Therefore, various parameters have been proposed as tools to distinguish NASH from NAFLD, or to determine fibrosis stage. Various serum biochemical markers, including indicators of oxidative stress, insulin resistance, inflammation, and apoptosis, have been used for this purpose.[1-6]

With regard to glucose metabolism and insulin resistance, a 75-g OGTT may help clinicians to identify high-risk patients for more intensive monitoring and treatment because blood glucose and insulin levels in the OGTT are important factors for the diagnosis of NAFLD and prediction of fibrosis.[11,12,24-26] Studies in which a 75-g OGTT was performed have shown that impaired glucose tolerance is common even in NAFLD patients without overt DM,[11,12,24-26] and that postprandial hyperglycemia is associated with advanced fibrosis.[12,24,26] Postprandial hyperinsulinemia is also observed in nearly all NAFLD patients, even those with normal glucose tolerance.[11,12] Kimura *et al.*[11] reported that postprandial hyperinsulinemia, as indicated by an OGTT, became more marked as fibrosis stage advanced.

Indirect calorimetry provides important information about energy expenditure, npRQ, and the rate of oxidation of three major macronutrients (carbohydrates, fat and protein) based on respiratory gas exchange and urinary nitrogen excretion. Indirect calorimetry is considered the gold standard for assessing energy expenditure and aids in the delivery of the highest quality of nutritional care.[7] The advantages of this modality are that it is non-invasive, portable enough to be done at bedside, easy to operate and inexpensive.[7] Many studies have used this modality to estimate the nutritional state of cirrhotic patients with chronic liver disease. Tajika *et al.*[8] have reported that low npRQ derived from PEM is associated with survival in patients with viral liver cirrhosis. We have previously used indirect calorimetry in cirrhotic patients to evaluate the effects of nutritional treatment with branched-chain amino acids.[19,27-29] In diabetic patients, indirect calorimetry is often used to estimate glucose oxidation rate and it has been reported that both glucose oxidation and non-oxidative disposal are impaired during hyperinsulinemic clamping in type 2 DM patients.[30] Although indirect calorimetry is used for assessment in several metabolic diseases, this is the first report to examine indirect calorimetry data from patients with NAFLD. The objective of the present study was to determine how energy metabolism, as estimated by indirect calorimetry, is related to the clinicopathogenesis of NAFLD with glucose intolerance.

We found that npRQ decreased in severity with increased fibrosis stage in NAFLD patients. This observation raised the question of the mechanism underlying decreased npRQ in patients with advanced fibrosis. As fibrosis progressed, npRQ decreased significantly, glucose intolerance worsened and insulin resistance increased (Tables 2,3). In fact, negative correlations were seen between npRQ and several parameters of glucose intolerance: AUC glucose, HOMA-IR, and fasting glucose and insulin levels. Thus, we speculated that decreased npRQ in NAFLD results from glucose intolerance due to insulin resistance, which worsened with fibrosis stage. Decreased npRQ can reflect reduced glucose oxidation and enhanced lipid oxidation.[8,19,27-29] It was reported that peripheral insulin resistance reduces glucose oxidation and glucose uptake in peripheral skeletal muscle.[30] This reduction in glucose uptake may reflect hyperglycemia and decreased glucose oxidation, because the amount of free cellular glucose available for oxidation is reduced.[31] However, it was also speculated that the low glucose oxidation rate seen in viral cirrhosis is a result of reduced glucose production due to decreased hepatic glycogen.[32] In our study, glycogen levels in the liver were not measured directly and thus a definitive statement cannot be made. However, npRQ was low even in the one patient with mild (stage 1) fibrosis, who was found not to be in a state of malnutrition as determined by anthropometry and in whom glycogen storage was likely not decreased to a large extent. Therefore, it is unlikely that the low npRQ in these patients primarily reflects decreased glycogen stores. However, we do not suggest that low npRQ in NAFLD patients is solely due to glucose intolerance because whole-body energy metabolism is a complex process, and thus it is possible that other factors also contribute to low npRQ.[30] Yokoyama *et al.*[33] have reported that the glucose oxidation rate of subjects with type 2 diabetes is inversely correlated with BMI, body fat percentage and plasma fatty acid levels, suggesting that decreased glucose oxidation and increased fat oxidation may be potently affected by adiposity. In our study, npRQ showed no correlation with lipid parameters, including serum total cholesterol, triglyceride and NEFA, or anthropometric parameters such as body fat percentage and VFA. Thus, low npRQ was speculated to be associated with decreased glucose oxidation due to glucose intolerance, but not with increased fat oxidation, which occurs in the maintenance and development

of hyperglycemia during decompensation.[30] Decreased npRQ was correlated with glucose intolerance and fibrosis stage, suggesting that the clinicopathogenesis of NAFLD is closely associated with glucose intolerance, and that early intervention for glucose intolerance is important in clinical practice.

