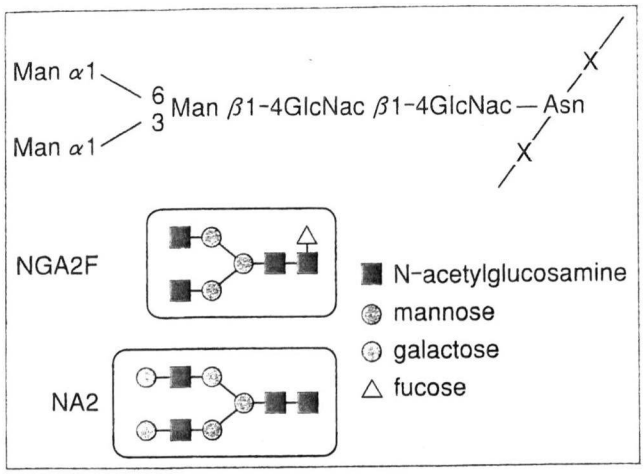

図 4　N 型糖鎖の基本構造とその一部

ら，血清 TNF-α 濃度は NASH のバイオマーカーとして有用と考えられ，その一塩基多型は NAFLD の進展予測にも有用である可能性がある。

③レプチン：脂肪細胞や肝星細胞に発現し，中枢神経に作用して強力な食欲抑制作用を示す。肥満をきたすとレプチンの血中濃度は上昇し，同時にレプチン抵抗性も生じ，もともとの糖・脂質代謝改善作用は減弱する。性と BMI をマッチさせた対照群と比較して NASH 患者では血中レプチン値が高く，血中レプチン値は肝の脂肪化と相関するが，肝炎や肝線維化とは相関しないこと[15]から，単純性脂肪肝と NASH の鑑別には有用ではない可能性がある。

④高感度 C reactive protein（高感度 CRP）：高感度 CRP は心血管イベントの予測因子として知られている。脂肪組織での高感度 CRP 発現は血中アディポネクチン発現と負の相関があり，アディポネクチンノックアウトマウスの脂肪組織では CRP の mRNA が亢進していることから，動脈硬化の進展にはアディポネクチン低下を介した高感度 CRP 高値が関連している可能性が示されている[16]。また，高感度 CRP は NASH と単純性脂肪肝との鑑別に有用であると報告されている[17]。

d　サイトケラチン-18（CK-18）断片

上皮系細胞において，アポトーシスが誘導されると Caspase-3 によりサイトケラチン 18（CK-18）が 3 つに切断されることが知られている。最近，CK-18 断片濃度が NAFLD と健康人の鑑別，NAFLD における NASH と単純性脂肪肝の鑑別に有用であり[18]，NAFLD における肝線維化とも相関することが報告されている[19]。

e　その他

血管内皮細胞間接着分子である intercellular adhesion molecule-1（ICAM-1）や pentraxin 3 が NASH の診断に有用であると報告されている[20,21]。また，N-glycan（N 型糖鎖）は糖蛋白質の糖鎖のうち，蛋白質のアスパラギン残基に結合している糖鎖の総称である（**図 4**）が，N-glycan のうち agalacto core-α-1,6-fucosylated biantennary glycan（NGA2F）濃度は NAFLD における NASH の診断に有用であることが報告されている[22]。さらに，bigalacto biantennary glycan（NA2）と NGA2F との比（NGA2F/NA2）は，NASH における肝線維化の重症度の予測に有用である可能性がある[22]。

● おわりに

NASH は進行した病態でなければ，一般的な血液検査などで単純性脂肪肝と鑑別することは容易ではない。しかし，病態に関連した種々のバイオマーカーの測定による NASH と単純性脂肪肝との鑑別が試みられており，それぞれの有用性が報告されている。また，肝組織所見を用いた NAFLD の活動性スコア（NAS score：5 点以上は NASH と診断）[23]も NASH の診断に用いられてきており，今後，そのスコアとバイオマーカーとの相関も検討が必要である。NASH 患者は増加傾向にあり，決して予後良好な疾患ではない。このため，一般臨床で簡便・迅速に測定できるような NASH 診断法の確立が強く望まれる。

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Original Article

Value of the extracellular water ratio for assessment of cirrhotic patients with and without ascites

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Aims: Ascites, which often complicates liver cirrhosis, is reported to be a factor that worsens the outcome. The aims of this study were to quantify body water compartment changes in cirrhotic patients, with and without ascites, and to elucidate the value of body water analysis for predicting the development of ascites.

Methods: A total of 109 cirrhotic patients, with and without ascites, and 65 controls were studied. Intra- and extracellular water (ECW) in the whole body and in the arm, leg and trunk were measured using the recently developed 8-electrodes multiple-frequency bioelectrical impedance analyzer. Furthermore, patients without ascites were followed to an episode of ascites or death.

Results: Patients with liver cirrhosis had significantly higher ECW ratios than controls. ECW ratios were increased in cirrhotic patients with moderate and severe disease. The ECW ratio of the trunk showed highly significant changes in cirrhotic patients with ascites. The ECW ratio correlated with age, serum albumin, and prothrombin time. A relative expansion of ECW and low albumin were predictive of further episodes of ascites (log-rank 6.94, $P < 0.01$). In multivariate analysis, the ECW ratio was independently associated with the development of ascites.

Conclusion: Liver cirrhosis was characterized by a redistribution of body water. The ECW ratio is a reliable tool for quantification of redistribution of body water and can predict the development of ascites.

Key words: ascites, bioelectrical impedance analysis, extracellular water, intracellular body water, liver cirrhosis, total body water

INTRODUCTION

PATIENTS WITH CIRRHOSIS often have an abnormal body composition with clinical signs of protein-energy malnutrition and a relative increase in body weight due to ascites or edema.[1–3] Ascites is a condition that is becoming treatable with diuretics, albumin preparations, ascetic reperfusion, and transjugular intrahepatic portosystemic shunting, but the prognosis of patients with ascites remains poor.[4,5] Heuman *et al.* studied 507 cirrhotic patients referred for consideration of liver transplantation and concluded that the presence of persistent ascites is an important independent predictor of high mortality risk.[6] On the other hand, some authors have reported an increase in body water even before ascites and edema are obvious.[3] For the clinical management of these patients, it would be useful if body water measurements could be made in a simple manner.

Recent methods for the determination of the body composition include dual-energy X-ray absorptiometry, deuterium dilution and bioelectrical impedance analysis (BIA).[7,8] Single-frequency BIA is a safe, non-invasive, rapid, and inexpensive method of assessing total body water (TBW) in healthy individuals.[9] However, in situations where there are clinically important changes in the intracellular and extracellular distribution of water, such as cirrhosis, the value of single-frequency BIA is limited.[5,10,11] Previous studies have indicated an improved prediction of body water compartments by BIA if measurements are made while the frequency of

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the applied current is changed.[12–14] At low frequencies, the current passes through the extracellular fluids because of the capacitance effect of cell membranes and tissue interfaces, whereas at higher frequencies, the current is conducted through both the intra- and extracellular fluids. More recently, technological advances include increasing the number of the contact electrodes from four to eight and software development to provide accurate estimations of water distribution within the whole body and various segments.[15–17] It is proposed that this technique may be of particular use in patients with cirrhosis because they have an altered distribution of body water even in the absence of ascites.[3] Moreover, separate measurements of the trunk and limbs may improve the precision of body water estimates in patients with ascites. However, it is not certain whether this approach will improve the accuracy of body water estimation.[18]

Thus, the purpose of this study was to assess the accuracy of 8-electrode multiple-frequency BIA for the measurement of whole body and segmental water compartments in cirrhotic patients with and without ascites. Then, patients without ascites were followed to elucidate the significance of the evaluation of the extracellular water (ECW) ratio for predicting the onset of ascites.

