heatmap visualization, and GraphPad Prism version 6.0.1 (Intuitive Software for Science, San Diego, CA) for ROC curve analysis and box-plot visualization.

3. Results

3.1. *Patient characteristics and virological responses*

The characteristics of patients who showed NVR and SVR are shown in Table 1. All of the patients had HCV genotype 1b infection with a high viral load exceeding 5 logIU/ml. Ten patients showed NVR and 10 achieved SVR. There were no significant differences in sex, age, height, weight, aspartate aminotransferase, ALT or GGT between the two patient groups. The only factor that differed significantly between the NVR and SVR patients was the prevalence of single nucleotide polymorphism of IL28B ($p = 0.011$); in the NVR group, a higher proportion of patients (6/10, 60%) carried the IL28B heterozygote or homozygote with minor alleles of rs8099917 than in the SVR group (0/10, 0%).

3.2. *Principal component analysis of patients before and after pegIFN/RBV therapy*

Principal component analysis demonstrated no significant differences in the pretreatment metabolomics profiles among patients with chronic hepatitis C examined before the start of PegIFN/RBV therapy, even between those who subsequently showed NVR and SVR (Fig. 1). When principal component (PC) analysis was applied to compare the changes in the metabolomics profiles before pegIFN/RBV therapy with those after the therapy, significant differences were evident, the first PC being significantly decreased and the third PC significantly increased in both the NVR and SVR patients (Fig. 2). The change in the distribution area of samples reflected these results, becoming narrower after treatment than before treatment, particularly in SVR cases (Fig. 3). This indicated a

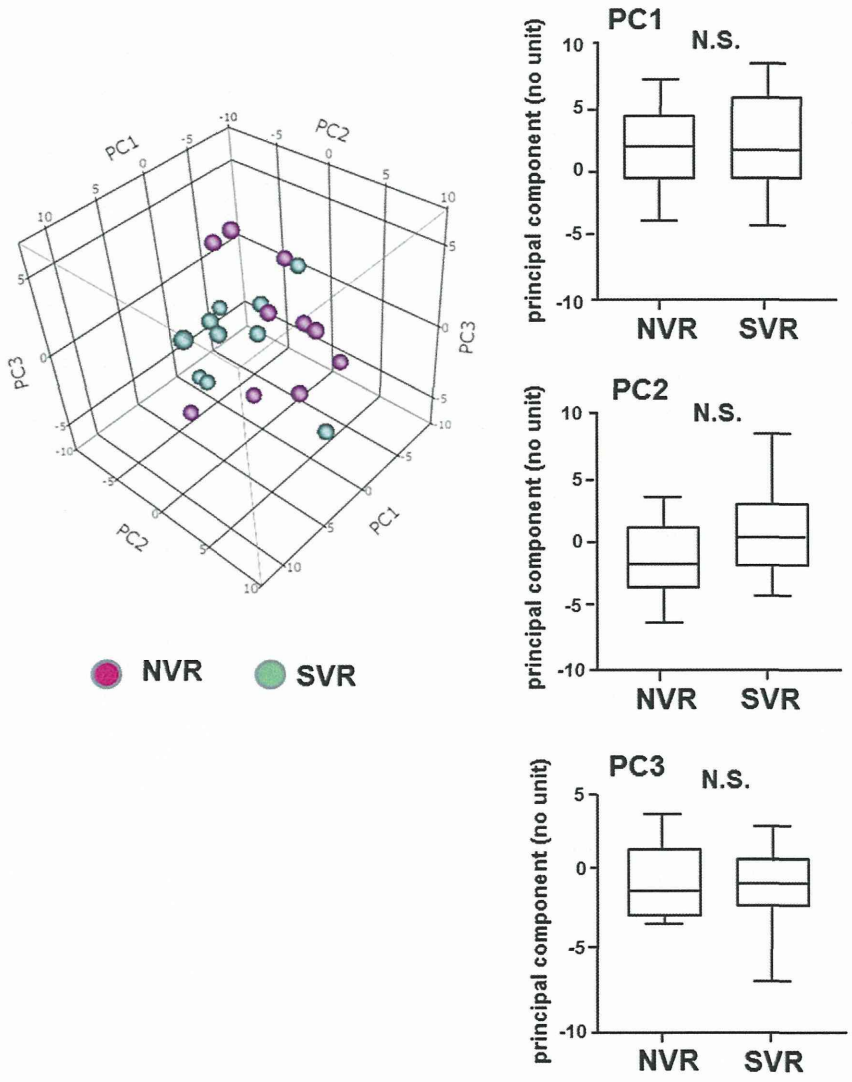

Fig. 1 – Principal component analysis of serum metabolites in the enrolled patients. No significant differences in the pretreatment metabolomics profiles were found between patients who subsequently showed a non-virological response (NVR) and those who achieved a sustained virological response (SVR). Mann–Whitney *U*-test.

