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【 ORIGINAL ARTICLE 】

Serum Copeptin and Zinc-α2-glycoprotein Levels Are Novel Biomarkers of Tolvaptan Treatment in Decompensated Cirrhotic Patients with Ascites

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Abstract:

Objective The efficacy of tolvaptan, an orally active vasopressin V2-receptor antagonist, has recently been reported in patients with massive ascites unresponsive to conventional diuretics. However, the effect of tolvaptan varies among patients. Recently, the prognostic role of the tolvaptan response in cases of decompensated liver cirrhosis (LC) has been attracting increasing attention. Using serum copeptin (vasopressin precursor), zinc-α2-glycoprotein (ZAG), cystatin C (renal biomarker), neutrophil gelatinase-associated lipocalin (NGAL) and liver-type fatty acid-binding protein (L-FABP), we explored which factors portend a good response to tolvaptan in LC patients with ascites.

Methods We enrolled 113 LC patients and divided them into the tolvaptan treatment group and non-treatment group. Tolvaptan (3.75 or 7.5 mg/day) was administrated to 38 LC patients with ascites, and a follow-up assessment was performed after a 7-day tolvaptan treatment regimen.

Results We determined the predictive ability for kidney and/or liver damage of serum copeptin, ZAG, cystatin C, NGAL and L-FABP levels in all patients. After 7-day tolvaptan treatment, 19 patients had lost more than 1.5 kg of body weight (Responders), while 19 showed no marked change in their body weight (Non-responders). Basal blood urea nitrogen (BUN) (p=0.0014), serum copeptin (p=0.0265) and serum ZAG levels (p=0.0142) were significantly higher in the Non-responders than in the Responders. BUN (odds ratio 7.43, p =0.0306), copeptin (odds ratio 9.12, p=0.0136) and ZAG (odds ratio 7.43, p=0.0306) were determined to be predictive factors of drug responsiveness using a multivariate logistic regression analysis.

Conclusion Serum BUN, copeptin and ZAG levels predict the patient response to tolvaptan, even when measured prior to treatment.

Key words: copeptin, zinc-α2-glycoprotein (ZAG), tolvaptan, liver cirrhosis, ascites

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Introduction

Arginine vasopressin (AVP) is a potent antidiuretic hormone in the human body. Despite the clinical relevance of AVP in maintaining fluid balance and vascular tone, measurement of the mature form of AVP is difficult due to its small size (9 amino acids), short half-life and ability to bind platelets (1). Copeptin, a 39-amino acid glycopeptide that comprises the C-terminal part of the AVP precursor, was found to be a stable and sensitive surrogate marker for AVP release (2). Previous reports have demonstrated that a high serum copeptin level predicts the survival of hospitalized liver cirrhosis (LC) patients, independent of liver-specific scoring systems (3), suggesting that copeptin may be a useful biomarker of disease progression and the prognosis in LC (4).

We found that the serum copeptin level was a predictor for ascites retention and hepatic encephalopathy as well as portosystemic shunt formation associated with portal hyper-

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tension in chronic liver disease (5). Zinc-α2-glycoperotein (ZAG) is a 41-kDa glycoprotein synthesized by epithelial cells and adipocytes and plays a role in lipid metabolism, cell cycling and cancer progression. The serum ZAG levels are reportedly useful biomarkers of renal injury and can predict mortality in hemodialysis patients (6). However, the changes in serum ZAG levels have not been reported in LC patients with ascites.

Several markers of the renal function have been established, such as the serum levels of cystatin C, neutrophil gelatinase-associated lipocalin (NGAL) and liver-type fatty acid binding protein (L-FABP). Urea NGAL is established as a clinical biomarker of acute renal injury (7) based on the function of NGAL as a kidney protector (8). Urine L-FABP levels derived from proximal tubular epithelial cells are elevated in renal tubular injury episodes and are therefore used as markers of several kidney diseases, including acute kidney injury and chronic kidney diseases (9). We previously reported that serum NGAL and serum L-FABP levels are prognostic factors for the survival in chronic liver diseases (10, 11).

Ascites is the most frequent complication of LC, and refractory ascites has a negative prognostic implication in the natural progression of LC. Tolvaptan is a novel, orally administered, selective vasopressin V_2-receptor antagonist that downregulates the expression of aquaporin-2 in the renal collecting duct. Research has uncovered a promising role for tolvaptan as an add-on treatment in patients with hepatic ascites resistant to furosemide and/or spironolactone, as it is able to decrease body weight and alleviate edema (12, 13). However, the effect of tolvaptan is highly variable, with only roughly half of the patient cohort reportedly responding to the drug (12). Blood urea nitrogen (BUN) (12) and C-reactive protein (CRP) (13) are useful for differentiating diagnoses between Responders and Non-responders of tolvaptan, but these factors are insufficient, so additional biomarkers are needed.

Recently, in addition to the aforementioned studies, several studies have further found that a response to tolvaptan led to an improvement in the long-term survival rates among LC patients with ascites (14, 15). Therefore, the present study assessed the correlations between the efficacy of tolvaptan and several treatment-related factors, including serum copeptin, ZAG, cystatin C, NGAL and L-FABP levels, in LC patients with ascites.

Materials and Methods

Human samples

The study protocol was approved by the Clinical Research Ethics Review Committee of Mie University Hospital. This study was performed retrospectively using stored samples, and patients were allowed to opt out of their data being used.

We enrolled 113 LC patients (69 men and 44 women) with a median age of 68.0 (60.0-75.0) years old whose data were available to be analyzed between November 2013 and September 2016 at Mie University Hospital. Patients positive for hepatitis B surface antigen were diagnosed with hepatitis B virus infection, whereas those positive for anti-hepatitis C virus (HCV) were diagnosed with HCV infection. Alcoholic cirrhosis was defined as the presence of alcohol consumption >60 g/day. Nonalcoholic steatohepatitis was diagnosed based on pathological findings or fatty liver without any other evident causes of chronic liver diseases (viral, autoimmune, genetic, etc.).