SUBJECTS AND METHODS

Study population

A TOTAL OF 109 consecutive patients with ($n = 48$) or without ($n = 61$) ascites were studied. The diagnosis of cirrhosis was based on histological findings and/or the evidence of portal hypertension by esophageal varices at endoscopy and/or splenomegaly at computed tomography. Clinically apparent infection, malabsorption, endocrinopathy, the presence of hepatocellular carcinoma (HCC) outside the Milan criteria,[19] and renal impairment (creatinine ≥ 2 mg/dL) were exclusion criteria. The causes of cirrhosis were alcohol abuse ($n = 12$), viral hepatitis ($n = 85$), and cryptogenic factors ($n = 12$). Fifteen of the 61 patients without ascites and 24 of the 46 patients with ascites were on diuretic treatment with spironolactone alone or a combination of spironolactone and furosemide. Patients were classified according to the severity of cirrhosis using the Child-Pugh score.[20] Thirty-four non-obese (body mass index, BMI < 30 kg/m^2), apparently healthy volunteers were enrolled as controls. All had normal alanine aminotransferase (ALT < 40 IU/L) and no evidence of viral infection (antibodies to hepatitis C virus and hepatitis B surface antigen negativity). Thirty-one patients suffering from chronic hepatitis, but without cirrhosis, also were enrolled as controls. Chronic hepatitis patients were characterized by the presence of antibodies to hepatitis C virus or hepatitis B surface antigen. The distinction between chronic hepatitis and liver cirrhosis was made using histological finding or the indirect index based on four clinical variables: gammaglobulin, hyaluronate, platelet counts, and gender.[21] When a positive result was calculated using this equation, the patient was excluded from the control group.

After 12 hours overnight fasting, venous blood samples were drawn to determine platelet counts, albumin, total bilirubin, prothrombin time, ALT, sodium and creatinine. These parameters were measured using standard techniques from clinical chemistry laboratories. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Body water analysis

Height and weight were obtained with a precision of 0.1 cm and 0.1 kg, respectively. BMI was calculated as weight (kg)/height (m) squared. BIA was performed by the whole body 8-electrode approach using a multifrequency impedance analyzer applying alternating electric currents of ≤ 100 μA at 1 kHz, and of ≤ 500 μA at 5, 50, 250, 500, and 1000 kHz (InBody 720, Soul, Korea). In accordance with the manufacturer's guidelines, patients were instructed to stand upright and to grasp the handles of the analyzer, thereby providing contact with a total of 8 electrodes (2 for each hand and foot). Intracellular body water (ICW), ECW, TBW, and ECW ratio (ECW/TBW) were measured in the whole body and in the arm, leg and trunk. ECW and TBW were estimated from area, volume, length, impedance, and a constant proportion (specific resistivity). Segmental body water data were similar for corresponding right and left measurements and therefore are reported as mean values. All body water data were normalized for height (m).

Follow-up

For patients without ascites and diuretic treatment at entry ($n = 46$), the follow-up was maintained until an episode of ascites, death, or the end of the observation period. During follow-up, cirrhotic patients without ascites underwent clinical assessment, renal and standard liver biochemical tests every month. Ultrasonography or computed tomography was performed three times a year, or more frequently, and the presence of

Table 1 Clinical characteristics of patients with cirrhosis and controls

	Normal volunteers ($n = 34$)	Chronic hepatitis ($n = 31$)	Cirrhosis ($n = 109$)
Age (years)	62 ± 8	60 ± 12	67 ± 9*,****
Male/Female	15/19	12/19	61/48
HBV/HCV/Alcohol/other	-	1/30/0/0	7/78/12/12
Laboratory test			
Platelet count ($\times 10^3/\mu L$)	208 ± 54	187 ± 61	115 ± 221*
Albumin (g/dL)	4.2 ± 0.4	4.3 ± 0.2	3.1 ± 0.6**,****
Total Bilirubin (mg/dL)	0.6 ± 0.2	0.7 ± 0.3	1.3 ± 1.5*,***
Prothrombin time (%)	85 ± 28	102 ± 14	71 ± 15*,****
ALT (IU/L)	24 ± 12	44 ± 47	67 ± 183
Sodium (mEq/L)	142 ± 2	139 ± 2*	139 ± 3**
Child-Pugh A/B/C	-	-	40/58/11
Ascites none/grade 1/grade 2	0/0/0	31/0/0	61/38/10
HCC, number (%)	-	0 (0)	28 (26)
Height (m)	1.605 ± 0.075	1.587 ± 0.108	1.586 ± 0.091
BMI (kg/m^2)	23.0 ± 0.7	23.8 ± 3.6	22.8 ± 4.2

*$P < 0.05$ vs. normal volunteers; **$P < 0.01$ vs. normal volunteers; ***$P < 0.05$ vs. chronic hepatitis; ****$P < 0.01$ vs. chronic hepatitis. ALT, alanine aminotransferase; BMI, body mass index; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

ascites was confirmed. The grade of ascites was classified according to International Ascites Club, i.e. grade 1 (mild ascites only detectable by radiological examination), grade 2 (moderate symmetrical distension of abdomen) or grade 3 (marked abdominal distension).[22]

Statistical analysis

All data are given as mean ± standard deviation or as numbers of cases. Statistical differences between groups were analyzed using the Mann–Whitney *U*-test or the Kruskal–Wallis test when applicable. To identify intergroup differences after significant differences in the Kruskal–Wallis test, multiple comparisons were performed using Scheffe's *F*-test. Correlations were analyzed using Spearman's rank correlation test. Multiple regression analysis was performed to identify independent predictors of ECW ratio as continuous dependent variables. Factors that reached statistical significance in the univariate analysis were regarded as explanatory variables. Receiver-operating characteristic (ROC) curves were used to determine the optimum cut-off for the prediction of the risk of developing ascites. Kaplan–Meier analysis was used for univariate analysis and Cox regression for multivariate analysis of variables associated with development of ascites. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Study population (Table 1)

POPULATION DATA ARE shown in Table 1. The cirrhosis group consisted of 109 subjects and the majority of patients (78%) had a viral etiology. The number and percentage of patients within each Child–Pugh grade was 40 (37%), 58 (53%), and 11 (10%) in grades A, B, and C, respectively. Clinical or radiological evidence of ascites was detectable in 48 (44%) patients. Twenty-eight (26%) patients exhibited an HCC. All patients were clinically stable at the time of assessment. The control group consisted of 34 healthy volunteers and 31 patients with chronic hepatitis. Patients with liver cirrhosis were significantly ($P < 0.05$, $P < 0.01$) older than control subjects. Platelet counts were significantly ($P < 0.05$) lower in the cirrhotic patients than the healthy volunteers. Serum albumin and prothrombin time were significantly ($P < 0.01$, $P < 0.05$) lower in the cirrhotic patients than the control subjects. Total bilirubin values were significantly ($P < 0.05$) elevated in the cirrhotic patients than the control subjects. Serum sodium values were significantly ($P < 0.05$, $P < 0.01$) lower in the subjects with hepatitis and cirrhosis than in the healthy volunteers. There were no differences in ALT or BMI levels among the three groups.

Table 2 Body water compartments of patients with cirrhosis and controls

	Normal volunteers ($n = 34$)	Chronic hepatitis ($n = 31$)	Cirrhosis ($n = 109$)
ICW (L/m)	11.94 ± 1.89	11.76 ± 2.08	11.81 ± 1.85
ECW (L/m)	7.82 ± 1.68	7.49 ± 1.28	7.85 ± 1.28
TBW (L/m)	19.54 ± 2.90	19.25 ± 3.33	19.66 ± 3.08
ECW ratio (ECW/TBW)	0.389 ± 0.009	0.389 ± 0.009	0.399 ± 0.012*,**

*$P < 0.01$ vs. normal volunteers; **$P < 0.01$ vs. chronic hepatitis.
ECW, etracellular water; ICW, intracellular water; TBW; total body water.