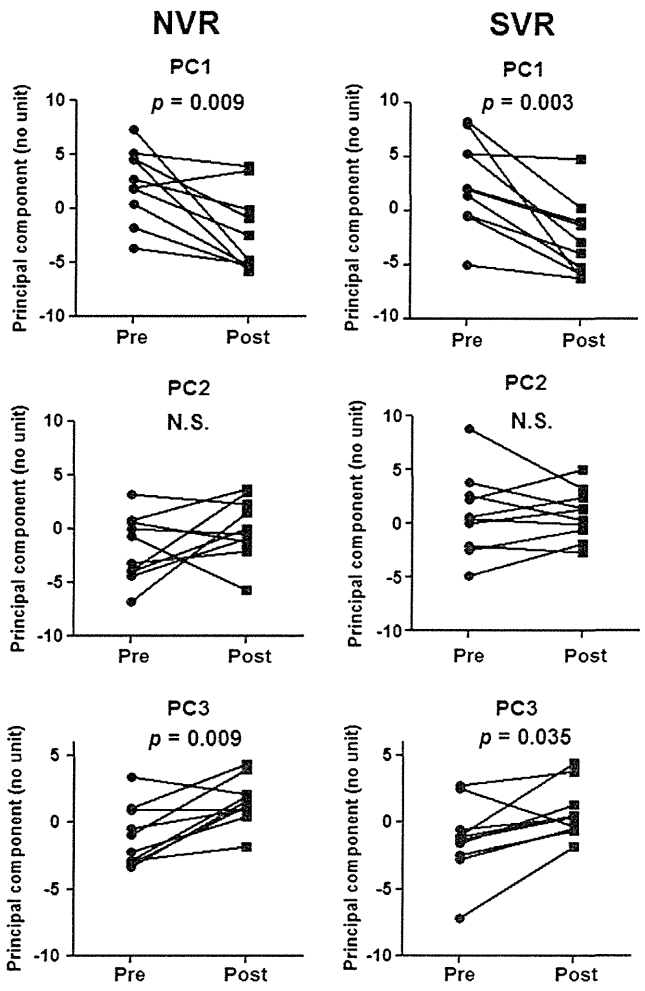

Fig. 2 – Changes in metabolomics profiles 24 weeks after pegIFN/RBV therapy compared with those before therapy. Pre and post indicate pretreatment and post-treatment, respectively. Principal component (PC) 1 was significantly decreased and PC 3 was significantly increased in both the NVR and SVR patients. Wilcoxon matched-pairs signed rank test.

loss of variation in cell metabolism among sustained virological responders, reflecting the fact that cell function had reached a state close to equilibrium as a result of successful HCV eradication.

3.3. *Comparison of metabolite levels in pretreatment serum between non-virological responders and sustained virological responders*

The pretreatment serum level of each metabolite in patients who received PegIFN/RBV therapy was compared between the NVR and SVR groups. We conducted ROC curve analysis to calculate the area under the ROC curve (AUC) values for all metabolites, and only four metabolites – tryptophan, glycine, γ-butyrobetaine and guanidoacetate – showed significant ($p < 0.05$) discrimination abilities (Supplementary Figure 1). Serum concentrations of all four of these metabolites were significantly higher in sustained virological responders than in non-virological responders ($p < 0.05$) (trypytophan: 39.7 ± 4.3 vs. 46.4 ± 6.4, p = 0.010; glycine: 168.7 ± 34.8 vs. 232.6 ± 77.0, p = 0.018; γ-butyrobetaine: 1.5 ± 0.2 vs. 1.8 ± 0.4, p = 0.049; guanidoacetate: 1.7 ± 0.5 vs. 2.1 ± 0.6, p = 0.049, NVR vs. SVR, mean ± standard deviation) (Fig. 4). The AUC values of the four metabolites for discriminating SVR from NVR were 0.84 (95% confidential interval (CI), 0.66–1.02, p = 0.010) for tryptophan, 0.78 (95% CI, 0.57–0.99, p = 0.034) for glycine, 0.76 (95% CI, 0.53–0.99, p = 0.049) for γ-butyrobetaine and 0.76 (95% CI, 0.55–0.98, p = 0.049) for guanidoacetate, all being significant (Fig. 5). We also evaluated the discrimination ability of combinations of multiple serum metabolites using MLR analysis. Among all metabolites, tryptophan and γ-glutamate-arginine were selected for the MLR model by stepwise feature selection methods. The ROC curve for the MLR model incorporating the pretreatment levels of these two metabolites for discriminating SVR from NVR showed a high and significant AUC value (AUC = 0.92, 95% CI, 0.79–1.05, p = 0.002) (Fig. 6). The mean AUC values obtained by bootstrap analysis remained high (AUC = 0.94, 95% CI, 0.93–0.95), indicating that the MLR model showed better accuracy for discriminating SVR from NVR in patients receiving PegIFN/RBV therapy for chronic hepatitis C.

3.4. *Comparison of serum levels of tryptophan between non-virological responders and sustained virological responders carrying an IL28B homozygote for major alleles of rs8099917*

The IL28B genotype is a strong host factor influencing the virological response of HCV to PegIFN/RBV therapy [21,22]. Patients carrying an IL28B homozygote for the major alleles of rs8099917 show a greater propensity to achieve SVR than those carrying an IL28B heterozygote or homozygote for its minor allele. To investigate whether the pretreatment serum level of tryptophan, which was the factor found to differ most significantly between NVR and SVR patients by analyses, was influenced by IL28B genotype, the pretreatment levels of tryptophan in patients with the IL28B homozygote for the major alleles of rs8099917 were compared between four of the 10 non-virological responders and all of the 10 sustained virological responders. The serum levels of tryptophan were significantly higher in the sustained virological responders harboring the major homozygote than in non-virological responders harboring the major homozygote (NVR vs. SVR, 37.8 ± 5.0 vs. 46.4 ± 6.4, p = 0.023) (Fig. 7). Thus the pretreatment level of tryptophan was not influenced by the IL28B genotype.

3.5. *Changes in levels of metabolites influencing the viral response after PegIFN/RBV therapy*

Changes in the serum levels of four metabolites – tryptophan, glycine, γ-butyrobetaine and guanidoacetate – whose pretreatment levels had been shown to differ significantly between the NVR and SVR groups, were examined at 24 weeks after completion of PegIFN/RBV therapy. The serum levels of all four metabolites after PegIFN/RBV therapy did not differ significantly from those before therapy in either NVR or SVR patients. Notably, there was little difference in the

serum level of tryptophan before and after the treatment (SVR: 46.4 ± 6.4 vs. 48.3 ± 12.0, p = 0.88, NVR: 39.7 ± 4.3 vs. 39.9 ± 8.1, p = 0.71, pretreatment vs. post-treatment). This suggests that the pretreatment levels of these metabolites, particularly tryptophan, may help to predict the therapeutic effect of this therapy.