We divided the 113 LC patients into 3 groups: without ascites (n=46), with ascites not receiving tolvaptan (n=22) and with ascites receiving tolvaptan (n=45). LC was diagnosed based on morphologic changes in the liver, such as hypertrophy of the left lateral and caudate lobes or atrophy of the right posterior hepatic lobe on ultrasonography, and through blood tests and/or computed tomography (CT), magnetic resonance imaging, FibroScan (Echosens, Paris, France) results, and esophageal varix by endoscopy, as is the general protocol. The FibroScan cannot be used on individuals with ascites, so it was used to assess liver fibrosis mainly in cases of chronic hepatitis or early cirrhosis. The definition of idiopathic portal hypertension/portal-sinusoidal vascular disorder was based on the absence of characteristic cirrhosis and known causes of liver disease. Decompensated cirrhosis was diagnosed in cases with a series of clinical and laboratory signs (e.g., ascites, encephalopathy, gastrointestinal bleeding, thrombocytopenia or hypoalbuminemia). Hepatocellular carcinoma (HCC) was diagnosed based on histological findings or typical imaging characteristics. The body mass index (BMI) was calculated as the weight (kg)/height (m) squared and was not adjusted for ascites. We performed a body composition analysis by measuring the visceral fat area (VFA), subcutaneous fat area (SFA) and psoas muscle index (PMI) due to the limited utility of BMI in patients with ascites. Using cross-sectional CT at the level of the transverse process of lumbar vertebra L3, the bilateral psoas muscle area was manually determined. The PMI (cm^2/m^2) was defined by normalizing the psoas muscle area (cm^2)/height (m) squared. The SFA (cm^2) and VFA (cm^2) near the umbilicus were automatically measured based on the standard fat attenuation range using the Fat Scan software program (PSP Corporation, Tokyo, Japan). Patients who had other malignancies within the past three years, spontaneous bacterial peritonitis, hepatic encephalopathy (coma scale score ≥II), heart failure (New York Heart Association category ≥II), human immunodeficiency virus infection, pregnancy or psychiatric problems were deemed unsuitable for inclusion in this clinical study.

Algorithm for the treatment of ascites and definition of Responders and Non-responders

As a general rule, the follow-up examinations included routine physical examinations and biochemical tests as well as diagnostic imagining studies, including ultrasonography.

All treatments of ascites were performed following the Japanese practical guidelines for LC in 2015 as much as possible (16): Patients with grade 1 mild ascites do not need diuretics and a low sodium diet. For patients with grade 2 moderate ascites, treatment begins with the administration of spironolactone at 25-50 mg/day, and in the absence of an effect, furosemide is added at 20 mg/day. Tolvaptan is recommended in combination with other diuretics, including 25-50 mg/day spironolactone and/or 20-40 mg/day furosemide, especially in patients with sustained hyponatremia. Although the administration of 7.5 mg/day tolvaptan may be more beneficial for the improvement in ascites volume, we have adopted 3.75 mg/day to balance the efficacy and side effects.

We divided the patients into two tolvaptan treatment groups: Responders and Non-responders. The Non-responder group was defined as patients with weight loss of <1.5 kg/week after receiving tolvaptan or performing paracentesis within the first week (13). All patients continued to take the same prescribed doses of furosemide and spironolactone within the first week (17).

Serum preparation

In the groups without ascites and with ascites but without treatment, serum samples were collected when patients presented at the hospital. In the group with ascites and treatment, serum collection was performed immediately before the administration of tolvaptan. Albumin (g/dL), total bilirubin (mg/dL), sodium (mEq/L), creatinine (mg/dL), BUN (mg/dL), CRP (mg/dL), α-fetoprotein (AFP; ng/mL) and des-γ-carboxy prothrombin (DCP; mAU/mL) were measured. Serum samples were kept at -80 °C until copeptin (pmol/L), ZAG (μg/dL), cystatin C (mg/dL), NGAL (ng/mL) and L-FABP (ng/mL) measurements were performed using an automated copeptin immunofluorescent assay kit (Thermo Fisher Scientific Inc., Tokyo, Japan), ZAG enzyme-linked immunosorbent assay (ELISA) kit (BioVendor, Brno, Czech Republic), cystatin C ELISA kit (R&D Systems, Minneapolis, MN, USA), NGAL ELISA kit (R&D Systems) and high-sensitivity human L-FABP ELISA kit (CMIC Holdings, Tokyo, Japan), respectively.

The Child-Pugh score, albumin-bilirubin (ALBI) score (18), fibrosis index based on 4 factors (FIB-4) (19), model for end-stage liver disease (MELD) score, MELD-Na score and estimated glomerular filtration rate (eGFR) with Cockcroft and Modification of Diet in Renal Disease (MDRD) formula were calculated.

Statistical analyses

All data are expressed as the median and range. Data were analyzed using the Mann-Whitney *U* test in two groups and one-way analysis of variance for comparison of continuous variables. The relationship between the serum copeptin, ZAG, cystatin C, NGAL and L-FABP levels and clinical data were examined using Spearman's rank correlation coefficient. For each continuous variable, the optimal cut-off value that maximized the sum of the sensitivity and specificity was selected using a receiver operating characteristic (ROC) analysis for the survival. A logistic regression analysis was used for the multivariate analysis to evaluate the relationship between the effect of tolvaptan and clinical data. Only variables deemed to be significant ($p<0.1$) in the univariate analysis were included in the subsequent multivariate analysis. The statistical analyses were performed using the JMP software program (SAS Institute, Cary, NC, USA) for univariate and multivariate logistic regression analyses. Differences were considered to be significant at $p<0.05$.