Body water compartments

The results of analysis of body water compartments in the cirrhosis and control groups are shown in Table 2. No significant differences in ICW, ECW, or TBW were observed among the three groups. Cirrhotic patients had significantly ($P < 0.01$) higher ECW ratios than healthy volunteers and hepatitis patients. Among the cirrhotic patients, no significant differences in ICW or TBW were observed between Child-Pugh A, B, and C (Table 3). Child-Pugh B or C cirrhotic patients trended to have higher ECW than grade A, but the difference was not statistically significant. Child-Pugh grade B or C cirrhotic patients had significantly ($P < 0.01$, $P < 0.05$) higher ECW ratios than grade A. In addition, no significant differences in ICW, ECW, or TBW were observed between patients without, and those with, ascites (Table 4). Cirrhotic patients with grade 2 ascites had significantly ($P < 0.01$) higher ECW ratios than those without ascites. We also studied the differences between patients with cirrhosis on diuretic treatment and those who were not (Table 5). There were no differences in body water compartments, including the ECW ratio, between the two groups.

Figure 1 shows the segmental ECW ratios of patients with and without ascites. Cirrhotic patients with grade 1 or 2 ascites had significantly ($P < 0.05$, $P < 0.01$) higher ECW ratios of the trunk than those with no ascites. Likewise, cirrhotic patients with grade 2 ascites had significantly ($P < 0.01$) higher ECW ratios of the leg than

Table 3 Body water compartments of patients with cirrhosis grouped by Child-Pugh grade

	Child-Pugh A ($n = 40$)	Child-Pugh B ($n = 58$)	Child-Pugh C ($n = 11$)
ICW (L/m)	11.91 ± 1.66	11.79 ± 1.93	11.57 ± 2.20
ECW (L/m)	7.72 ± 1.04	7.94 ± 1.41	7.85 ± 1.40
TBW (L/m)	19.63 ± 2.68	19.73 ± 3.30	19.42 ± 3.46
ECW ratio (ECW/TBW)	0.393 ± 0.008	0.402 ± 0.011*	0.405 ± 0.022**

*$P < 0.01$ vs. Child-Pugh B; **$P < 0.05$ vs. Child-Pugh A.
ECW, etracellular water; ICW, intracellular water; TBW; total body water.

Table 4 Body water compartments in cirrhotic patients with and without ascites

	No ascites ($n = 61$)	Grade 1 ascites ($n = 38$)	Grade 2 ascites ($n = 10$)
ICW (L/m)	11.92 ± 1.98	11.91 ± 1.74	10.85 ± 1.06
ECW (L/m)	7.81 ± 1.34	7.99 ± 1.23	7.59 ± 1.06
TBW (L/m)	19.72 ± 3.29	19.90 ± 2.92	18.45 ± 2.02
ECW ratio (ECW/TBW)	0.396 ± 0.010	0.402 ± 0.011	0.411 ± 0.018*

*$P < 0.01$ vs. no ascites.
ECW, etracellular water; ICW, intracellular water; TBW; total body water.

Table 5 Body water compartments of patients with cirrhosis on diuretic treatment and those who are not

	Not on diuretic treatment ($n = 70$)	On diuretic treatment ($n = 39$)
ICW (L/m)	12.04 ± 1.85	11.42 ± 1.82
ECW (L/m)	7.99 ± 1.32	7.59 ± 1.18
TBW (L/m)	20.03 ± 3.12	19.02 ± 2.95
ECW ratio (ECW/TBW)	0.399 ± 0.012	0.399 ± 0.012

ICW, intracellular water; ECW, etracellular water, TBW; total body water.

those with no ascites. However, no significant differences in ECW ratios of the arm were observed among the three groups.

Factors associated with the ECW ratio

Among the 109 cirrhotic patients, age ($r = 0.393$, $P < 0.001$), low albumin ($r = -0.497$, $P < 0.001$), and low prothrombin time ($r = -0.293$, $P < 0.01$), evaluated as continuous variables, were associated with higher ECW ratio. There were no significant correlations among ECW ratio and platelet count ($r = 0.109$), total bilirubin ($r = 0.102$), ALT ($r = -0.149$), sodium ($r = 0.028$), and BMI ($r = -0.185$). In the multiple regression analysis using age, albumin, prothrombin time, and BMI, age ($r = 0.299$, $P < 0.01$), albumin ($r = -0.280$, $P < 0.01$), and prothrombin time ($r = -0.302$, $P < 0.01$) remained independent variables in the model.

ECW ratio for the prediction of the risk of developing ascites

Fifteen of 61 patients without ascites were on diuretic treatment at time of initial assessment and these patients were excluded from follow-up. Time zero was that of the initial body water analysis and the end points were an episode of ascites or death. The mean follow-up period was 7.7 months. During the period of observation, two patients died due to progressive HCC. The ROC curve analysis of the ECW ratio for the prediction of developing ascites showed an area under the curve value of 0.80. With a cut-off of 0.398, the sensitivity was 85.7% and specificity was 64.9%. Among the 44 cirrhotic patients, 19 showed abnormalities in the results of ECW ratio, i.e. more than 0.398. Table 6 summarizes the results of laboratory tests for both groups. Serum albumin was significantly ($P < 0.05$) lower in cirrhotic patients with an altered distribution of body water than in those with a normal distribution. HCC was present in 3 (16%) of 19 patients with an altered distribution of body water, and a normal distribution was found in five cases (20%). There was no statistically significant difference between groups in the incidence of HCC.

In all, seven patients exhibited an episode of ascites during follow-up. Two patients developed HCC during follow-up, but no patients developed ascites as a result of advanced HCC. Six (86%) of the 7 who developed ascites showed a relative expansion of ECW, defined using the ECW ratios from the initial assessments, whereas only one (14%) patient showed normal distribution of body water according to the ECW ratio. In univariate analysis, the variables associated with a higher risk of developing ascites were relative expansion

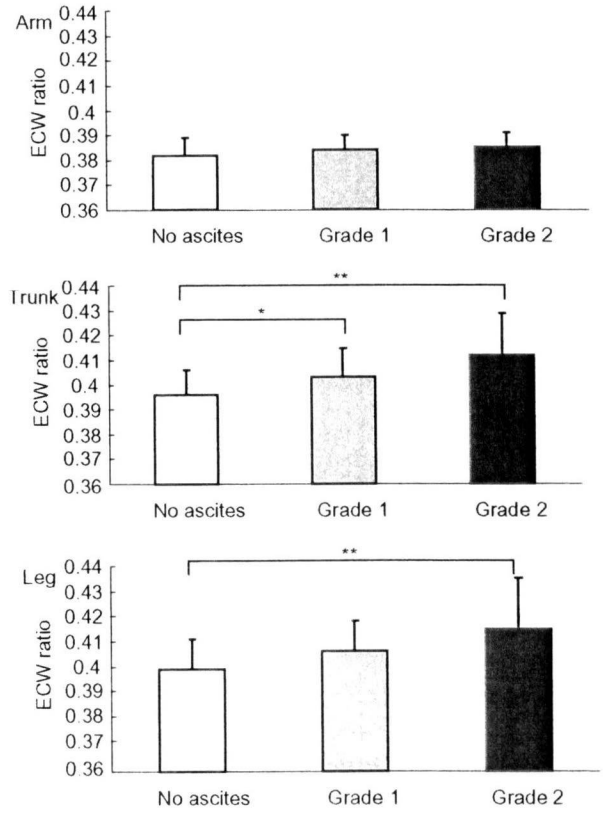

Figure 1 Segmental body water compartments in cirrhotic patients with and without ascites. No significant differences in extracellular water (ECW) ratios of the arm were observed among the three groups (upper). Cirrhotic patients with grade 1 or 2 ascites had significantly higher ECW ratios of the trunk than those with no ascites (middle). Cirrhotic patients with grade 2 ascites had significantly higher ECW ratios of the leg than those with no ascites (lower). *, $P < 0.05$; **, $P < 0.01$.