3.6. *Dynamics of serum metabolite levels in patients with chronic hepatitis C before and after PegIFN/RBV therapy*

The dynamics of serum metabolite profile in patients with chronic hepatitis C before and after PegIFN/RBV therapy are shown as a heatmap in Fig. 8. Specific metabolites that showed changes in their serum levels as a result of PegIFN/RBV therapy were determined. The serum levels of four γ-glutamyl dipeptides (γ-Glu-His, γ-Glu-Lys, γ-Glu-Phe, γ-Glu-Val), glutamic acid, 5-oxoproline, glucosamine and methionine sulfoxide were decreased significantly ($p < 0.05$) at 24 weeks after completion of PegIFN/RBV therapy in both non-virological responders and sustained virological responders (Supplementary Figure 2). The serum levels of GGT were decreased significantly at 24 weeks after completion of PegIFN/RBV therapy in sustained virological responders (pretreatment vs. post-treatment: 43.1 ± 31.6 vs. 23.4 ± 8.0, p = 0.028). On the other hand, those of 5-methoxy-3-indoleacetate, glutamine, kynurenine and lysine were increased significantly ($p < 0.05$) in both groups at the same time point (Supplementary Figure 3).

4. Discussion

In this study, we demonstrated that PegIFN/RBV therapy for chronic hepatitis C altered the metabolism of cells in the liver of treated patients. These changes were confirmed by both principal component analysis of the overall metabolome and the dynamics of specific metabolites in serum associated with oxidative stress.

Principal component analysis of metabolites has been shown to be a powerful bioinformatics tool for providing an overall picture of cell metabolic status in individual patients [18–20]. Three-dimensional principle component analysis of metabolites demonstrated no differences in such profiling among the patients enrolled in this study. Twenty four weeks after completion of PegIFN/RBV therapy, principal component analysis demonstrated convergence of these metabolites in the score plots, suggesting that cell function became more homogeneous after completion of the therapy in comparison with the situation before therapy. In particular, the score plot area of the metabolites became narrower in patients who achieved successful HCV eradication. These data suggest that PegIFN/RBV therapy is able to modify the cell function of such patients, so that it approximates the normal function seen in healthy individuals.

The effect of PegIFN/RBV therapy on the viremia in patients with chronic hepatitis C is affected by various host factors. Therefore we considered that it would be informative to

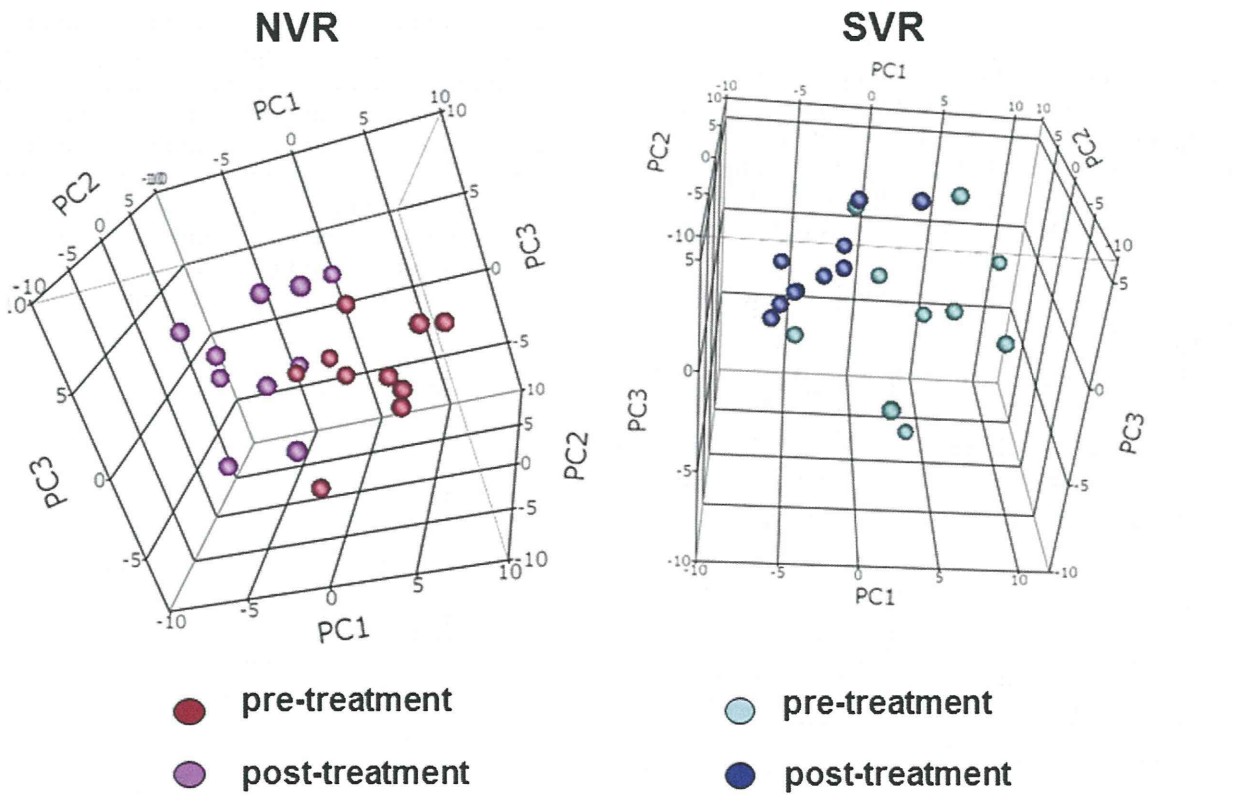

Fig. 3 – Principal component analysis of changes in serum metabolites 24 weeks after pegIFN/RBV therapy (post-treatment) compared with those before therapy (pretreatment). The distribution area of samples became narrower after therapy relative to that before therapy, and this change was particularly obvious in SVR cases.