To examine the utility of npRQ as a marker of disease progression in NAFLD, we compared AUROC for npRQ with those for various other parameters and scoring systems in three patterns to discriminate NASH from NAFLD (stage 0 vs stages 1–3), significant fibrosis (stages 0–1 vs stages 2–3) and advanced fibrosis (stages 0–2 vs stage 3). As the decrease in npRQ became significant at stage 2 (Fig. 2a), the AUROC for npRQ for differentiation of stages 2–3 from stages 0–1 was superior to other parameters. Therefore, our results indicate that decreased npRQ can be used to detect NASH, including relatively early stage NASH in many NAFLD patients. In addition to npRQ, AUROC for HOMA-IR, AUC glucose, fasting insulin and NAFIC score were each approximately 0.850 and also showed differences for each of the three differentiation patterns. Therefore, these parameters also have the ability to detect NASH from the early stages to the development of severe fibrosis. NAFIC score, the scoring system for fibrosis proposed by Sumida *et al.*,[14] comprises three measurements (serum ferritin, insulin and type IV collagen 7S) and is easy to calculate. A validation study by the Japan Study Group of NAFLD (JSG-NAFLD) reported that NAFIC score was superior to other several previously established scoring systems in detecting NASH with fibrosis among Japanese NAFLD patients, and also for predicting severe fibrosis.[14] In the present study, AUROC for NAFIC score for differentiation of stage 3 from stages 0–2 was 0.9200 and was the highest among the various parameters, supporting the conclusions of the JSG-NAFLD report.[14] NAFLD fibrosis score,[15] which consists of six variables (age, BMI, hyperglycemia, platelet count, albumin and AST/ALT ratio) has been reported to reliably predict advanced fibrosis. In a meta-analysis by Angulo *et al.*,[15] NAFLD fibrosis score had an AUROC of 0.85 for predicting advanced fibrosis (stages 3–4). The fact that subjects with stage 4 were excluded from the present study but were included as "advanced stage" in previously reported studies,[14,15,34] may also contribute to the lower value of AUROC in the present study. In addition, it is uncertain whether a scoring system established using data from Caucasian populations is applicable to Asian patients, because Asian patients tend to develop NASH and other metabolic complications at a lower BMI than Caucasians.[3] The FIB-4 index was developed as a scoring system for estimation of liver fibrosis in subjects with HIV and hepatitis C virus co-infection.[16] It relies on patient age, AST, ALT and platelet count. The advantage of FIB-4 index is that it is easy to calculate and does not require the use of BMI. In a validation study of JSG-NAFLD,[34] FIB-4 index was superior to other fibrosis scoring systems in Japanese NAFLD patients for excluding advanced fibrosis. In our study, the AUROC for FIB-4 index for discrimination of stage 3 from stages 0–2 was not satisfied (0.7143) and was inferior to NAFIC score. As described above, the lower AUROC for FIB-4 index than in previous reports[34] may be due to the fact that stage 4 patients were excluded from the present study, but were included in previous reports. As the number of subjects in the present study was small, we cannot conclude which parameters and scoring system is best for the estimation of severity of NAFLD, nor we can definitively state that npRQ is the best method for differentiation of NASH from NAFLD. However, Table 4 shows that npRQ, HOMA-IR, AUC glucose, fasting glucose, insulin and NAFIC scores all could provide useful information for the detection of NASH, including patients in the early fibrosis stage, whereas NAFLD fibrosis score and type IV collagen were useful for identification of advanced fibrosis. It is important to select these parameters and scoring systems according to the purpose: to differentiate early-stage NASH from NAFLD, or to detect advanced fibrosis.