Table 6 Clinical characteristics of patients with normal distribution of body water and those with altered distribution

	ECW ratio < 0.398 ($n = 25$)	ECW ratio ≥ 0.398 ($n = 19$)
Age (years)	64 ± 12	73 ± 10*
Male/Female	15/10	61/48
HBV/HCV/Alcohol/other	4/15/5/1	0/12/2/5
Laboratory test		
Platelet count (× 10^3/μL)	97 ± 57	123 ± 68
Albumin (g/dL)	3.6 ± 0.5	3.2 ± 0.4**
Total Bilirubin (mg/dL)	0.9 ± 0.4	1.1 ± 0.6
Prothrombin time (%)	76 ± 15	73 ± 15
ALT (IU/L)	52 ± 37	50 ± 56
Sodium (mEq/L)	141 ± 3	140 ± 3
Child-Pugh A/B/C	16/9/0	8/11/0
HCC, number (%)	5 (20)	3 (16)

*$P < 0.01$ vs. < 0.398; **$P < 0.05$ vs. < 0.398.
ALT, alanine aminotransferase; ECW, extracellular water; HBV, hepatitis B virus;HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

of ECW > 0.398 (log-rank 6.94; $P < 0.01$) (Fig. 2) and low albumin < 3.5 g/dL (log-rank 5.45; $P < 0.05$). There were no statistically significant associations with age, platelets, bilirubin, prothrombin time, ALT, sodium, or BMI. In a Cox multivariate regression analysis, the independent predictor of developing ascites was ECW ratio (ECW ratio, hazard ratio 4.04, 95 % CI 0.04–4.82, $P < 0.05$; albumin, hazard ratio 2.66, 95% CI −3.83–0.32, $P = 0.10$).

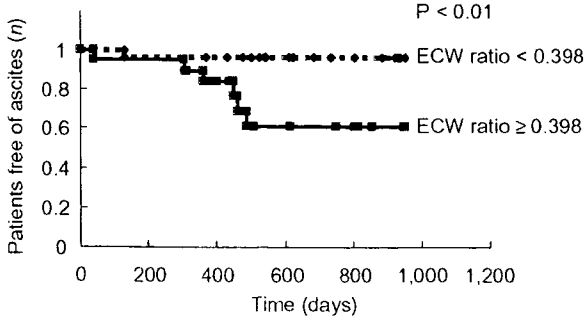

Figure 2 Extracellular water (ECW) ratio in the prediction of the risk of developing ascites. According to Kaplan–Meier analysis, a relative expansion of ECW (i.e. more than 0.398 in the initial assessment) was associated with a higher risk of developing ascites (long-rank test 6.94, $P < 0.01$).

DISCUSSION

ESTIMATION OF BODY water compartments is important for the assessment and monitoring of cirrhotic patients.[23] Previous studies of animals and healthy humans indicated that measurements of body water using multiple-frequency BIA are reliable.[10] However, reports of the use of multiple-frequency BIA for the assessment of body water compartments in cirrhotic patients with ascites are limited.[24] Lehnert *et al.* found that ECW and TBW (as determined by isotope methods) were predicted accurately by multiple-frequency BIA ($r = 0.73$ and 0.89, respectively).[13] However, they noted that the 95% confidence interval for the limits of agreement between the multiple-frequency BIA and isotope methods was acceptable for the control group (± 5%) but was slightly higher for the cirrhotic group (± 9%).[13] The reasons why multiple-frequency BIA does not measure water compartments accurately in patients with cirrhosis, and particularly those with ascites, are unknown. The body water compartments are determined with BIA by tissue cellularity, tissue hydration and membrane potential. The known altered distribution of water in cirrhotic patients may alter the capacitant effect of all membranes on conductance through the fluid compartments. A limitation of this study is that we did not validate body water compartments, because other methods for the determination of the body water were not available for our patients.

In this study, the changes in ICW, ECW, or TBW did not correlate with the severity of liver disease. Similarly, Müller *et al.* reported no differences in the sizes of the TBW compartments between groups of patients with Child–Pugh A, B, or C.[25] Conversely, Figueiredo *et al.* showed that ECW was increased, and ICW decreased, in patients with cirrhosis.[23] This discrepancy may be explained by whether or not adjustments were made for physical characteristics. In our study, height was used for normalization because it is not affected by fluid retention. However, it has not been established fully whether height is suitable for normalization, and further study is needed.

One of the important conclusions of our study is that the ECW ratio was the most sensitive indicator, detecting redistribution of body water in patients with liver cirrhosis with and without ascites. In addition, the ECW ratios of our patients were also related to development of ascites. Recently, Planas *et al.* followed up 263 cirrhotic patients after their first significant episode of ascites and found that the overall survival rate at 5 years

for their cirrhotic patients with ascites was 56.5%.[26,27] However, the probability of survival decreased markedly in those patients who developed refractory ascites during follow-up.[26] The natural history of cirrhotic patients with ascites may improve with the implementation of strict management if body water measurements could be made in a simple manner.

Medical treatment based on sodium-restricted diet, spironolactone, and furosemide achieves a response rate in up to 90% of patients without renal failure in controlled clinical trials.[28,29] Indeed, we found that there were no differences in body water compartments, including the ECW ratio, between patients on diuretic treatment and those who were not. This finding suggests that diuretics strongly affect body water compartments and it is therefore necessary to consider whether diuretics have been administered when performing body water measurements.

Our study demonstrated that ECW ratios of the trunk and leg are correlated with the grade of ascites. In patients with grade 1 ascites, the ECW ratio of the trunk seems to be reliable to assess body water compartments. An explanation of this finding may be the presence of leg edema in a relevant number of patients with ascites, whilst none of the patients had edema of the arms. We conclude that segmental body water measurements may be superior to commonly used whole body information for the detailed assessment of cirrhotic patients with ascites.

In conclusion, the present study shows that the ECW ratio is a reliable indicator for assessing body water compartments in patients with cirrhosis, even in those with an advanced disease and ascites. Inclusion of segmental ECW ratios should be considered for the detailed assessment of cirrhotic patients. In addition, ECW ratio measurements may be an objective parameter for assessment of therapeutic regimens (i.e. sodium restriction or oral supplementation with branched-chain amino acids).[30,31] Early management before ascites and edema are obvious is not recommended at this time because there are no studies showing a positive influence of treatment on quality of life, or survival. Further interventional studies may be needed to determine whether treatment could change the natural history of this disease entity.

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Patients achieving clearance of HCV with interferon therapy recover from decreased retinol-binding protein 4 levels

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SUMMARY. Retinol-binding protein 4 (RBP4) is a recently identified adipokine that is elevated in the blood in several insulin-resistant states. We investigated the association between plasma RBP4 and histological and biochemical characteristics of chronic hepatitis C (CHC), as well as changes in RBP4 levels following interferon therapy. Eighty-one patients with CHC infected with genotype 1 received treatment with peginterferon plus ribavirin. Histological data were available for 41 out of 81 patients before treatment, and the degree of fibrosis, inflammation and steatosis was assessed. Plasma levels of RBP4 were determined in serial samples (before, at the end of treatment, and at 6 months post-treatment). RBP4 levels were lower in CHC patients than in control subjects (34.6 ± 12.3 μg/mL *vs* 46.2 ± 10.5 μg/mL; $P \leq 0.001$). Higher RBP4 levels were linked to lower alanine aminotransferase (ALT) ($P < 0.01$), higher cholinesterase ($P < 0.01$), hyperlipidaemia ($P < 0.01$), hyperglycaemia ($P < 0.05$), and higher platelet ($P < 0.01$) count in CHC patients. Plasma RBP4 levels tended to decrease concomitantly with the grade of histological fibrosis, activity, and steatosis. RBP4 levels at baseline were not a predictor of the response to antiviral therapy in CHC patients. After peginterferon plus ribavirin therapy, only patients who had achieved clearance of hepatitis C virus had higher post-treatment RBP4 levels. This study suggests that an association between RBP4 levels and abnormal metabolic features, and that liver function may determine RBP4 levels in CHC patents. This is further supported by the observation that RBP4 levels increased significantly after treatment only in sustained virological response (SVR) patients and reached levels comparable to those of healthy subjects.