Results

Clinical characteristics of patients with or without tolvaptan

In this study, we divided the 113 LC patients into 3 groups: one without ascites, one with ascites not receiving tolvaptan and one with ascites receiving tolvaptan. Table 1 shows the comparison of the baseline clinical characteristics and laboratory variables between the group with ascites not receiving tolvaptan and the group with ascites receiving tolvaptan. In the group with ascites receiving tolvaptan, the aspartate aminotransferase, total bilirubin, CRP, the Child-Pugh score, ALBI score and FIB4-index were significantly increased, while the serum albumin and sodium levels were reduced, suggesting that patients treated with tolvaptan had more advanced liver diseases than patients treated without tolvaptan. In the group with ascites receiving tolvaptan, the dose of diuretics (furosemide and/or spironolactone) was significantly higher than in the group with ascites not receiving tolvaptan. In contrast, there were no obvious differences in age, gender, body weight, BMI, SFA, VFA, PMI, prevalence of HCC, alanine aminotransferase, prothrombin time, MELD score, MELD-Na score, creatinine, BUN, eGFR (Cockcroft and MDRD-6), copeptin, ZAG, NGAL, L-FABP or cystatin C values between the group with ascites not receiving tolvaptan and the group with ascites receiving tolvaptan (Table 1).

Correlation of copeptin, ZAG, cystatin C, NGAL and L-FABP to clinical parameters in all patients

The correlations between copeptin, ZAG, cystatin C, NGAL and L-FABP and clinical parameters in LC patients are shown in Table 2. Copeptin was mainly strongly correlated with the hepatic function, including the albumin level ($p=0.0007$; Fig. 1A), Child-Pugh score ($p<0.0001$), ALBI score ($p=0.0003$) and CRP ($p=0.0047$; Fig. 1B). There was a weak or no correlation between copeptin and the body weight, BMI, creatinine and BUN values (Fig. 1C-F). ZAG was not correlated with the albumin level (Fig. 1G), but the serum ZAG levels were significantly correlated with the CRP ($p<0.0001$; Fig. 1H), body weight ($p=0.0092$; Fig. 1I), BMI ($p=0.0001$; Fig. 1J) and SFA values ($p=0.0003$; Ta-

Table 1. Characteristics of 113 Liver Cirrhotic Patients.

Parameters	All	Without ascites	With ascites		p†
			Not receiving tolvaptan	Receiving tolvaptan	
	n=113	n=46	n=22	n=45	
Age (years)	68.0 (60.0-75.0)	66.5 (58.0-72.8)	74.0 (66.3-80.8)	68.0 (61.0-76.0)	0.1117
Gender, Male : Female	69 : 44	29 : 17	14 : 8	26 : 19	0.6939
Bodyweight (kg)	58.1 (51.3-66.7)	62.3 (51.3-72.7)	57.0 (53.4-61.6)	56.6 (51.0-63.6)	0.7620
BMI (kg/m^2)	23.6 (20.8-25.3)	24.0 (22.5-25.8)	22.3 (20.1-25.0)	22.1 (19.5-24.3)	0.7010
SFA (cm^2)	125.3 (68.1-204.0)	180.9 (117.1-223.8)	98.4 (50.4-177.7)	110.0 (54.2-163.3)	0.6397
VFA (cm^2)	80.7 (54.3-115.5)	96.8 (78.8-129.9)	67.4 (48.6-109.4)	70.3 (48.0-91.0)	0.9068
PMI (cm^2/m^2)	5.6 (4.7-7.0)	6.1 (4.7-7.4)	5.6 (4.8-6.4)	5.2 (4.2-6.2)	0.2651
HCV/HBV/HCV+AL/AL/NASH/others	56/7/4/23/13/10	28/2/0/10/2/4	11/0/1/3/4/3	17/4/3/10/7/4	-
presence of HCC, yes : no	61 : 52	23 : 23	10 : 12	28 : 17	0.6045
Ratio of using diuretic drugs (%)		26	45	100	-
Dose of diuretic drugs (n)					
Tolvaptan (0mg/3.75mg/7.5mg)	68 / 37 / 8	46 / 0 / 0	22 / 0 / 0	0 / 37 / 8	-
Furosemide (0mg/10mg/20mg/40mg/80mg)	62/4/28/14/5	40/0/4/2/0	16/1/4/0/1	6/3/20/12/4	**<0.0001**
Spironolactone (0mg/25mg/50mg/100mg)	55 / 26 / 28 / 4	38 / 4 / 3 / 1	14 / 4 / 3 / 1	3 / 18 / 22 / 2	**<0.0001**
Grade of ascites (1 / 2 / 3)	37 / 22/ 8	0 / 0 / 0	17 / 2 / 3	20 / 20 / 5	**-**
AST (U/L)	45.0 (31.0-70.0)	37.0 (28.3-54.5)	35.5 (28.0-62.5)	55.0 (42.0-87.0)	**0.0014**
ALT (U/L)	29.0 (17.0-41.0)	28.0 (17.0-41.8)	29.5 (15.0-34.3)	29.0 (23.0-42.0)	0.2421
Albumin (g/dL)	3.2 (2.8-3.7)	3.6 (3.3-4.1)	3.3 (3.0-3.7)	2.9 (2.6-3.1)	**0.0010**
Total Bilirubin (mg/dL)	1.2 (0.7-1.9)	1.1 (0.7-1.4)	0.8 (0.7-1.8)	1.5 (0.8-2.7)	**0.0210**
Prothrombin time (%)	72.0 (60.9-82.4)	75.6 (64.1-84.0)	74.2 (61.5-85.5)	67.4 (55.1-78.4)	0.1297
Child-Pugh score	7.0 (6.0-9.0)	5.5 (5.0-6.8)	7.5 (6.3-9.0)	8.0 (8.0-10.0)	**0.0083**
ALBI score	-1.9 (-2.4- -1.5)	-2.4 (-2.6- -1.9)	-2.0 (-2.7- -1.3)	-1.5 (-1.8- -1.2)	**0.0004**
FIB4-index	6.3 (3.8-9.3)	5.2 (3.5-7.9)	5.0 (3.7-8.4)	8.0 (5.5-10.6)	**0.0343**
MELD score	16.0 (14.6-18.2)	15.0 (14.0-16.1)	16.5 (15.8-20.0)	16.9 (15.0-19.3)	0.6643
MELD-Na score	18.3 (15.5-21.5)	15.6 (13.5-17.4)	17.6 (16.5-21.7)	20.3 (18.4-23.5)	0.0557
Creatinine (mg/dL)	0.8 (0.6-1.0)	0.7 (0.6-0.8)	0.9 (0.8-1.1)	0.8 (0.7-1.0)	0.2143
BUN (mg/dL)	15.5 (11.7-22.0)	14.0 (10.0-18.0)	18.0 (13.5-27.2)	18.5 (13.0-25.0)	0.6594
eGFR (mL/min/1.73m^2)	69.0 (59.0-104.4)	75.1 (59.3-93.6)	58.8 (34.4-74.5)	61.1 (46.7-87.7)	0.4999
GFR (MDRD-6) (mL/min)	84.3 (70.4-126.4)	99.6 (80.4-128.2)	75.5 (53.0-95.0)	77.2 (54.0-95.7)	0.5887
Serum sodium (mEq/L)	138 (134-140)	140 (139-141)	138 (136-139)	134 (131-138)	**0.0043**
CRP (mg/dL)	0.3 (0.1-1.3)	0.1 (0.0-0.3)	0.2 (0.1-1.0)	1.2 (0.4-3.4)	**<0.0001**
Copeptin (pmol/L)	6.6 (3.6-14.2)	4.2 (2.6-6.7)	8.0 (5.2-15.2)	10.7 (5.4-19.7)	0.5002
ZAG (μg/mL)	29.2 (24.3-37.6)	27.4 (22.5-32.0)	37.9 (26.9-45.6)	32.1 (24.7-41.1)	0.1797
NGAL (ng/mL)	61.0 (30.1-104.2)	30.3 (18.4-53.3)	88.9 (43.9-114.5)	82.9 (54.8-149.8)	0.6595
Cystatin C (mg/dL)	1.1 (0.9-1.3)	1.0 (0.8-1.2)	1.2 (1.0-1.5)	1.1 (0.9-1.4)	0.2854
L-FABP (ng/mL)	8.1 (3.8-13.8)	5.3 (2.2-13.3)	8.4 (3.8-10.8)	9.7 (5.5-19.3)	0.2119