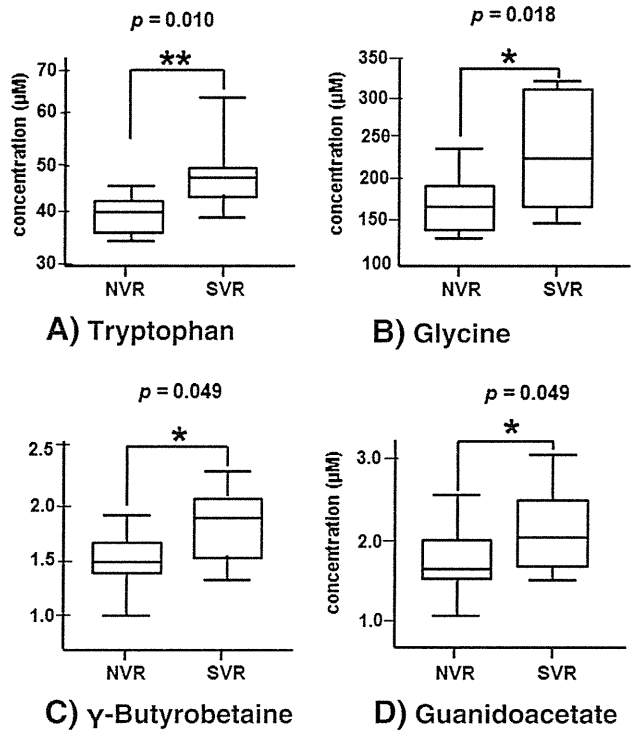

Fig. 4 – Comparison of metabolite levels in serum before treatment between the NVR and SVR patients. The serum levels of tryptophan, glycine, γ-butyrobetaine and guanidoacetate were significantly higher in SVR patients than in NVR patients ($p < 0.05$). Mann–Whitney U-test.

investigate whether the cell metabolic status of patients before the start of treatment would affect the viral response to PegIFN/RBV therapy. The serum levels of several specific metabolites, including tryptophan, glycine, γ-butyrobetaine and guanidoacetate, were significantly higher in the sustained virological responders than in non-virological responders,

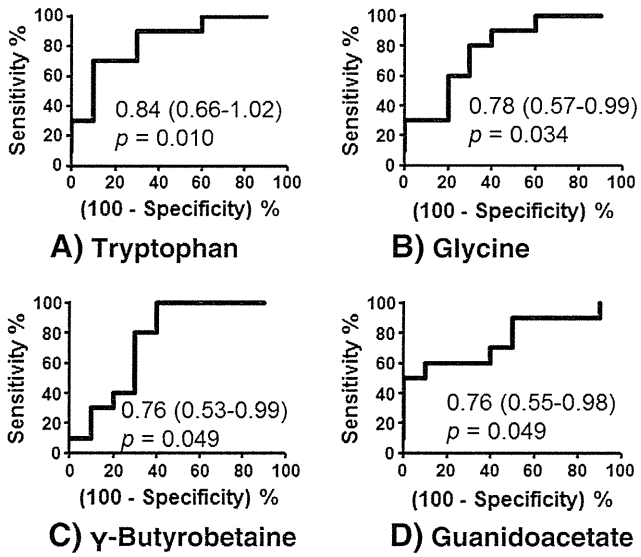

Fig. 5 – ROC curve analyses of four metabolites that were able to discriminate SVR from NVR.

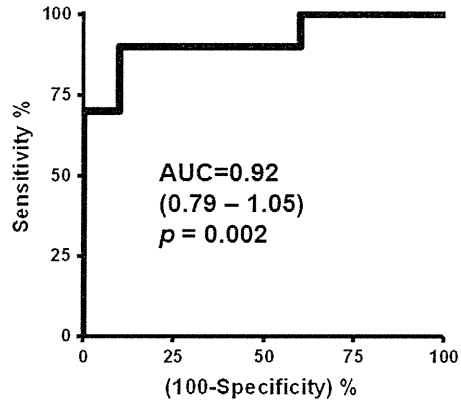

Fig. 6 – ROC curve of multiple logistic regression model incorporating the pretreatment levels of tryptophan and γ-glutamate-arginine for discriminating SVR from NVR.

although principal component analysis revealed no significant difference in the total metabolite profile between them. Our MLR analysis selected two metabolites – tryptophan and γ-glutamate-arginine – that were able to predict the viral response with some degree of accuracy. Although a further study with a larger cohort will be needed in order to confirm whether the serum levels of these metabolites are indeed associated with antiviral effect of PegIFN/RBV therapy, some of them, particularly tryptophan, have already been suggested to have such a relationship.

Tryptophan is a source of kynurenine derivatives, and the pathway responsible is dependent on indoleamine 2,3-dioxygenase (IDO) [23,24]. IDO is inducible in a large variety of cells by inflammatory cytokines such as IFN-γ, and therefore tryptophan degradation is accelerated by infection or malignant diseases that are accompanied by cellular immune activation [25,26]. IDO plays an important role in suppression of the cellular immune response [27,28], and can inhibit T-cell responses, thereby inducing immunological tolerance [29]. Genetic variants affecting serum metabolite levels may play a functional role in the liver [30]. Although no previous study

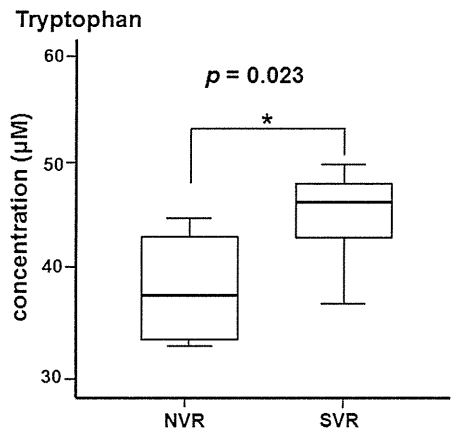

Fig. 7 – Comparison of the serum levels of tryptophan between NVR and SVR patients carrying IL28B homozygotes for the major alleles of rs8099917. Mann–Whitney U-test.

has investigated genetic differences associated with the degree of IDO induction, non-virological responders with low tryptophan levels due to active tryptophan degradation may be in a state of down-regulation of the immune response in the presence of an increased amount of inducible IDO, which in turn may be partly associated with low responsiveness to IFN-based antiviral therapy. The difference in the serum levels of tryptophan between the NVR and SVR groups in the present study was not related to IL28B genotype. These data suggest that the metabolic status of liver cells represented by differences in the serum metabolite profiles between NVR and SVR may predict the therapeutic effect of PegIFN/RBV on chronic hepatitis C.