Keywords: chronic hepatitis C, insulin resistance, interferon, retinol-binding protein 4, steatosis, sustained virological response.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. It is thought that about 80% of acute HCV infection cases become chronic, roughly 20% will develop liver cirrhosis, and 1–6% will develop hepatocellular carcinoma annually [1]. Treatment with interferon, alone or in combination with ribavirin, can eradicate HCV infection in some patients, leading to sustained normalization of liver function, improvement of hepatic inflammation and fibrosis, and a decreased risk for the development of hepatocellular carcinoma [2,3]. However, only 54–63% of patients achieve a sustained virological response (SVR) to therapy even with the most advanced treatment regimen, which combines peginterferon with ribavirin [4,5].

Clinical data have revealed that several metabolic disturbances, and especially insulin resistance (IR), are significant risk factors for decreased SVR to antiviral therapy with a combination of peginterferon and ribavirin in chronic hepatitis C (CHC) patients [6]. Although IR has been found to be associated with elevated body mass index (BMI) and central adiposity in genotype 1 CHC, in some circumstances it depends on the presence of HCV [7–9]. Experimental and clinical studies support a role of HCV infection in the development of IR. Patients with mild CHC have a higher homeostasis model of assessment (HOMA) IR than healthy

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CHC, chronic hepatitis C; FPG, fasting plasma glucose; GTP, glutamyltransferase; HCV, hepatitis C virus; HOMA, homeostasis model of assessment; IR, insulin resistance; RBP4, retinol-binding protein 4; RXR, retinol X receptor; SVR, sustained virological response.

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controls matched for age and BMI [10]. Moreover, HCV infection has been shown to cause steatosis through HCV-core antigen-related inhibition of mitochondrial beta-oxidation of fatty acids [11]. IR has also been implicated in fibrosis progression and steatosis development associated with CHC infection [12,13]. These findings suggest that two pathways to IR, involving host-related metabolic disorders and HCV, coexist in a large proportion of cases.

Recently, the role of retinol-binding protein 4 (RBP4) in the pathogenesis of IR has received attention [14]. This protein is secreted mainly by hepatocytes (80%), but also by adipose tissue (20%). It is the only specific transport protein for retinol and, by interacting with nuclear retinol X receptor (RXR), it plays a role in controlling metabolic cell functions, including steatogenesis [15]. Animal and human studies have highlighted a pathogenic link among IR, diabetes, and high levels of RBP4 [16,17]. A recent study found a direct relationship between hepatic fat content and circulating RBP4 levels in healthy subjects, whereas other studies have linked RBP4 levels to the inflammatory response in obese, insulin-resistant patients [17,18].

In the past year, two research groups have investigated blood levels of RBP4 in chronic liver disease with respect to the patients' histological and biochemical characteristics [19,20]. An association was reported between increased blood RBP4 and hepatic steatosis in one study [19], yet in the other, this association was not observed [20]. Aside from these two conflicting studies, no other studies to date have reported on changes in RBP4 levels in CHC patients during treatment with a combination of peginterferon and ribavirin. Accordingly, the aim of the present study was to evaluate whether RBP4 levels could be used to assess the presence of various biochemical and histological disease features, including steatosis, as well as the response rates to treatment in a group of 81 genotype one CHC patients. Furthermore, we compared the plasma levels of RBP4 at baseline, at the end of treatment, and after 6 months of treatment in patients with CHC.

METHODS

Subjects

The study population consisted of 81 CHC patients diagnosed according to the following criteria: no excessive alcohol intake (more than 20 g/day); a high viral load (≥100 KIU/mL) by quantitative analysis of HCV-RNA with polymerase chain reaction (Amplicor HCV monitor; Roche Diagnostic Systems, Tokyo, Japan); infection with HCV genotype 1b alone; negativity for hepatitis B surface antigen; lack of co-infection with human immunodeficiency virus; no hepatocellular carcinoma; an absence of other forms of chronic liver disease; and no previous treatment with antiviral drugs, immunosuppressive drugs, or steatosis-inducing drugs within 6 months prior to enrolment. Nineteen non diabetic, non obese (BMI ≤ 30 kg/m^2), apparently healthy subjects were enrolled as controls. All had normal alanine aminotransferase (ALT, ≤35 IU/L), normal cholesterol (≤220 mg/dL), triglycerides (≤160 mg/dL) and fasting plasma glucose (FPG, ≤110 mg/dL), and no evidence of viral infection (HCV and hepatitis B surface antigen negativity). Written informed consent was obtained from each subject. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. BMI was calculated as weight (kg) divided by square of value of height expressed in metres (m^2). After a 12-h overnight fast, venous blood samples were drawn to determine ALT, aspartate aminotransferase (AST), γ-glutamyltransferase (γ-GTP), cholinesterase, total cholesterol, triglycerides, FPG, insulin, ferritin, prothrombin time, hyaluronic acid, and platelet count. These parameters were measured using standard clinical biochemistry laboratory techniques. Plasma RBP4 levels were measured in duplicate by a human competitive enzyme-linked immunosorbent assay kit (AdipoGen, Seoul, Korea) according to the manufacturer's instructions. IR was determined by the HOMA method, using the following equation: HOMA-IR = fasting insulin (μU/mL) × FPG (mg/dL)/405 [21]. The HOMA score was calculated only in patients without overt diabetes (FPG > 126 mg/dL). Table 1 summarized the profiles of the patients.

Liver histology

Liver biopsies were performed in 41 out of 81 patients and evaluated blindly by an experienced pathologist. Formalin-fixed and paraffin-embedded liver biopsy specimens were stained with haematoxylin-eosin and Masson's trichrome. The degree of hepatic fibrosis was scored as follows: F0 = none, F1 = portal expansion, F2 = bridging fibrosis, F3 = bridging fibrosis with lobular distortion, and F4 = cirrhosis. The degree of inflammation was scored as follows: A0 = none, A1 = mild, A2 = moderate and A3 = severe. Steatosis was assessed as the percentage of hepatocytes containing fat droplets and classified as absent–minimum <5%; mild <30%; moderate–severe ≥30%.

Treatment outcomes

All patients received peginterferon alfa-2b at a median dose of 1.5 μg/kg (range, 0.8–1.8 μg/kg) subcutaneously every week in combination with ribavirin at a dose of 600, 800, or 1000 mg/day, according to body weight for 48 weeks. In a randomly selected group of 45 patients, RBP4 was measured at baseline, at the end of treatment, and after 6 months of treatment. SVR was defined as serum HCV-RNA negativity 6 months after the conclusion of treatment.

Statistical analysis

Data are presented as mean ± SD or as the number of cases. Correlations were analysed using Spearman's rank

Table 1 Baseline characteristics in the study subjects

Characteristics	CHC (n = 81)	Characteristics	CHC (n = 41)
Gender (M/F)	40/41	Histology at biopsy	
Age (years)	57.0 ± 8.7	Stage of fibrosis	
BMI (kg/m^2)	23.1 ± 3.3	0–1	17
ALT (IU/L)	64 ± 52	2	11
AST (IU/L)	54 ± 37	3–4	13
γ-GTP (IU/L)	49 ± 84	Grade of activity	
Cholinesterase (ΔpH)	0.92 ± 0.24	0–1	19
Total cholesterol (mg/dL)	171 ± 37	2	18
Triglycerides (mg/dL)	102 ± 52	3	4
FPG (mg/dL)	90 ± 12	Steatosis	
HOMA score	2.1 ± 1.9	<5%	22
Ferritin (ng/mL)	160 ± 160	≥5% to <30%	14
Prothrombin time (%)	95.1 ± 10.7	≥30%	5
Hyaluronic acid (ng/mL)	69.9 ± 65.7		
Platelet count (×10^4/μL)	16.9 ± 5.7		
HCV-RNA (kIU/mL)	1593 ± 1245		
RBP4 (μg/mL)	34.6 ± 12.3		

Data are given as mean ± SD or as number of cases.
BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyltransferase; FPG, fasting plasma glucose; HOMA, homeostasis model assessment; HCV, hepatitis C virus; RBP4, retinol-binding protein 4.

correlation test. Stepwise regression analysis was performed to identify independent predictors of plasma RBP4 levels as continuous dependent variables. Factors that reached statistical significance in the univariate analysis were regarded as explanatory variables. Categorical data were analysed using the χ^2 test. Mann–Whitney U-test was used for two-group comparisons. The analysis of variance was performed using Scheffe's F-test for multiple comparisons among the three groups. Paired t-test was used to compare mean values before and after treatment with peginterferon plus ribavirin. A P value of <0.05 was considered statistically significant.