†Mann–Whitney *U* test (Non-treatment vs. Treatment). BMI: body mass index, SFA: subcutaneous fat area, VFA: visceral fat area, PMI: Psoas muscle index, HCV: hepatitis C virus, HBV: hepatitis B virus, AL: alcohol, NASH: nonalcoholic steatohepatitis, HCC: hepatocellular carcinoma, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALBI: albumin-bilirubin, FIB4-index: fibrosis-4, MELD: model for end-stage liver disease, BUN: blood urea nitrogen, eGFR: estimated glomerular filtration rate, MDRD-6: the 6-variable modification of diet in renal disease, CRP: C-reactive protein, ZAG: zinc-α2-glycoprotein, NGAL: neutrophil gelatinase-associated lipocalin, L-FABP: liver-type fatty acid–binding protein. All data are expressed as median (first quartile-third quartile).

ble 2). Furthermore, ZAG was strongly correlated with the renal function, including the creatinine (p<0.0001; Fig. 1K), BUN (p<0.0001; Fig. 1L) and GFR (MDRD-6) (p<0.0001) (Table 2). Cystatin C was significantly correlated with the age (p=0.0191), renal parameters (creatinine, BUN and eGFR: p<0.0001), copeptin (p=0.0008), NGAL (p<0.0001) and L-FABP (p<0.0001) (Table 2). NGAL and L-FABP were significantly correlated with indicators of both the renal function and hepatic function (Table 2). The MELD-Na score was significantly correlated with all markers, copeptin (p=0.0008), ZAG (p=0.001), cystatin C (p<0.0001), NGAL (p<0.0001) and L-FABP (p=0.0003).

The background comparison between the Responders and Non-responders to tolvaptan

In this study, we excluded 7 of 45 cases for whom the tolvaptan efficacy could not be determined due to a hospital transfer, lack of weight data or presence of other treatments,

Table 2. Correlation between Serum Copeptin, ZAG, Cystatin C, NGAL, L-FABP Levels and Clinical Parameters in 113 Liver Cirrhotic Patients.