This study identified for the first time the specific metabolites in serum whose levels were altered in patients with chronic hepatitis C receiving PegIFN/RBV therapy. We found that PegIFN/RBV therapy reduced the serum levels of four γ-glutamyl dipeptides (γ-Glu-His, γ-Glu-Lys, γ-Glu-Phe, γ-Glu-Val), glutamic acid, 5-oxoproline, glucosamine, and methionine sulfoxide. The γ-glutamyl dipeptides are formed by binding of glutamic acid to various amino acids catalyzed by γ-glutamylcysteine synthetase, and are produced as a by-product of glutathione, which has a protective effect against oxidative stress [15,31,32]. The γ-glutamyl cycle is activated by glutathione production in patients with liver diseases such as hepatitis, the glutathione being consumed to neutralize generated ROS, in turn leading to activation of γ-glutamylcysteine synthetase, and resulting in the biosynthesis of glutathione together with γ-glutamyl dipeptides [15]. This would suggest that PegIFN/RBV therapy removes oxidative stress and alters cell metabolism towards a more normal range as a result of reduced glutathione production, as was shown by principal component analysis in the present study. In addition, the serum level of GGT has been widely used as a marker of liver dysfunction, alcohol intake, or metabolic syndrome, and it can be also used as an indicator of amelioration of oxidative stress [16]. To investigate the γ-glutamyl dipeptide biosynthetic pathway, we previously performed trace analyses of γ-Glu-X and γ-Glu-X-Gly peptides by intraperitoneal injection of labeled threonine and acetaminophen [15]. The rapid decrease of labeled threonine and the gradual increasing of the labeled γ-Glu-X-Gly/γ-Glu-X ratio indicated that γ-glutamyl peptides were synthesized in the order γ-Glu-X > γ-Glu-X-Gly [15], suggesting that γ-Glu-X was synthesized rather than being a product of γ-Glu-X-Gly catalysis by GGT. Previous studies have reported that an improvement of the serum GGT level leads to a decrease of free radical production, and that the baseline level of GGT is associated with the response to IFN-based antiviral therapy in chronic hepatitis C [33–35]. In the present study, the pretreatment serum levels of GGT in non-virological responders had a tendency to be higher than those of sustained virological responders, and they were significantly decreased after successful treatment. These results suggest that this therapy helps remove oxidative stress.

On the other hand, the serum levels of 5-methoxy-3-indoleacetate, glutamine, kynurenine and lysine were increased significantly 24 weeks after completion of PegIFN/RBV therapy in both non-virological responders and sustained virological responders. These changes may have resulted from the improvement of cell metabolism by the therapy. The removal of the oxidative stress reflected production of glutamine and lysine. Improvement of tryptophan metabolism through the anti-inflammatory effect of PegIFN/RBV therapy is thought to normalize the production of kynurenine from tryptophan [23–26]. Also, the improvement of liver steatosis as a result of reduced expression of HCV core protein may generate an increase of 5-methoxy-3-indoleacetate, which binds to, and activates, peroxisome proliferator-activated receptor-γ [36,37]. Oxidative stress plays an important role in the progression of liver inflammation and hepatocarcinogenesis [8,38], including that associated with HCV infection [10–12]. The results of the present study suggest that PegIFN/RBV therapy can slow the progression of liver disease in patients with chronic HCV infection through reduction of oxidative stress.

In conclusion, the present study has shown that the pretreatment serum levels of low-molecular-weight metabolites, including tryptophan, are associated with the virological response to PegIFN/RBV therapy, and that such therapy can reduce the level of oxidative stress in patients with chronic HCV infection, as well as modifying the state of cell metabolism. However, further studies are needed to validate the present findings in a larger cohort of patients.

Author contributions

Study design, data analysis and interpretation, writing manuscript: T Saito and Sugimoto equally contributed to this work as the lead author of this manuscript; Study conduct: Soga; Study design and interpretation: M Tomita, Ueno; analysis and interpretation: Igarashi, K Saito, Shao; data collection: Katsumi, K Tomita, Sato, Okumoto, Nishise, Watanabe.

