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E. GRZEBYK[1], M. PAZGAN-SIMON[2], J. JAGAS[3], J. ZUWALA-JAGIELLO[1], J. GORKA-DYNYSIEWICZ[1]

LEFT VENTRICULAR FUNCTION IS RELATED WITH AMPHIREGULIN AND FIBROSIS MARKERS IN CIRRHOTIC CARDIOMYOPATHY

[1]Wroclaw Medical University, Department of Pharmaceutical Biochemistry, Wroclaw, Poland;
[2]Medical University, Clinic of Infectious Diseases, Liver Diseases and Acquired Immune Deficiency, Wroclaw, Poland;
[3]Cardiology Department County Hospital, Wroclaw, Poland

The relationship between left ventricle (LV), extracellular matrix remodeling and fibrosis-linked amphiregulin (ARG) in cirrhotic cardiomyopathy (CCM) is unknown. The aim of the study was to investigate the associations between markers of extracellular matrix remodeling and ARG in cirrhosis and their association with indicators of ventricular remodeling and LV functional parameters. In hepatitis C virus (HCV) patients with cirrhosis, who underwent echocardiography, the presence of left ventricular diastolic dysfunction (LVDD) was determined by having gradable diastolic dysfunction in accordance with modified 2020 Cirrhotic Cardiomyopathy Consortium criteria. A total of 87 cirrhotic patients were consecutively analyzed. Based on detailed echocardiographic assessment - 35 HCV patients with cirrhosis had normal left ventricular diastolic function (non-CCM group), whereas 52 patients had LVDD (CCM group). ARG was measured by enzyme-linked immunosorbent assay. The ARG levels were significantly increased in the CCM group compared to the non-CCM group (P<0.001). ARG levels in all HCV patients were independently associated to the presence of CCM, and showed significant correlations with LVDD. The close relationship between ARG levels and the direct serum marker of fibrosis, and selected markers of extracellular matrix (*i.e.* transforming growth factor-beta1 (TGF-β_1), and carboxyterminal propeptide of type I collagen (PICP), amino-terminal propeptide of type III procollagen (PIIINP), tissue inhibitor of matrix proteinase-1 (TIMP-1), respectively), ventricular remodeling (*i.e.* N-terminal pro B-type natriuretic peptide (NT-proBNP), high-sensitivity cardiac troponin-T (hs-TnT)), and LV functional parameters suggest an active role in the myocardial injury. Using ROC analysis, the best marker for the diagnosis of CCM was NT-proBNP with AUROC = 0.796. The area under the curve of ARG (AUROC = 0.709) for predicting CCM was greater than this for PICP (AUROC = 0.662) and similar to this hs-TnT (AUROC = 0.753). The simultaneous monitoring of serum ARG and markers of extracellular matrix and ventricular remodeling can be helpful for the alterations in myocardial function control in HCV patients with cirrhosis.

Key words: *cirrhotic cardiomyopathy, left ventricle, fibrosis, amphiregulin, hepatitis C virus, transforming growth factor beta 1, tissue inhibitor of matrix proteinase-1, proinflammatory cytokines*

INTRODUCTION

Hepatitis C virus (HCV) persistently infects the majority of patients exposed to it and can cause irreversible fibrosis, leading to the onerous clinical sequelae of cirrhosis. Most authors favor the opinion that HCV infection is frequently associated with myocarditis and cardiomyopathy (1). Alterations of the structure and composition of the myocardium appear to play a central role in the pathogenesis of cirrhotic cardiomyopathy (CCM) (2). The presence of left ventricle (LV) hypertrophy and diastolic dysfunction in HCV patients during the pre-cirrhotic stage suggests a possible role of HCV in this structural abnormality of the heart (3).

All circulating molecules proposed as markers of myocardial fibrosis in humans including the collagen-related peptides, matrix metalloproteinases, tissue inhibitors of metalloproteinases, selected circulating micro-RNAs and a wide array of non-collagen related peptides either have evidence that weighs against a clinically relevant correlation between serum levels and collagen volume fraction or the presence or absence of such a relationship is unknown (4). Among these many candidate molecules, amphiregulin (ARG), a member of the epidermal growth factor (EGF) family, potentially provides information reflecting the integrated effects of fibrosis, injury, and inflammation (5). In this regard, findings from several experimental studies suggest that ARG may participate in organ fibrosis diseases, including those of the lung, kidney, and liver (6-8). Additionally, ARG, an autocrine growth factor secreted by a variety of cells, including endothelial cells, macrophages, and fibroblasts, has an important role in cardiac fibrogenesis in experimental models of heart failure (9, 10). In a physiological state, activation of EGF receptor (EGFR) in the heart induces major intracellular signaling cascades governing cardiac fibroblasts proliferation, migration, and collagen and extracellular matrix synthesis. However, prolonged activation of EGFR in a chronic stress