Parameter	Copeptin		ZAG		Cystatin C		NGAL		L-FABP	
	r	p*	r	p*	r	p*	r	p*	r	p*
Age	-0.0127	0.8930	0.1691	0.0734	0.2202	**0.0191**	0.1902	**0.0436**	0.0945	0.3194
Gender	-0.0623	0.5120	0.1474	0.1191	-0.0729	0.4429	-0.0762	0.4223	0.1380	0.1448
Bodyweight	-0.0417	0.6683	-0.2497	**0.0092**	-0.1195	0.2182	-0.0907	0.3507	-0.1156	0.2336
BMI	-0.0504	0.6100	-0.3675	**0.0001**	-0.0453	0.6460	-0.0796	0.4197	-0.1695	0.0839
SFA	-0.0921	0.3501	-0.3429	**0.0003**	-0.1340	0.5655	-0.1340	0.1730	-0.1863	0.0571
VFA	-0.1717	0.0799	-0.1487	0.1301	0.0357	0.7176	-0.1121	0.2547	-0.0806	0.4187
PMI	-0.1605	0.1070	-0.0610	0.5425	-0.1211	0.2252	-0.0484	0.6293	-0.0676	0.4998
AST	0.1947	**0.0388**	-0.2462	**0.0086**	-0.0894	0.3464	0.0226	0.8120	0.3504	**0.0001**
ALT	0.0521	0.5835	-0.1792	0.0575	-0.0647	0.4959	-0.0707	0.4567	0.4015	**<0.0001**
Total bilirubin	0.1580	0.0947	-0.3090	**0.0009**	-0.1947	**0.0388**	-0.0392	0.6804	-0.0671	0.4800
Albumin	-0.3152	**0.0007**	-0.0022	0.9819	-0.1280	0.1766	-0.4398	**<0.0001**	-0.2312	**0.0137**
Prothrombin time	-0.1141	0.2311	0.2386	**0.0113**	-0.1582	0.0957	-0.0752	0.4308	-0.0918	0.3357
Creatinine	0.2197	**0.0199**	0.4322	**<0.0001**	0.5714	**<0.0001**	0.4085	**<0.0001**	0.3497	**0.0001**
BUN	0.2575	**0.0059**	0.5037	**<0.0001**	0.5074	**<0.0001**	0.3703	**<0.0001**	0.4430	**<0.0001**
eGFR	-0.2490	**0.0078**	-0.3451	**0.0002**	-0.6086	**<0.0001**	-0.4481	**<0.0001**	-0.2788	**0.0028**
GFR (MDRD-6)	-0.3018	**0.0012**	-0.3731	**<0.0001**	-0.6077	**<0.0001**	-0.4949	**<0.0001**	-0.3412	**0.0002**
Serum sodium	-0.1200	0.2181	-0.1232	0.2062	-0.0285	0.7711	-0.2553	**0.0080**	-0.1790	0.0650
CRP	0.2701	**0.0047**	0.3677	**<0.0001**	0.0518	0.5944	0.4291	**<0.0001**	0.2281	**0.0176**
Copeptin	-	**-**	0.1969	**0.0366**	0.3104	**0.0008**	0.3182	**0.0006**	0.1709	0.0703
ZAG	0.1969	**0.0366**	-	**-**	0.2701	**0.0038**	0.3634	**<0.0001**	0.1735	0.0662
Cystatin C	0.3104	**0.0008**	0.2701	**0.0038**	-	-	0.4913	**<0.0001**	0.4468	**<0.0001**
NGAL	0.3182	**0.0006**	0.3634	**<0.0001**	0.4913	**<0.0001**	-	-	0.3105	**0.0008**
L-FABP	0.1709	0.0703	0.1735	0.0662	0.4468	**<0.0001**	0.3105	**0.0008**	-	-
Child-Pugh score	0.3819	**<0.0001**	-0.0271	0.7754	0.1468	0.1208	0.3876	**<0.0001**	0.2143	**0.0227**
ALBI score	0.3366	**0.0003**	-0.0921	0.3322	0.0656	0.4901	0.3750	**<0.0001**	0.2078	**0.0272**
MELD score	0.3057	**0.0010**	0.3338	**0.0003**	0.5595	**<0.0001**	0.4498	**<0.0001**	0.2839	**0.0024**
MELD-Na score	0.3187	**0.0008**	0.3135	**0.0010**	0.4342	**<0.0001**	0.5188	**<0.0001**	0.3450	**0.0003**

*Spearman's rank correlation coefficient. BMI: body mass index, SFA: subcutaneous fat area, VFA: visceral fat area, PMI: psoas muscle index, AST: aspartate aminotransferase, ALT: alanine aminotransferase, BUN: blood urea nitrogen, eGFR: estimated glomerular filtration rate, MDRD-6: the 6-variable modification of diet in renal disease, CRP: C-reactive protein, ZAG: zinc-α2-glycoprotein, NGAL: neutrophil gelatinase-associated lipocalin, L-FABP: liver-type fatty acid–binding protein, ALBI: albumin-bilirubin, MELD: model for end-stage liver disease

such as albumin transfusion, large-volume paracentesis or cell-free, concentrated ascites reinfusion therapy. We enrolled 38 decompensated LC patients with ascites (24 men and 14 women) with a median age of 66.0 (59.5-73.0) years old. We divided the 38 patients into 2 tolvaptan treatment groups: Responders and Non-responders. There were no obvious differences in the age, gender, body weight, BMI, SFA, VFA, PMI, presence of HCC, AFP, DCP, dose of diuretic drugs, albumin, total bilirubin, Child-Pugh score, ALBI score, FIB4-index, MELD score/MELD-Na score, creatinine, eGFR, serum sodium, cystatin C, NGAL or L-FABP between these groups (Table 3; Fig. 2A-C).

There was no significant difference in the efficacy between tolvaptan doses of 3.75 mg and 7.5 mg (Table 3). In contrast, the BUN, copeptin and ZAG levels were significantly higher in the Non-responders than in the Responders (respectively, p=0.0014, p=0.0265, p=0.0142) (Table 3; Fig. 2D-F). We further examined the population after excluding HCC patients, as HCC patients have a pro-inflammatory condition. BUN (p=0.015) levels were significantly higher in Non-responders (n=7) than in Responders (n=8) among patients without HCC. In contrast, the copeptin and ZAG levels were not higher in Non-responders without HCC.

Predictors contributing to the effect of tolvaptan in treatment for ascites

We calculated the cut-off values, area under the ROC curve, sensitivity and specificity of BUN, copeptin and ZAG using an ROC analysis. The cut-off values ascertained from our analyses of BUN, copeptin and ZAG were 18.3 mg/dL, 10.1 pmol/L and 32.4 μg/mL, respectively (Table 4). The contributions of HCC, BUN, copeptin and ZAG were evaluated using a multivariate logistic regression analysis. We found that serum BUN (odds ratio 7.43, p=0.0306), copeptin (odds ratio 9.12, p=0.0136) and ZAG (odds ratio 7.43, p=0.0306) levels were significant predictors contributing to the efficacy of tolvaptan for treating ascites; however, the presence of HCC was not selected as a predictor (Table 4).