RESULTS

Factors associated with RBP4 levels

The control subjects had a mean age of 41.3 ± 12.4 years, and 89.5 % were women. Their mean BMI was 22.4 ± 3.8 kg/m^2. All had normal ALT (17 ± 7 IU/L), cholesterol (185 ± 26 mg/dL), triglycerides (80 ± 25 mg/dL), and FPG (96 ± 8 mg/dL). None had arterial hypertension. The characteristics of the study patients are summarized in Table 1. The cohort included 40 men and 41 women. The mean BMI was 23.1 kg/m^2 and only one was obese (≥30 kg/m^2).

Retinol-binding protein 4 levels were lower in CHC patients than in control subjects (34.6 ± 12.3 μg/mL *vs* 46.2 ± 10.5 μg/mL; $P \leq 0.001$). Low ALT ($P < 0.01$), high cholinesterase ($P < 0.01$), high cholesterol ($P < 0.05$), high triglycerides ($P < 0.01$), high FPG ($P < 0.05$), and high platelet count ($P < 0.01$), evaluated as continuous variables, were associated with higher RBP4 levels in CHC patients. There were no significant correlations among RBP4 and age, BMI, AST, γ-GTP, HOMA score, ferritin, haemoglobin and HCV-RNA levels (Table 2). Stepwise regression analysis showed that triglycerides (step 1), ALT (step 2), and cholesterol (step 3) independently predicted RBP4 levels (Table 2).

Association of RBP4 with liver histology

At liver biopsy, steatosis was present in 19 out of 41 patients with CHC, but a grade of moderate-to-severe was identified in only five cases. Plasma RBP4 levels tended to decrease in parallel with the grade of histological fibrosis, activity, and steatosis in CHC patients, but the difference was not statistically significant (Fig. 1). In addition, the majority of patients who presented with steatosis tended to also have more severe fibrosis and activity (Table 3).

Factors associated with response to peginterferon plus ribavirin antiviral treatment

Sustained virological response (SVR) was achieved in 52% (42/81) of CHC patients. Univariate analysis identified only three parameters that influenced SVR: gender (male;

Table 2 Factors associated with RBP4 levels

Parameters	*r*	*P*
Spearman's rank correlation		
Age	−0.149	0.178
BMI	0.075	0.514
ALT	−0.333	0.003
AST	−0.170	0.162
γ-GTP	0.084	0.491
Cholinesterase	0.324	0.008
Total cholesterol	0.276	0.023
Triglycerides	0.409	0.001
FPG	0.472	0.045
HOMA score	0.013	0.958
Ferritin	−0.141	0.248
Prothrombin time	0.406	0.070
Hyaluronic acid	−0.230	0.090
Platelet count	0.289	0.017
Haemoglobin	−0.001	0.995
HCV-RNA	0.076	0.512
Parameters	*r*	*β*
Stepwise regression analysis (including ALT, cholinesterase, total cholesterol, FPG, triglycerides, and platelet count)		
Step 1		
Triglycerides	0.538	0.465
Step 2		
Triglycerides	0.605	0.477
ALT		−0.331
Step 3		
ALT	0.997	−0.245
Total cholesterol		0.339
Triglycerides		0.391

RBP4, retinol-binding protein 4; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyltransferase; FPG, fasting plasma glucose; HOMA, homeostasis model assessment; HCV, hepatitis C virus.

Table 3 Factors associated with steatosis

Characteristics	Steatosis <5% (n = 23)	Steatosis ≥5% (n = 18)	*P*
Stage of fibrosis 0–2/3–4	18/5	9/9	0.058
Grade of activity 0–1/2–3	13/10	6/12	0.139

Data are given as number of cases.

$P < 0.05$), high haemoglobin concentration ($P < 0.05$), and no hepatocyte steatosis ($P < 0.05$) (Table 4). SVR was not associated with HOMA score, RBP4 levels, or HCV viral load. Furthermore, neither fibrosis levels nor necro-inflammatory activity were associated with SVR rate.

Changes in the levels of RBP4 and other metabolic markers (cholesterol, triglycerides, and FPG) after peginterferon plus ribavirin therapy

In 45 patients, the levels of RBP4 were measured before treatment, at the end of treatment, and at 6 months after the end of treatment. Figure 2 shows the RBP4 values among sustained responders. RBP4 levels increased at the end of treatment in patients who had achieved clearance of HCV-RNA ($P < 0.05$) and continued to increase out to 6 months; RBP levels at 6 months were significantly higher than at baseline ($P < 0.01$; *t*-test). RBP4 levels did not change after the end of treatment in SVR patients (41.1 ± 13.5 μg/mL at end of treatment *vs* 45.1 ± 16.5 μg/mL at 6 months). Moreover, RBP4 levels at 6 months were similar in those in control subjects (46.2 ± 10.5 μg/mL). In nonresponders and relapsers, RBP4 levels remained unchanged during the follow-up period (31.9 ± 6.7 μg/mL at baseline *vs* 34.2 ± 5.6 μg/mL at end of treatment; 42.6 ± 13.5 μg/mL at baseline *vs* 40.2 ± 8.7 μg/mL at 6 months).

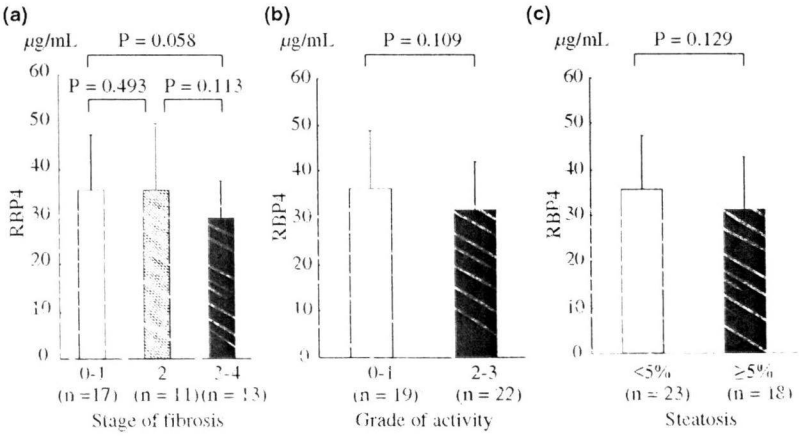

Fig. 1 Plasma RBP4 levels in relation to the degree of fibrosis, activity, and steatosis. RBP4 = retinol-binding protein 4.