state characterized by continuous elevation of ARG can enhance cardiac hypertrophy (10). ARG also plays a role in mediating proinflammatory effects alongside cardiac fibrosis. Given preclinical evidence, it remains plausible that ARG is expressed by a number of activated cells including eosinophils, basophils, CD4+ T cells, and CD8+ T cells under different inflammatory conditions (11). Several studies have identified the factors and signaling pathways that mediate ARG secretion from various immune cells as well as downstream signaling associated with ARG/EGFR interaction (6, 11). According to Zhu *et al*. (12) the cardiac TIR-domain-containing adapter-inducing interferon-β (TRIF) triggers the activation of EGFR signaling by nuclear factor kappaB (NF-κB) transcriptional regulation and downstream EGFR ligands ARG and epiregulin. In this context, the TRIF/EGFR axis partially explained the molecular mechanism of ARG-induced cardiac inflammation, fibrosis, hypertrophy and dysfunction in the heart. Collectively, ARG combined with other signal molecules to modulate the cellular cell survival, apoptosis and proliferation which eventually implicated cardiac fibrosis. Thus, ARG offer information above and beyond that which we can derive by current routine methods for assessment of cardiac fibrosis.

The goal of this study was to test the hypothesis that left ventricular diastolic dysfunction is related to myocardial fibrosis due to alterations in circulating amphiregulin level in cirrhotic cardiomyopathy. In addition, we evaluated whether proinflammatory cytokine are associated with this process.

PATIENTS AND METHODS

Patients

A total of 87 HCV patients diagnosed with cirrhosis were consecutively recruited from the Department of Clinic of Infectious Diseases, Liver Diseases and Acquired Immune Deficiency, and Cardiology Department County Hospital between April 2017 and October 2019. The HCV patients with cirrhosis included in this study were aged from 49 to 69 years. For this study, we selected patients fulfilling following inclusion criteria: 1) confirmed diagnosis of chronic HCV infection and cirrhosis; 2) echocardiography-proven left ventricular diastolic dysfunction (LVDD); 3) absence of co-existing diseases like an HIV infection, chronic kidney disease, hypertension or diabetes mellitus, cardiac diseases (heart failure, coronary artery disease, and atrial fibrillation), acute myocardial infarction, and ischemic stroke, and presence of hepatocellular carcinoma at the time of serum collection; 4) no evidence of metabolic, toxic or autoimmune liver disease and at least 1 year of alcohol abstinence; 5) adequate frozen serum sample available at inclusion. The patients with cirrhosis provided a complete medical history on the day of admission, from which clinical information, such as sex, age, presence of ascites, esophageal varices, and encephalopathy was obtained. All the patients underwent rigorous analysis by a comprehensive physical and clinical examination, including electrocardiography and echocardiography. The diagnosis of chronic HCV infection was based on persistently increased alanine aminotransferase values, anti-HCV and HCV-RNA positivity and liver histology features. The HCV inflammation was confirmed by measurement of HCV-Ab and HCV-RNA in the serum, using the EIA methods and RT PCR - Cobas Amplicor Roche methods, respectively. Diagnosis of cirrhosis was established according histological criteria when liver biopsy was performed, or by the combination of clinical, biochemical and ultrasound imaging data (presence of irregular margins on ultrasound, portal hypertension with laboratory evidence of chronic liver disease) consistent with such a diagnosis. Patients were grouped according to Child-Pugh classification. Three biochemical variables (serum albumin, bilirubin, and prothrombin time (international normalized ratio, INR)) in addition to the presence or absence of ascites determine the Child-Pugh score. At the time of the study no Child-Pugh A patients showed clinical features of decompensated liver cirrhosis (ascites or hepatic encephalopathy). At enrollment, esophageal varices were detected by endoscopy in 31% of patients, ascites and hepatic encephalopathy grade I were present by physical examination in 44 (53.8%) and 14 (17.1%) patients, respectively. Presence of ascites was assessed by ultrasonography. The model for end-stage liver disease (MELD) score was also calculated; it is the most commonly used alternative prognostic indicator for cirrhotic patients to the Child-Pugh score (13).