Although we believed that npRQ is useful for the estimation of disease severity in NAFLD patients, unfortunately, npRQ measurement is not always possible in clinical practice. This is because indirect calorimetry for measurement of npRQ was primarily used in inpatient and research settings[7] and not all clinicians are familiar with the equipment used. In addition, patients must be tested in the early morning after fasting, and 24-h urine specimens must be collected for calculation of npRQ. Okumura *et al.*[35] investigated the use of serum biochemistry to predict npRQ in patients with viral cirrhosis. That report concluded that serum NEFA can be used to predict npRQ. However, in the present study, npRQ was not correlated with NEFA. This may be because the subjects in the present study were NAFLD patients without cirrhosis, and not viral cirrhosis patients with decreased glycogen storage.[35] In NAFLD patients with glucose intolerance, HOMA-IR may be useful for prediction of npRQ without calorimetry, because these two parameters were negatively correlated.

In conclusion, npRQ is useful for the estimation of disease severity in NAFLD patients with glucose intolerance. It enables the detection of NASH with relatively

early-stage fibrosis among NAFLD patients. As this measurement can provide useful information without the burden of blood collection, it should be included in clinical practice in addition to anthropometry and blood analysis. When an NAFLD patient exhibits low npRQ, the patient should undergo further examination such as a liver biopsy to allow for early intervention.

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ORIGINAL RESEARCH

Treatment of nonalcoholic steatohepatitis with vitamins E and C: a pilot study

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Background: Nonalcoholic steatohepatitis (NASH) is a common liver disease that can progress to cirrhosis. Oxidative stress is one of the central mechanisms causing hepatocellular injury in the disease. In this study, antioxidant therapy using both vitamins C and E was conducted in patients with NASH.

Methods: Vitamin E 300 mg/day and vitamin C 300 mg/day were administered orally to 23 patients with NASH for 12 months. Body mass index was measured during therapy. Serum levels of alanine aminotransferase, thioredoxin (an oxidative stress marker), and high-sensitivity C-reactive protein were measured before treatment and after 12 months in all patients. Ten of the 23 patients underwent liver biopsy before and after treatment.

Results: Body mass index remained unchanged during treatment with vitamins C and E. Serum alanine aminotransferase, thioredoxin, and high-sensitivity C-reactive protein levels were decreased significantly at 12 months compared with pretreatment. Liver biopsies showed improved necroinflammatory activity in eight cases and improved fibrosis staging in 4.

Conclusion: Serum alanine aminotransferase, thioredoxin, and high-sensitivity C-reactive protein levels, and liver histology were clearly improved with vitamin C and E therapy. These findings suggest that combination therapy using these vitamins may be useful in patients with NASH to minimize damage from oxidative stress and slow the processes leading to cirrhosis.

Keywords: vitamin E, vitamin C, nonalcoholic steatohepatitis, oxidative stress

Introduction

Nonalcoholic steatohepatitis (NASH) is a very common chronic liver disease that resembles alcoholic liver disease clinically and histologically, but occurs in individuals in the absence of a history of significant alcohol consumption. NASH is frequently associated with clinical conditions such as obesity, type 2 diabetes mellitus, hyperlipidemia, and hypertension. These background characteristics indicate that NASH may be part of the spectrum of "metabolic syndrome". More recently, NASH has been proposed as a possible cause of cryptogenic cirrhosis.[1] The pathogenesis of NASH is multifactorial, involving abnormal lipid metabolism, production of reactive oxygen species, increased hepatic lipid peroxidation, activated stellate cells, and abnormal patterns of cytokine production. Oxidative stress appears to be a key factor in the progression from steatosis to NASH and potentially to cirrhosis.[2–5]

No universally effective treatment for NASH has been identified. Vitamin E refers to a group of naturally occurring compounds with antioxidant properties. A pilot trial of vitamin E aimed at decreasing oxidative stress in pediatric patients with presumed