Table 4 Factors associated with SVR

Characteristics	SVR (n = 42)	Non-SVR (n = 39)	P
Gender (M/F)	26/16	14/25	0.019
Age (years)	55.9 ± 9.5	58.3 ± 7.7	0.398
BMI (kg/m^2)	23.1 ± 3.6	23.1 ± 2.9	0.706
ALT (IU/L)	60 ± 54	68 ± 50	0.379
AST (IU/L)	45 ± 30	63 ± 42	0.094
γ-GTP (IU/L)	34 ± 28	64 ± 116	0.169
Cholinesterase (ΔpH)	0.90 ± 0.19	0.93 ± 0.28	0.838
Total cholesterol (mg/dL)	173 ± 39	166 ± 35	0.585
Triglycerides (mg/dL)	100 ± 59	104 ± 45	0.288
FPG (mg/dL)	88 ± 12	94 ± 11	0.229
HOMA score	2.3 ± 2.2	1.6 ± 0.4	0.926
Ferritin (ng/mL)	160 ± 153	160 ± 169	0.811
Prothrombin time (%)	95.5 ± 10.5	94.6 ± 11.6	1
Hyaluronic acid (ng/mL)	62.9 ± 64.5	78.4 ± 67.3	0.179
Platelet count (×10^4/μL)	17.6 ± 6.4	16.2 ± 4.8	0.532
Haemoglobin (g/dL)	14.5 ± 1.1	13.9 ± 1.7	0.012
HCV-RNA (kIU/mL)	2236 ± 4586	2225 ± 1842	0.989
RBP4 (μg/mL)	34.5 ± 13.5	34.8 ± 11.1	0.419
	SVR (n = 21)	Non-SVR (n = 20)	P
Stage of fibrosis 0–2/3–4	12/9	16/4	0.116
Grade of activity 0–1/2–3	10/11	9/11	0.867
Steatosis <5%/≥5%	12/9	5/15	0.037

Data are given as mean ± SD or as number of cases.
SVR, sustained virological response; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyltransferase; FPG, fasting plasma glucose; HOMA, homeostasis model assessment; HCV, hepatitis C virus; RBP4, retinol-binding protein 4.

Cholesterol and triglycerides levels at 6 months after the end of treatment (192 ± 39 mg/dL; 146 ± 96 mg/dL, respectively) were significantly ($P \leq 0.01$; $P \leq 0.05$, respectively) higher than at baseline in SVR patients. FPG levels remained unchanged during the follow-up period. High cholesterol ($r = 0.640$, $P \leq 0.01$) and high triglycerides ($r = 0.692$, $P \leq 0.01$) were closely associated with higher RBP4 levels at 6 months in SVR patients.

DISCUSSION

Recent data from healthy volunteers and patients with obesity indicate a novel function for RBP4 in the development of metabolic abnormalities such as dyslipidaemia [22,23]. In our patients with CHC, the observed correlations between RBP4 and total cholesterol and triglycerides before and after peginterferon plus ribavirin therapy were in general agreement with recent literature [22]. However, it is not entirely clear whether RBP4 affects lipid metabolism in hepatocytes. A possible explanation is that RBP4 may influence the transactivation of retinol-sensitive transcription factors such as RXR. RXRs bind to DNA as obligate heterodimers with peroxisome-proliferator activated receptors that regulate the transcription of genes involved in fatty acid metabolism [24]. Another possible mechanism is that this protein is the only specific transport protein for retinol and changes in retinoid metabolism may alter the tissue level of retinol. A previous study reported that normalization of circulating RBP4 by synthetic retinoid improves IR and glucose intolerance [14]. In addition, the results of the correlation analysis showed that the plasma RBP4 levels were negatively correlated with ALT levels. Clinical studies have reported a direct association between adipose or serum expression of RBP4 and adipose or systemic inflammation [18,25]. On the other hand, Petta *et al.* [19] showed that RBP4 was inversely associated with hepatic necro-inflam-

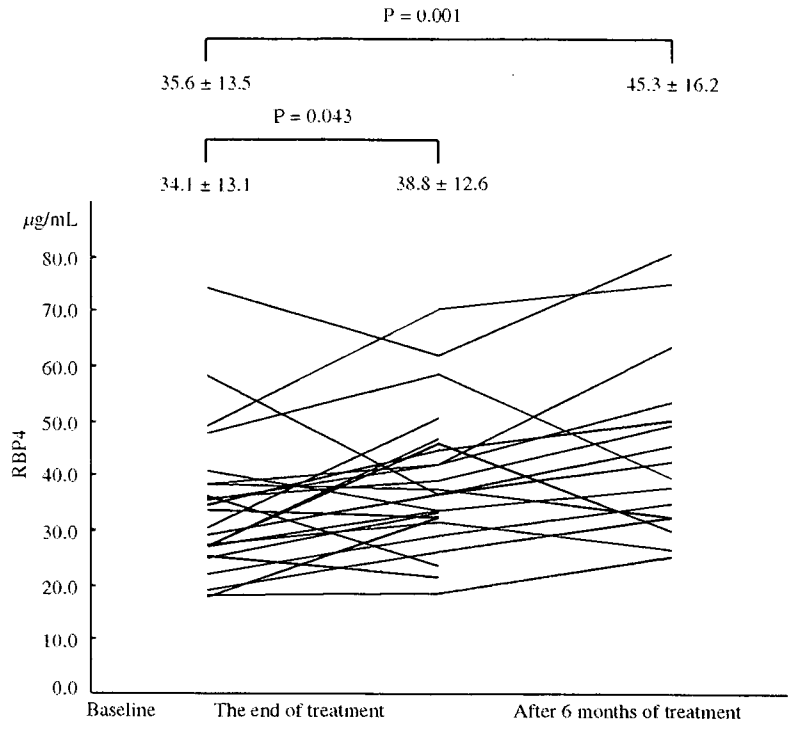

Fig. 2 Retinol-binding protein 4 levels after treatment in sustained responders. Retinol-binding protein levels at the end of treatment and at 6 months were significantly higher than at baseline.

matory activity in patients with CHC. The effect of RBP4 on liver cells may differ from that on adipose tissue and on systemic inflammation, especially in CHC patients.

It has been reported that RBP4, which is normally expressed in liver and hepatocytes, is the principal source of circulating RBP4 under normal conditions while adipose tissue is the organ that expresses the second highest level of RBP4 [26]. Janke *et al.* [27] found no significant relationship between RBP4 expression in adipose tissue and serum RBP4 levels in postmenopausal women, and Stefan *et al.* [17] showed that circulating RBP4 levels were not associated with the amount of visceral and subcutaneous abdominal fat. The findings above suggest that the liver is the major source of circulating RBP4 in humans. Recently, Yagmur *et al.* [20] found that serum RBP4 was significantly reduced in patients with liver cirrhosis as compared with healthy control subjects and was closely linked to biomarkers of liver synthesis function: e.g., cholinesterase activity, serum albumin, and prothrombin time. In addition, their analysis of an animal model corroborated high gene expression of RBP4 in hepatic tissue and a reduction after induction of experimental cirrhosis [20]. This finding is in agreement with our observation in CHC patients that RBP4 levels were associated with cholinesterase activity and platelet count and showed a trend for a weak association with the grade of histological fibrosis. More recently, hepatic RBP4 production rates were assessed by measuring the arterial hepatic venous concentration in cirrhotic patients, and it was reported that RBP4 in cirrhosis is decreased because of reduced hepatic production [28]. These studies suggest that liver function affects plasma RBP4, and it is therefore necessary to consider liver function when examining plasma RBP4 levels.

Previous work in humans suggested that RBP4 is associated with whole-body insulin sensitivity [14]. This study performed in CHC patients confirms that this association is not detectable when using HOMA-derived indices. A recent study by Petta *et al.* [19] also failed to report an association between RBP4 and HOMA scores in CHC patients. Differences in material, age, and anthropometric indices among studies may explain this discrepancy. A majority of patients in our study had normal body weight and only one had obesity (BMI $\geq$ 30 kg/m^2). In contrast with a previous study in healthy volunteers and patients with obesity, our data suggest that the relationship with IR is not mediated by obesity because plasma RBP4 levels were not associated with parameters of body adiposity (e.g., BMI and hepatic steatosis) in CHC patients. Moreover, during the progression of CHC, fat droplets containing large amounts of vitamin A are lost. Vitamin A status, which is likely reduced in advanced fibrosis, would influence the effects of RBP4 on IR [29].