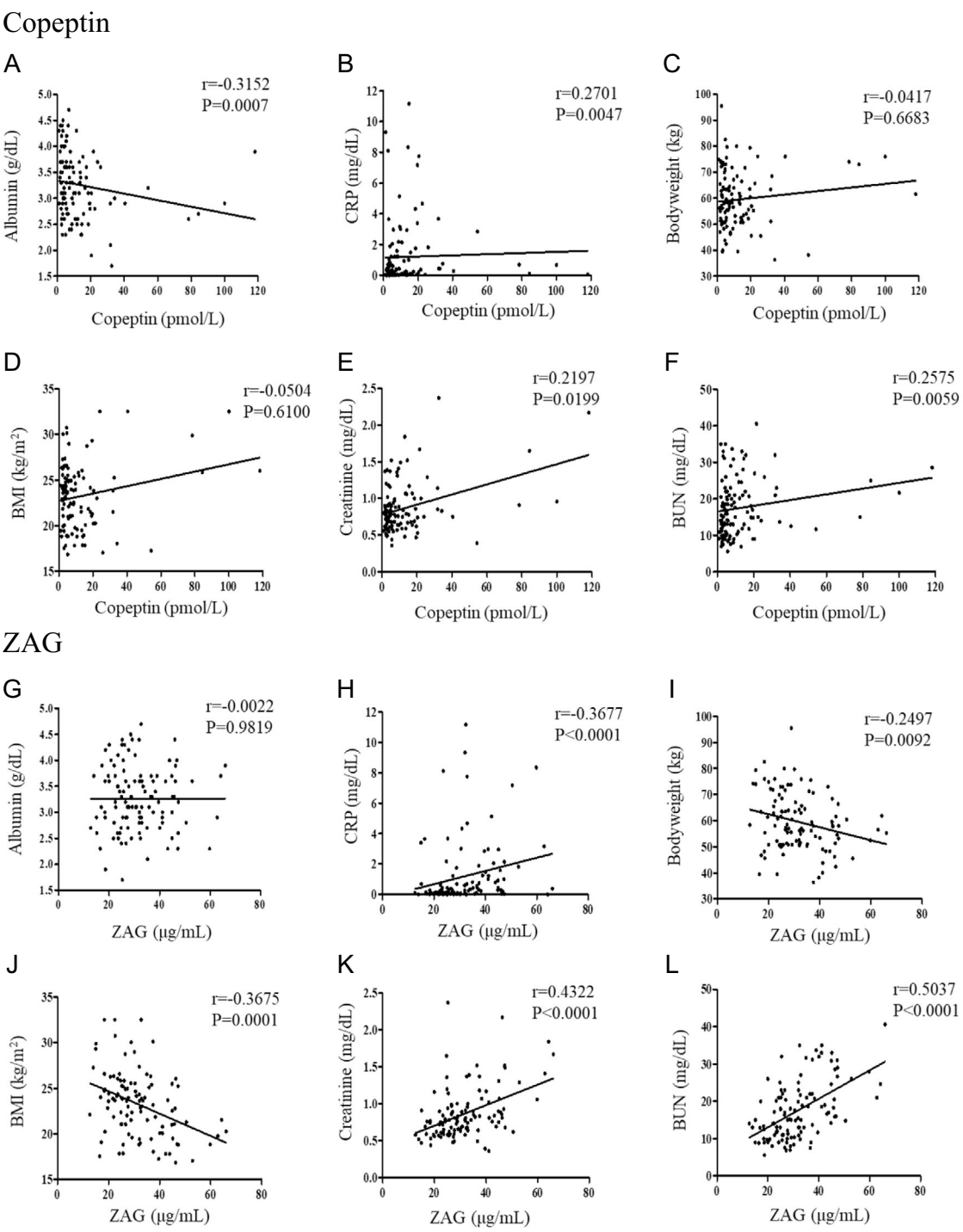

Figure 1. Correlation of copeptin and ZAG with the clinical parameters. Correlation between the serum copeptin levels and albumin (A), CRP (B), body weight (C), BMI (D), creatinine (E) or BUN (F). Correlation between the serum ZAG levels and albumin (G), CRP (H), body weight (I), BMI (J), creatinine (K) or BUN (L). ZAG: zinc-α2-glycoprotein, CRP: C-reactive protein, BMI: body mass index, BUN: blood urea nitrogen. Spearman's rank correlation coefficient.

Discussion

In this study, we revealed for the first time that serum copeptin and ZAG levels were significantly higher in the Non-responders receiving tolvaptan than in similar Responders. In addition, we found that serum BUN, copeptin and ZAG levels were independent predictors of the overall response to tolvaptan therapy. We further showed that the serum creatinine and cystatin C levels were not predictive of the patient response to tolvaptan, thus confirming the findings reported by others (12, 13).

In LC, arteriolar vasodilation causes underfilling of the systemic arterial vascular space, and the decrease in the ef-

Table 3. Characteristics of Patients Treated with Tolvaptan.

Parameters	Responders n=19	Non-responders n=19	p†
Age (years)	65.0 (60.5-74.0)	68.0 (58.0-73.5)	0.7589
Gender, Male : Female	13 : 6	11 : 8	0.5795
Bodyweight (kg)	61.2 (55.6-64.4)	57.0 (50.9-63.1)	0.4229
BMI (kg/m^2)	21.9 (19.3-24.8)	22.5 (19.2-24.7)	0.8315
SFA (cm^2)	104.30 (60.2-166.4)	118.6 (54.1-166.1)	0.8040
VFA (cm^2)	70.3 (43.1-89.0)	79.3 (54.0-114.4)	0.3280
PMI (cm^2/m^2)	5.7 (4.5-7.1)	5.3 (4.6-5.9)	0.5534
HCV/HBV/HCV+AL/AL/NASH/others	7/0/2/6/2/2	5/4/1/4/4/1	-
presence of HCC, yes : no	11 : 8	12 : 7	0.7862
Dose of diuretic drugs			
Tolvaptan (3.75 mg/7.5 mg)	15 / 4	17 / 2	0.6976
Furosemide (mg)	20 (5-35)	20 (20-40)	0.8217
Spironolactone (mg)	50 (25-50)	25 (25-50)	0.2548
Albumin (g/dL)	2.9 (2.6-3.1)	2.8 (2.7-3.2)	0.8490
Total Bilirubin (mg/dL)	1.8 (1.2-3.1)	1.4 (0.8-2.3)	0.1361
Child-Pugh score	8.0 (8.0-10.5)	8.0 (7.5-10.0)	0.3679
ALBI score	-1.4 (-1.8- -1.1)	-1.5 (-1.8- -1.3)	0.7371
FIB4-index	7.8 (6.3-10.1)	8.3 (5.0-10.4)	0.9534
MELD score	16.6 (15.0-19.5)	17.4 (15.8-20.0)	0.3655
MELD-Na score	20.3 (18.9-22.5)	21.3 (18.0-23.7)	0.9302
Creatinine (mg/dL)	0.8 (0.6-0.9)	0.9 (0.7-1.1)	0.2932
BUN (mg/dL)	13.0 (10.0-19.0)	23.0 (18.8-27.5)	**0.0014**
eGFR (mL/min/1.73m^2)	69.4 (50.2-81.8)	58.2 (45.4-76.6)	0.2093
GFR (MDRD-6) (mL/min)	82.8 (60.1-102.7)	60.2 (51.8-88.6)	0.0904
Serum sodium (mEq/L)	134 (132-137)	135 (132-139)	0.2909
CRP (mg/dL)	1.2 (0.4-2.7)	1.9 (0.4-3.8)	0.5018
Copeptin (pmol/L)	6.6 (4.0-14.0)	14.0 (9.0-27.2)	**0.0265**
ZAG (μg/mL)	27.4 (23.1-33.2)	37.4 (32.8-44.9)	**0.0142**
NGAL (ng/mL)	95.1 (59.5-162.0)	82.9 (61.2-140.6)	1.0000
Cystatin C (mg/dL)	1.1 (0.9-1.3)	1.1 (0.8-1.4)	0.9534
L-FABP (ng/mL)	8.5 (4.4-17.5)	11.5 (8.1-17.9)	0.2429
AFP (ng/mL)	9 (5-335)	8 (3-67)	0.4362
DCP (mAU/mL)	498 (39-8039)	2816 (157-9950)	0.5065