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NASH showed that serum alanine aminotransferase and aspartate aminotransferase levels normalized during this treatment.[6] We have previously evaluated the efficacy of vitamin E therapy in patients with NASH.[7] Significant improvements in serum alanine aminotransferase and gamma glutamyl transferase were observed during treatment. At the same time, levels of the oxidative stress markers, thioredoxin and thiobarbituric acid, were significantly decreased. Furthermore, Harrison et al recently reported that treatment with vitamins C and E improves liver fibrosis in patients with NASH.[8] In the present study, vitamins C and E were administered for 12 months at lower doses than those used by Harrison et al, and changes in levels of alanine aminotransferase, oxidative stress markers, and high-sensitivity C-reactive protein were measured before and after treatment to clarify the efficacy and mechanisms of action underlying vitamin C and E therapy. Thioredoxin is a stress-inducible thiol-containing protein that has been shown to be an indicator of oxidative stress in a variety of diseases. Weight reduction is reported to improve liver enzyme abnormalities and liver histology in obese patients with NASH.[9,10] However, many patients find it virtually impossible to maintain body weight loss. Therefore, this study evaluated the effects of vitamin C and E on serum alanine aminotransferase, thioredoxin, high-sensitivity C-reactive protein, and liver histology in patients with NASH in association with changes in body mass index during treatment.

Materials and methods

Patients

Twenty-three patients with NASH (ten men and 13 women, Matteoni classification 3 or 4, mean age 53.1 ± 14.9 years) were included in this study. Twenty patients had a body mass index > 25 kg/m^2. Nineteen patients had hypertension and 18 patients had hyperinsulinemia and dyslipidemia (Table 1). Before pretreatment, all patients had been diagnosed as having NASH by liver biopsy.

Table 1 Demographic feature of patients at baseline

Feature	n
N	23
Age	53.1 ± 14.9
Male/female	10/13
BMI (kg/m^2)	20
Hypertension	19
Hyperinsulinemia	18
Dyslipidemia	18
Stage 0/1/2/3	0/14/6/3
Grade 0/1/2/3	0/15/5/3

Diagnostic criteria

NASH was diagnosed based on the following criteria: negative results for hepatitis B surface antigen and hepatitis C virus RNA, and exclusion of autoimmune liver disease, drug-induced hepatic disorder, or metabolic liver disease (eg, Wilson's disease, hemochromatosis); alcohol intake ≤ 30 g/week; and presence of steatosis (>30%) or steatohepatitis. The pathological classification proposed by Matteoni et al[11] was used to diagnose histological types 1 and 2 as simple steatosis and types 3 and 4 as NASH. Fibrosis was graded from stage 0 to stage 4 in accordance with the staging system, and inflammatory activity in the liver was graded from grade 0 to grade 3, also according to the grading system proposed by Brunt et al.[12] Steatosis was defined according to the percent of hepatocytes affected, and divided into four grades: grade 0, 0%; grade 1, >0% but <33%; grade 2, 33%–66%; and grade 3, >66%. Some pathologists read the liver biopsy blinded. Diabetes mellitus was diagnosed based on the following criteria proposed by the Japan Diabetic Society: fasting blood glucose ≥ 126 mg/dL; two-hour post-75 g oral glucose tolerance test result ≥ 200 mg/dL; casual blood glucose ≥ 200 mg/dL; and glycosylated hemoglobin ≥ 6.1 or treatment with one or more antidiabetic agents. Dyslipidemia was defined as triglyceride > 150 mg/dL and/or a low-density lipoprotein cholesterol level > 140 mg/dL or treatment with one or more lipid-lowering drugs. Hypertension was defined as systolic/diastolic blood pressure ≥ 140/90 mmHg or treatment with one or more antihypertensives.

Changes in body mass index were observed monthly in all patients during vitamin C and E therapy. Serum thioredoxin[13] and high-sensitivity C-reactive protein[14] levels were determined using enzyme-linked immunosorbent assays (Mitsubishi Kagaku Yatoron, Tokyo, Japan). Serum thioredoxin levels were also estimated using an enzyme-linked immunosorbent assay kit (Ledox BioScience, Kyoto, Japan). Vitamin E (α-tocopherol, Eisai, Tokyo, Japan) and vitamin C (ascorbic acid, Sionogi, Tokyo, Japan) were each administered orally at 300 mg/day for 12 months.

During therapy, the lifestyle of all patients remained unchanged. Body mass index and serum alanine aminotransferase, thioredoxin, and high-sensitivity C-reactive protein levels were measured before and after treatment. Liver biopsy was performed for all patients before treatment, and ten patients from whom consent was able to be obtained underwent liver biopsy after treatment. Liver histology was evaluated by staging of fibrosis and grading of necroinflammatory activity and steatosis.