This study demonstrated no significant association between plasma RBP4 levels and hepatic steatosis in patients with CHC. Our finding is in accordance with a previous report by Yagmur *et al.* but conflicts with a recent report by Petta *et al.* [19,20]. An explanation for this discrepancy between the studies could be that moderate-to-severe steatosis was identified in only 5 (12%) out of 41 patients with CHC in our study, and in the majority of cases, steatosis was

associated with more severe fibrosis. The impact of increased steatosis on elevated RBP4 levels might not be fully evaluated and advanced fibrosis might also affect RBP4 levels. Lastly, RBP4 levels did not appear to influence the SVR rate in patients infected with genotype 1. This study identified gender, haemoglobin concentration, and histological steatosis as predictors of SVR. These findings may be attributable to the fact that plasma RBP4 levels were less sensitive to the degree of hepatic steatosis in our CHC patients. Further studies are required to explore the relationship between the severity of histological steatosis and RBP4 levels in patients with CHC.

A novel finding in this study was that the clearance of HCV minimized the reduction of RBP4 levels. It is well known that viral clearance during interferon therapy results in a significant improvement of liver function and fibrosis in patients with CHC, which supports a connection between RBP4 levels and hepatic function, and RBP4 increased when the virus was eradicated.

In conclusion, this study has shown that higher RBP4 levels were linked to hyperlipidaemia and that liver function might determine RBP4 levels in CHC patients. This is further supported by the fact that post-treatment RBP4 levels were significantly increased only in SVR patients, reaching values observed in healthy subjects. Moreover, we have found that elevated RBP4 levels are strongly associated with hyperlipidaemia after achieving clearance of HCV.

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Short Communication

Impaired regulation of serum hepcidin during phlebotomy in patients with chronic hepatitis C

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Aim: This study was conducted to determine the clinical relevance of hepcidin, a recently identified key iron regulatory hormone, in patients with chronic hepatitis C virus (C-HCV).

Methods: Serum hepcidin levels were measured in 9 C-HCV patients by surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS), and compared to those of healthy controls. Sequential changes of hepcidin were also investigated during phlebotomy.

Results: Serum hepcidin and ferritin were significantly higher in C-HCV than in controls ($P = 0.0002$), these two variables were strongly related to each other ($r = 0.658$; $P < 0.01$), and phlebotomy significantly decreased serum hepcidin in C-HCV ($P = 0.0007$); all these results recollect the hepcidin response to iron signal. Hepcidin/ferritin ratio, an index of the appropriateness of hepcidin expression relative to iron overload, was significantly lower in C-HCV than in controls (0.33 ± 0.41 vs. 0.73 ± 0.36, $P = 0.0068$). This relative impairment of hepcidin expression was not reversible after phlebotomy (P = NS).

Conclusions: Although the hepcidin expression responds to iron conditions in C-HCV, this response is relatively limited. This relative impairment of hepcidin expression may be relevant to disease progression, and thus correction of its regulation may be beneficial for these iron-overloaded C-HCV patients.

Key words: Chronic hepatitis C virus, Ferritin, Hepcidin, Iron-regulated genes, Phlebotomy, Surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS)

Abbreviations:
AU, arbitrary units; C-HCV, chronic hepatitis C; HCV, hepatitis C virus; SELDI-TOF-MS, surface-enhanced laser desorption/ionization time of flight mass spectrometry; SD, standard deviation; TfR, transferrin receptor.

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is a global health problem, affecting more than 170 million people worldwide.[1] Although the causative factor responsible for the initiation of liver disease is a single insult, i.e. HCV infection, it has become increasingly evident that the involvement of cofactors is critical in determining disease progression. Chronic hepatitis C virus (C-HCV) is often associated with disturbances in iron homeostasis, with serum ferritin and hepatic iron stores being elevated in approximately 50% of patients.[1–4] Because iron is a redox-active metal, catalyzing free radical reactions via the Fenton reaction, iron has been highlighted as an important element affecting the natural history of C-HCV. Further, it was also reported that the phlebotomy-induced iron reduction was effective for hepatocellular injury in C-HCV.[5–9] However, little is known about the causative mechanism of iron overload during chronic HCV infection.

Recent work has established the importance of the hepatocyte produced-hepcidin in iron homeostasis

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as a negative regulator of iron release into the circulation by duodenal enterocytes and reticuloendothelial macrophages.[10,11] Hepcidin binds to the iron exporter ferroportin, which results in ferroportin internalization and degradation.[12] Studies on animals,[13–15] and on hepcidin mRNA expression in human liver biopsies,[16] have led to the unifying concept that insufficient hepcidin production is the key pathogenetic feature of most types of iron overload diseases. Therefore, using a surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) assay,[17] we measured the serum hepcidin levels in patients with C-HCV and compared to normal subjects. The sequential changes of serum hepcidin during phlebotomy were also investigated.

PATIENTS AND METHODS

Patients

A TOTAL OF 9 C-HCV patients treated with phlebotomy at Mie University Hospital between September 2005 and June 2007 were enrolled (Table 1). At enrollment, they completed a questionnaire with specific items relevant to iron metabolism (i.e. any history of blood donation, previous pregnancy, menstrual losses, etc.) and were evaluated by laboratory studies including complete blood count, serum iron, transferrin saturation, ferritin, and liver function tests. No patients had received oral or intravenous iron-containing drug, or multiple blood transfusions within the last 5 years before the study. All patients were absence of the HFE mutations C282Y or H63D. Liver biopsy was performed in 8 patients before phlebotomy for clinical histological evaluation.[18] Phlebotomy was performed as described previously.[9] In brief, at the initial phase of iron depletion, all patients underwent weekly or biweekly phlebotomy of 200 or 400 gram until a state of iron deficiency was achieved (defined by either by a serum ferritin levels <10 ng/mL and/or a blood hemoglobin concentration of 10 g/dL). The iron deficiency state was maintained by additional phlebotomies during the study period: patients were followed-up every 1–2 months for the duration, and a phlebotomy was performed if the serum ferritin level exceeded 20 ng/mL. We also studied a group of healthy volunteers ($n = 10$) with normal serum iron status, no signs of anemia or liver disease, and without HCV infection as controls. Informed consent was obtained from each patient included in the study and the study protocol conformed

Table 1 Clinical characteristics of patients in this study

Characteristics	Chronic hepatitis C ($n = 9$)	Normal subject ($n = 10$)	*P* values
Age (years)	54.7 ± 12.4	37.2 ± 10.3	0.0055
Gender (M/F)	7/2	6/4	NS
Laboratory data			
ALT (IU/L)	100 ± 28.6	23.8 ± 9.0	0.0002
AST (IU/L)	92.9 ± 45.4	20.6 ± 5.3	0.0002
RBC ($\times 10^4$/mm^3)	383 ± 36	473 ± 62	0.0048
Hemoglobin (g/dL)	14.3 ± 0.7	14.3 ± 1.5	NS
Hematocrit (%)	40.2 ± 5.3	43.7 ± 4.3	NS
Platelet count ($\times 10^4$/mm^3)	14.6 ± 4.2	20.9 ± 4.9	0.0143
Serum iron (μg/dL)	149 ± 40	108 ± 42	0.0436
Transferrin saturation (%)	49.2 ± 16.7	31.0 ± 12.5	0.0222
Serum ferritin (ng/mL)	433 ± 210	54 ± 30	0.0002
Serum HCV RNA (KIU/mL)	689 ± 590	–	
Liver histology	($n = 8$)		
Inflammatory activity (0/1/2/3)†	0/6/2/0	–	
Fibrosis staging (0/1/2/3/4)‡	0/2/4/1/1	–	

Data are expressed as mean ±SD.
†Inflammatory activity was graded according to the intensity of necroinflammatory lesions (0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity). ‡Fibrosis staging was scored (0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; RBC, red blood cell count.