†Mann–Whitney *U* test, BMI: body mass index, SFA: subcutaneous fat area, VFA: visceral fat area, PMI: psoas muscle index, HCV: hepatitis C virus, HBV: hepatitis B virus, AL: alcohol, NASH: nonalcoholic steatohepatitis, HCC: hepatocellular carcinoma, ALBI: albumin-bilirubin, FIB4-index: fibrosis-4, MELD: model for end-stage liver disease, BUN: blood urea nitrogen, eGFR: estimated glomerular filtration rate, MDRD-6: the 6-variable modification of diet in renal disease, CRP: C-reactive protein, ZAG: zinc-α2-glycoprotein, NGAL: neutrophil gelatinase-associated lipocalin, L-FABP: liver-type fatty acid–binding protein, AFP: α-fetoprotein, DCP: des-γ-carboxy prothrombin. Responders were defined as decompensated liver cirrhosis patients in whom tolvaptan was an effective treatment for ascites. All data are expressed as median (first quartile-third quartile).

fective blood volume leads to a decrease in arterial pressure (20). Consequently, activation of the renin-angiotensin-aldosterone system and sympathetic nervous system and the release of antidiuretic hormone take place as the body attempts to restore normal blood pressure homeostasis (21). The main physiological function of AVP is antidiuresis, thereby regulating the systemic osmotic pressure. Plasma AVP levels are normally regulated by plasma osmotic pressure and have been found to be elevated in LC patients with ascites, as reported by Pérez-Ayuso (22). However, no study has confirmed whether the effect of tolvaptan is related to plasma AVP or pro-AVP (copeptin) levels in ascites patients. The Non-responder group was considered to be in a state of relative vascular underfilling with an increase in BUN and copeptin, thus suggesting intravascular dehydration (12). Furthermore, serum copeptin was correlated with multiple factors, including the renal function and CRP level.

Although NGAL and L-FABP are more useful markers than copeptin for acute-on-chronic liver failure (23) and acute kidney injury (24), respectively, this study indicated that serum NGAL and L-FABP levels were not useful for assessing the efficacy of the response to tolvaptan in LC patients with ascites. Serum ZAG levels, it has been determined, are increased in acute kidney injury (25) and minor