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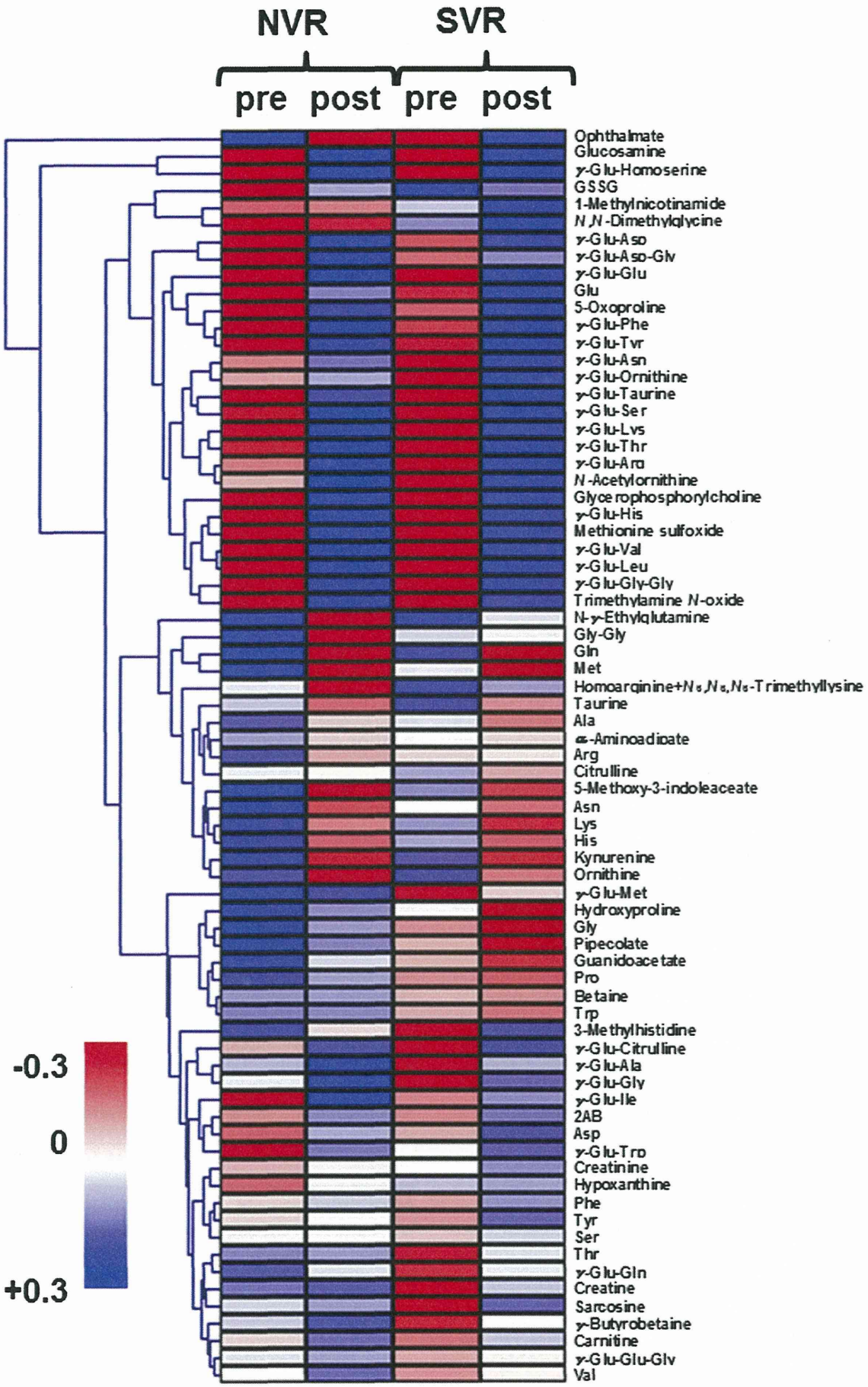
Fig. 8 – Heat map of observed metabolomic profiles. Pre and post indicate pretreatment and post-treatment, respectively. Red color indicates metabolite concentrations that were higher than average, while blue color indicates those that were lower than average. The order of the metabolites was arranged on the basis of clustering analysis.


NVR
SVR
pre
post
pre
post
Ophthalmate
Glucosamine
γ-Glu-Homoserine
GSSG
1-Methylnicotinamide
N,N-Dimethylglycine
γ-Glu-Asp
γ-Glu-Asp-Gly
γ-Glu-Glu
Glu
5-Oxoproline
γ-Glu-Phe
γ-Glu-Tyr
γ-Glu-Asn
γ-Glu-Ornithine
γ-Glu-Taurine
γ-Glu-Ser
γ-Glu-Lys
γ-Glu-Thr
γ-Glu-Arg
N-Acetylornithine
Glycerophosphorylcholine
γ-Glu-His
Methionine sulfoxide
γ-Glu-Val
γ-Glu-Leu
γ-Glu-Gly-Gly
Trimethylamine N-oxide
N-γ-Ethylglutamine
Gly-Gly
Gln
Met
Homoarginine+Nε,Nε,Nε-Trimethyllysine
Taurine
Ala
α-Aminoadipate
Arg
Citrulline
5-Methoxy-3-indoleacetate
Asn
Lys
His
Kynurenine
Ornithine
γ-Glu-Met
Hydroxyproline
Gly
Pipecolate
Guanidoacetate
Pro
Betaine
Trp
3-Methylhistidine
γ-Glu-Citrulline
γ-Glu-Ala
γ-Glu-Gly
γ-Glu-Ile
2AB
Asp
γ-Glu-Trp
Creatinine
Hypoxanthine
Phe
Tyr
Ser
Thr
γ-Glu-Gln
Creatine
Sarcosine
γ-Butyrobetaine
Carnitine
γ-Glu-Glu-Gly
Val
-0.3
0
+0.3

Conflict of interest

The authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.metabol.2013.07.002.

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RESEARCH ARTICLE Open Access

Impaired mitochondrial β-oxidation in patients with chronic hepatitis C: relation with viral load and insulin resistance

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Abstract

Background: Hepatic steatosis is often seen in patients with chronic hepatitis C (CH-C). It is still unclear whether these patients have an impaired mitochondrial β-oxidation. In this study we assessed mitochondrial β-oxidation in CH-C patients by investigating ketogenesis during fasting.

Methods: This study consisted of thirty patients with CH-C. Serum levels of insulin and hepatitis C virus (HCV) core protein were measured by chemiluminescence enzyme immunoassay. The subjects were then fasted, and venous blood samples were drawn 12 h and 15 h after the start of fasting. The levels of blood ketone bodies were measured by an enzymatic cycling method. The rate of change in total ketone body concentration was compared with that in eight healthy volunteers.

Results: The rate of change in total ketone body concentration between 12 h and 15 h after the start of fasting was significantly lower in CH-C patients than in healthy volunteers (129.9% (8.5-577.3%) vs. 321.6% (139.6-405.4%); P <0.01). The rate of change in total ketone body concentration in patients with a serum level of HCV core protein of 10000 fmol/L or higher was significantly lower than in patients with a level of less than 10000 fmol/L (54.8% (8.5-304.3%) vs. 153.6% (17.1-577.3%); P <0.05). The rate of change in total ketone body concentration in patients with a homeostasis model assessment of insulin resistance (HOMA-IR) of 2.5 or higher was significantly lower than in patients with a HOMA-IR of less than 2.5 (56.7% (8.5-186.7%) vs. 156.4% (33.3-577.3%); P <0.01).

Conclusions: These results suggest that mitochondrial β-oxidation is impaired, possibly due to HCV infection in patients with CH-C.