All patients gave written consent for blood sampling at the baseline visit; Bioethics Committee of the Wroclaw Medical University approval for the protocol was obtained. The studies were conducted in compliance with the ethical standards formulated in the Helsinki Declaration of 1975 (revised in 1983).

Echocardiography and cirrhotic cardiomyopathy criteria

Measurements were taken off-line from digitally recorded images by dedicated measurement software and consisted of the average of 5, usually consecutive, cardiac cycles. The operator that performed the examination obtained measurements. During off-line evaluation, the observer was aware of the identity of the patient, but numerical data were not visible on the screen at the time measurements were made. Patients were subject to repeated echocardiographic examination by 2 operators. Each patients was echocardiographically examined 12 times, once by each operator during mornings and afternoons of 3 nonconsecutive days. Measurement variation was defined by coefficients of variation. Of 50 within-day, between-day, and interoperator coefficients of variation, 35 were less than 10% and 44 were less than 15%. The following echocardiography parameters were measured: the early diastolic mitral annular velocity (E/e'), left atrial volume index (LAVI), septal early diastolic mitral annular velocity (e'), tricuspid regurgitation (TR) maximum velocity, and early to late diastolic transmitral flow velocity (E/A) ratio. In accordance with the recent Cirrhotic Cardiomyopathy Consortium criteria (14), left ventricular diastolic dysfunction (LVDD) grade was defined by 3 of the following 4 criteria were met: E/e'≥15, LAVI>34 mL/m^2, e'<7 cm/second, or TR maximum velocity >2.8 m/second in the absence of pulmonary hypertension and the presence of measurable E/A ratio (E/A 0.8–2 = grade II, E/A>2 = grade III). The presence of LVDD was determined by having gradable diastolic dysfunction. The subjects with evidence of LVDD and no obvious occult coronary artery disease were identified as cirrhotic cardiomyopathy.

Clinical and laboratory assessment

Peripheral venous blood from fasted HCV patients was drawn at the same day of liver biopsy procedures. The blood was allowed to clot for 30 min at 25°C and centrifuged at 2000×g for 15 min at room temperature, and the serum was then separated and aliquoted into tubes for storage. The tubes were stored frozen at –80°C until they were used to study different parameters. The concentrations of alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin, serum hs-CRP, hemoglobin, albumin, platelets, leukocytes, and creatinine were measured by standard clinical methods. The Model for End stage Liver Disease (MELD) score was calculated using

bilirubin, creatinine, and international normalized ratio (INR) (15). ARG levels were determined, using an ELISA method (Nori Human ELISA Kit Genorise Scientific, INC; USA), by following the instructions in the manual, provided by the manufacturer. Control samples and serum standards with concentrations that ranged from 0.031–2.0 ng/mL were included in each run. The minimum detectable dose of ARG was typically less than 6 pg/mL. The inter-assay and intra-assay coefficients of variation were less than 9.0% and 6.0%, respectively. The concentrations of fibrosis controlling markers and ventricular remodeling markers were determined in plasma using a commercially available ELISA tests as follows: amino-terminal propeptide of type III procollagen (PIIINP 2.69–63.56 ng/mL), carboxyl-terminal peptide of type I procollagen (PICP 0.78–50 ng/ml), high-sensitivity cardiac troponin-T (hs-TnT 125–8000 pg/ml), N-terminal pro B-type natriuretic peptide (NT-proBNP 0.78–50 ng/ml; all from Nori Human ELISA KIT Genorise Scientific, Inc., Berwyn, PA, USA); transforming growth factor β_1 (TGF-β_1 4.639–14.757 ng/mL) (Diaclone SAS, Besancon Cedex, France) and tissue inhibitor of matrix proteinase-1 (TIMP-1) (Diaclone, Human ELISA KIT and R&D Systems Inc., Minneapolis, MN, USA). Serum levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were assayed with ELISA kits (Diaclone; Human ELISA KIT and R&D Systems Inc., Minneapolis, MN, USA). All measurements were performed by technicians blinded to the sample status. Intra-assay and inter-assay coefficients of variation were <14% in two replicate measurements. CK by coupled-enzyme assay with a normal range of 38–174 U/L, CK-MB by immune inhibition assay with a normal range of 0–24 U/L. The laboratory analytical coefficient of variation is 2.2%.