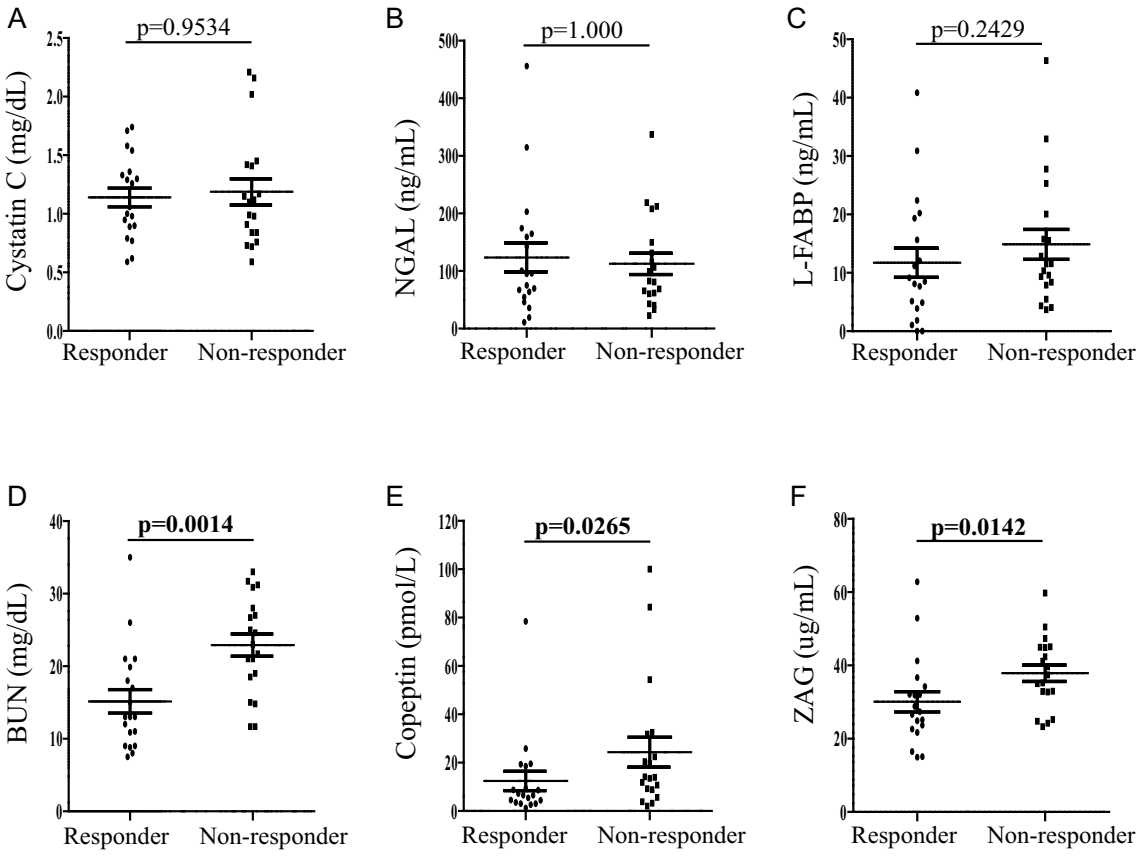

Figure 2. **Predictors contributing to the effect of tolvaptan for treating ascites. Cystatin C (A), NGAL (B), L-FABL (C), BUN (D), copeptin (E), and ZAG (F) levels among Responders and Non-responders to tolvaptan. BUN: blood urea nitrogen, ZAG: zinc-α2-glycoprotein, NGAL: neutrophil gelatinase-associated lipocalin, L-FABP: liver-type fatty acid-binding protein. Mann–Whitney *U* test.**

Table 4. **Predictors Contributing to the Effect of Tolvaptan in the Treatment for Ascites.**

Predictors	cutoff	ROCAUC	Sensitivity	Specificity	Univariate analysis			Multivariate analysis		
					OR	95%CI	p§	OR	95%CI	p§
BUN	18.3	0.8047	79	74	10.50	2.54 → 53.39	0.0009	7.43	1.20 → 64.69	**0.0306**
Copeptin	10.1	0.7119	68	74	6.07	1.57 → 27.02	0.0083	9.12	1.54 → 91.14	**0.0136**
ZAG	32.4	0.7341	79	74	10.50	2.54 → 53.39	0.0009	7.43	1.20 → 64.69	**0.0306**
HCC	-	-	-	-	1.24	0.34 → 4.69	0.7399	-	-	**-**

ROCAUC: receiver operating characteristic area under the curve

p§: Logistic regression analysis, OR: odds ratio, 95%CI: 95% confidence interval, BUN: blood urea nitrogen, ZAG: zinc-α2-glycoprotein, HCC: hepatocellular carcinoma

kidney injury caused by normo-albuminuric diabetic kidney disease (e.g., patients presenting with renal insufficiency, but no significant proteinuria) (26). Serum cystatin C accurately reflects the renal function, while ZAG reflects lipolysis and the renal function. Cachexia is a multifactorial syndrome characterized by systemic inflammation, weight loss and loss of SFA (27), and serum ZAG has been expected to be useful as a biomarker for cachexia (28). We confirmed the negative correlation between serum ZAG levels and SFA in this study.

Several reports have shown that body weight loss and elevated CRP levels were predictors of Non-responders to tolvaptan (13), and serum soluble CD14 and CRP levels have been shown to be useful predictors of a response to tolvaptan (29). In the current study, Responders with high BUN (≥18.3 mg/dL) accounted for 26% of the cohort and were associated with lower levels of serum CRP, copeptin and ZAG than Responders with low BUN. In contrast, Non-responders with low BUN (<18.3 mg/dL) accounted for 21% of the cohort and were associated with higher levels of serum CRP, copeptin and ZAG than Non-responders with high BUN. These results suggested that serum copeptin and ZAG levels were associated with serum CRP levels. Therefore, copeptin and ZAG may be useful biomarkers, even in patients with inflammation.

Based on the evidence outlined above, serum copeptin

levels may indicate the underlying pathological condition of a decreased osmotic pressure, intravascular dehydration and inflammation, whereas serum ZAG levels may reflect minor kidney injury, cachexia and inflammation, suggesting that copeptin and ZAG are independent predictors of the patient response to tolvaptan.

Although many patients with LC are complicated by HCC, whether or not coexistent HCC is linked to the response to tolvaptan therapy is unclear. A recent large-scale post-marketing surveillance study (n=1,111) reported that there was no difference in the mean weight reduction induced by tolvaptan treatment between patients with and without HCC (p=0.2248) (30). Both the American and European clinical practice guidelines recommended high-dose diuretic treatment against massive ascites (16). In contrast, the Japanese guideline recommends additional tolvaptan administration for massive ascites. Clinical trials involving a large number of patients are thus needed to determine the timing of tolvaptan initiation.

Several limitations associated with the present study warrant mention. This was a retrospective and single-center study with a small sample size and a short follow-up period. The confidence intervals of the predictors for tolvaptan treatment of ascites were thus huge due to the small sample size. A further study using a larger cohort, particularly one with many cirrhotic patients without HCC, will be required to investigate the interaction of copeptin, ZAG and BUN and the possible predictors of the patient survival.

In conclusion, serum BUN <18.3 mg/dL, copeptin <10.1 pmol/L and ZAG <32.4 μg/mL appear to be good predictors of an overall patient response to tolvaptan treatment.

Conclusion

Serum copeptin, ZAG and BUN levels may be used as novel biomarkers to determine the overall response to tolvaptan in patients presenting with LC and ascites. Future studies will be required to develop a new clinical prediction model using serum copeptin, ZAG and BUN that may help clinicians determine the effectiveness of tolvaptan treatment.

The study protocol was approved by the Clinical Research Ethics Review Committee of Mie University Hospital. This research complies with the guidelines for human studies and includes evidence that the research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The subjects (or their parents or legal guardians) have provided their written informed consent.

Author's disclosure of potential Conflicts of Interest (COI).
Motoh Iwasa: Honoraria, Otsuka Pharmaceutical.

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