Keywords: Ketogenesis, Fasting test, Hepatic steatosis, HCV

Background

Hepatitis C virus (HCV) infection is a major cause of chronic liver injury. Hepatic steatosis is one of histologic features of chronic HCV infection with a risk of progression of liver diseases [1]. Hepatic steatosis is caused by some mechanisms, which include an increase of fatty acids uptake and synthesis, a decrease of fatty acids β-oxidation, or low level of secretion of very-low density lipoprotein. HCV core protein-transgenic mice develop hepatic steatosis due to impaired β-oxidation caused by mitochondrial damage [2]. However, there has been no evidence of impaired β-oxidation in patients with chronic hepatitis C (CH-C) *in vivo*.

During starvation, mitochondria produces acetyl CoA, which is converted into ketone bodies by fatty acids β-oxidation. In patients with impaired hepatic mitochondrial β-oxidation, ketogenesis is expected to be inadequate. Adult-onset type 2 citrullinemia (CTLN2) has been demonstrated to present as non-alcoholic fatty liver disease (NAFLD) [3]. CTLN 2 is associated with mutations in the SLC25A13 gene encoding citrin, which is a component of the mitochondrial malate-aspartate shuttle. Functional defectiveness of citrin impairs not only transport of aspartate from mitochondria but that of NADH into mitochondria.

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This induces activation of the citrate-malate shuttle with compensatory production of acetyl CoA, which in turn stimulates fatty acid synthesis. In addition, mitochondrial accumulation of malonyl CoA in a high NADH/NAD^+ environment suppresses fatty acid oxidation. These circumstances lead to hepatic steatosis in patients with CTLN 2 [4]. Inui et al. have demonstrated that suppression of fatty acid oxidation is accompanied by impaired ketogenesis in such patients [5].

Based on this background, we measured the concentration of blood ketone bodies during fasting in order to evaluate mitochondrial β-oxidation in patients with CH-C, and thus to investigate a mechanism of steatosis associated with HCV infection. Here, we report for the first time that mitochondrial β-oxidation is impaired in patients with CH-C.

Methods

Patients

Thirty patients (14 male and 16 female, the mean age 54.2, ranging from 22 to 74 years old) with CH-C were studied. The patients were admitted to Yamagata University Hospital for treatment between March 2006 and May 2009. All of the patients had been positive for both serum anti-HCV and HCV RNA for more than 6 months, and had elevated levels of serum alanine aminotransferase (ALT). They were all negative for hepatocellular carcinoma, hepatitis B, autoimmune hepatitis, primary biliary cirrhosis, heart failure, renal insufficiency, a history of diabetes mellitus, excess alcohol intake (daily ethanol consumption >20 g) or drug abuse. None of the patients fulfilled the criteria for Metabolic Syndrome in Japan [6,7], i.e. the presence of at least two of the following three abnormalities in addition to visceral obesity (waist circumference: 85 cm or more in men, 90 cm or more in women): 1) triglycerides ≥150 mg/dl and/or HDL-cholesterol <40 mg/dl, or receiving treatment for this type of dyslipidemia; 2) systolic blood pressure ≥130 and/or diastolic blood pressure ≥85, or receiving treatment for hypertension; 3) fasting glucose ≥110 mg/dl or receiving treatment for diabetes. As a control group, eight volunteers (4 male and 4 female) were included (the mean age 30.5, ranging from 26 to 39 years old). All of them were healthy, with a BMI of <25 kg/m^2, without medication or severe disease. Written informed consent to participate was obtained from all subjects, and the study protocol was approved by The Yamagata University Hospital Ethics Committee.

Methods

Clinical and laboratory data

Body height, weight and waist circumference were measured at the time of admission. Body mass index (BMI) was calculated as: BMI = body weight (kg)/body height $(m)^2$. Venous blood samples were collected after a 12-h overnight fast for standard biochemical testing and determination of serum insulin levels, HCV genotype and HCV core protein. Serum insulin was determined by chemiluminescence enzyme immunoassay (Lumipulseprestoinsulin®, Fujirebio Inc., Tokyo, Japan). Insulin resistance was determined based on the homeostasis model assessment of insulin resistance (HOMA-IR). HOMA-IR was calculated using the formula: [fasting glucose (mg/dl) × fasting insulin (μU/ml)] / 405 [8]. Insulin resistance was defined as HOMA-IR >2.5. The amount of HCV core antigen and HCV RNA in serum were measured by a chemiluminescence enzyme immunoassay (Lumispot Eiken HCV antigen®, Eiken Chemical Co., Ltd., Tokyo, Japan) and an amplicor HCV RNA detection kit (Amplicor HCV v2.0®, Roche Diagnostics, Tokyo, Japan) or a real-time PCR assay (COBAS® TaqMan® HCV Test, Roche Diagnostics, Tokyo, Japan), respectively.

Fasting test

In general, ketone bodies are not detected during periods of feeding, but after the onset of fasting, glycogen is gradually consumed and ketone bodies are produced rapidly after about 12 hours of fasting. The rate of change in ketone body production between 12 and 15 hours represented the initial increase, and was interpreted as the initial velocity of ketogenesis.

Fasting tests were performed in both subjects and volunteers. They were permitted to drink water after their last meal, and blood samples were drawn to measure the proportion of ketone bodies, glucose, insulin, free fatty acid and triglyceride levels at 12 and 15 h after the last meal. Carnitine fractionation was also measured at 12 after the last meal, using an enzymatic cycling method (Total Carnitine Kainos®, Free Carnitine Kainos®, Kainos Laboratories, Inc., Tokyo, Japan). During fasting, urine organic acids were measured by gas chromatograph-mass spectrometry and then analyzed using software to determine the presence of disorders of organic acid metabolism. Serum ketone bodies were measured by an enzymatic cycling method (3-hydroxybutyrate Kainos®, Total ketone body Kainos®, ketone body standard reagent 2®, Kainos Laboratories, Inc. Tokyo, Japan). These measurements were performed by SRL Inc. (Tokyo, Japan). The rate of change in total ketone body concentration from 12 h to 15 h was calculated using the equation: (total ketone bodies at 15 h – total ketone bodies at 12 h)/ total ketone bodies at 12 h × 100.

Histological assessment

Liver biopsies were performed under sonographic guidance in 25 patients who provided informed consent. The liver tissues were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin and silver stain. Liver histology was graded and staged by a pathologist based on the international classification [9]. The grade of steatosis was modified as follows: grade 0 = no steatosis and between 0%

and 5% of hepatocytes containing visible macrovesicular steatosis, grade 1 = between 5% and 33%, grade 2 = between 33% and 66%, and grade 3 = more than 66% according to the non-alcoholic fatty liver disease Activity Score [10].

Statistical analysis

Results are expressed as the mean ± standard deviation (SD) or the median and range (in parenthesis). Student's *t* test was used for normally distributed non-paired continuous variables. The rate of change in total ketone body concentration was assessed as a parametric value because the value was distributed parametrically after logarithmic transformation. Wilcoxon's signed-ranks test was used for paired continuous variables. Comparisons between more than two groups were made by one-way analysis of variance. All P-values were based on a two-sided test of statistical significance. Differences at $P < 0.05$ were considered to be statistically significant.

Results

Subject characteristics

The characteristics of thirty patients with chronic hepatitis C were shown in Table 1. The mean level of BMI was less than 25 kg/m^2, and that of fasting plasma glucose was within normal range. The mean level of ALT was greater than the upper limit of normal range. Nine patients had a status of insulin resistance in whom HOMA-IR showed a level of 2.5 or greater. All patients were positive for HCV RNA in whom the mean level of serum HCV core antigen showed a 6505 fmol/L. Of the 30 patients, 20 (66.7%) were infected with the HCV genotype 1b, 6 (20.0%) with genotype 2a, and 4 (13.3%) with genotype 2b. Fifteen (60%) had a liver fibrosis grade of F1, 7 (28%) had F2, and 3 (12%) had F3. Six (24%) had a liver steatosis grade of 0, 18 (72%) had grade 1, 1 (4%) had grade 2, and none had grade 3.

Table 1 Patient characteristics

		Patients	Reference value
Age (yr) [b]		54.2 ± 10.6	
Male/Female (ratio)		14/16 (0.47)	
BMI (kg/m^2) [b]		23.7 ± 2.7	18.5 - 25
ALT (IU/L) [a]		47.5 (17–167)	8 - 42
γGTP (IU/L) [a]		41 (13–152)	10 - 47
ChE (IU/L) [b]		312.9 ± 88.2	185 - 431
Fasting plasma glucose (mg/dL) [b]		95.5 ± 9.7	70 - 109
Insulin (μIU/mL) [a]		8.7 (4.5 - 20.4)	1.84 - 12.2
HOMA-IR [a]		2.0 (1.2 - 5.1)	
HOMA-IR > 2.5 (%)		9 (30)	
Triglyceride (mg/dL) [b]		91.4 ± 31.1	30 - 149
Total cholesterol (mg/dL) [b]		175.2 ± 25.4	129 - 219
Acylcarnitine (μmol/L) [a]		9.25 (5.7 - 21.1)	6 - 23
HCV RNA (LogIU/mL) [b]		6.1 ± 0.9	
HCV core antigen (fmol/L) [a]		6505 (<20–23200)	
HCV Genotype	1b / 2a / 2b	20 / 6 / 4	
Inflammation	A0 / A1 / A2 / A3	0 / 15 / 10 / 0	
Fibrosis	F0 / F1 / F2 / F3 / F4	0 / 15 / 7 / 3 / 0	
Steatosis	G0 / G1 / G2 / G3	6 /18 / 1 / 0	

BMI, body mass index; ALT, alanine aminotransferase; γGTP, gamma-glutamyltranspeptidase; ChE, cholinesterase; HOMA-IR, homeostasis model assessment of insulin resistance.
Data are [a]medians (min-max), [b]the means ± standard deviation.

Free fatty acids concentration during fasting in CH-C patients and healthy volunteers

The concentration of free fatty acids increased by fasting in CH-C patients and healthy volunteers. The rate of change in free fatty acids concentration between 12 h and 15 h was similar in both groups (CH-C patients 48.6% ± 45.0 vs. healthy volunteers 70.3% ± 95.2; ns), as shown in Figure 1.

Ketone body concentration during fasting in CH-C patients and healthy volunteers

The levels of total blood ketone bodies were elevated in both CH-C patients and healthy volunteers 15 h after the start of fasting, but the rate of change between 12 h and 15 h after fasting was significantly different between them. The rate of change in total ketone body concentration between 12 h and 15 h was shown in Figure 2. It was significantly lower in CH-C patients than in healthy volunteers (129.9% (8.5-577.3%) vs. 321.6% (139.6-405.4%); $P < 0.01$). The rates of change in both acetoacetate (Figure 3A) and 3-hydroxybutyrate (Figure 3B) between 12 h and 15 h after fasting were also significantly lower in CH-C patients than in healthy volunteers (acetoacetate : 109.5% (−5.8-514.3%) vs. 254.8% (145.5-341.7%) , 3-hydroxybutyrate : 130.8% (−3.8-606.7%) vs. 337% (135.5-495%) ; $P < 0.01$).

There was a significant positive correlation between the concentration of total ketone body and the levels of acylcarnitine (rs 0.56, $P < 0.01$) at 12 h after fasting, as shown in Figure 4A, similar to the pattern of free fatty acids (rs 0.54, $P < 0.01$, Figure 4B). The level of acylcarnitine was significantly lower in CH-C patients than in healthy volunteers (9.25 μmol/L (5.7-21.1 μmol/L) vs. 11.65 μmol/L (9.3-17 μmol/L); $P < 0.05$).

Relationship between the rate of change in total ketone body concentration and clinical parameters in CH-C patients

We stratified CH-C patients into two groups based on the clinical parameters. The rate of change in total ketone body