Size calculation and power of study

A our pilot study was first carried out using 20 subjects per group. Based on these preliminary results, with a confidence interval of 95%, an estimated P value <0.05, and a power of 80%, the present sample size was derived. 5 subjects were added in each group to account for the high level of variation seen in the levels of serum markers.

Statistical analysis

Continuous variables are expressed as median (interquartile range; IQR) or mean ± standard deviation, and categorical variables as number (percentage). Frequency data were compared using the χ^2 test or the Fischer's exact test when necessary. Because many of the variables analyzed did not have a normal distribution as determined by the Kolmogorov-Smirnov test, nonparametric Mann-Whitney U-test was used for comparison of data. Regression analysis to determine significant correlations among different parameters was performed using the simple linear regression or Spearman correlation coefficient. Multivariate analysis by conditional logistical regression with a forward stepwise method was performed to find independent variables associated with the presence of CCM. To compare the diagnostic efficiency of different parameters, receiver operating characteristic curve (ROC) and area under the curve (AUROC) were constructed, and the diagnostic sensitivity, specificity, and accuracy of each variable were calculated. The Youden index was used to detect the best cutoff value. The empirical non-parametric method according to DeLong (16) was used to compare the ROC of full parameters. All *P* values were two-tailed, and *P*-values <0.05 were considered significant. Data processing and analysis were performed using Statistica, version 13.3 software.

RESULTS

Baseline characteristics of the study population

Echocardiographic and biochemical data of HCV patients with cirrhosis classified according to the presence or not of CCM are shown in *Table 1*. A total of 87 HCV patients with liver cirrhosis were consecutively analyzed. Based on detailed echocardiographic assessment - 35 HCV patients with cirrhosis had normal left ventricular diastolic function (non-CCM group), whereas 52 patients had LVDD (CCM group) (*Table 1*). By definition (14), HCV patients with LVDD had higher E/e' ratio (*P*<0.0001), TR maximum velocity (*P*<0.001), E/A ratio (P<0.01) and larger LAVI (*P*<0.001). No statistically significant difference was found between these groups of HCV patients regarding their LVEF % (a marker of left ventricular systolic function) (*Table 1*). The prevalence of CCM particularly tended to increase with significantly higher MELD score (*P*<0.001). One further factor tended to be related to CCM: presence of ascites (61%) (*Table 1*). No differences between CCM group and non-CCM group were noted regarding the presence of varices and encephalopathy. Albumin, total bilirubin and hemoglobin levels, platelet counts, as well as the Child-Pugh score were similar among these groups. On the other hand, CCM was associated with higher median values of white blood cells (WBC) counts. (*P*<0.01). Only one further factor tended to be related to non-CCM: high median values of AST activity (*P*<0.01) (*Table 1*).

Amphiregulin in the degrees of left ventricular diastolic dysfunction in hepatitis C virus patients with cirrhosis

In cirrhotic patients, who underwent echocardiography, the LVDD grade was grade I for 22 cases (42.3%), grade II for 19 cases (36.5%), and grade III for 11 cases (21.2%) (*Table 1*). ARG significantly correlated with the LVDD grade (rho=0.32, *P*<0.01), and the ARG levels were significantly higher among cirrhotic patients with a severe degree of LVDD (grade II–III) compared to those with grade 0–I of LVDD (*P*<0.001) (*Fig. 1*).

Comparison of markers of extracellular matrix, ventricular remodeling, inflammation, and amphiregulin between HCV patients with and without cirrhotic cardiomyopathy

Serum concentrations of ARG were statistically significantly higher in all HCV patients with cirrhosis when compared with a fully age- and gender-matched control group of 35 healthy subjects (median 0.135 ng/mL; IQR, 0.0–0.214 ng/mL, *P*<0.001). ARG levels were significantly lower in the non-CCM group (n=35) (median 0.281 ng/mL; IQR, 0.239–0.370 ng/mL) than in the CCM group (n=52) (median 0.875 ng/mL (IQR, 0.439–1.96 ng/mL) (*Table 2*). CCM was associated with significantly higher levels of selected markers of ventricular remodeling (hs-TnT, NT-proBNP; *P*<0.001), *P*<0.0001, respectively), inflammatory markers (TNF-α, sCD163; *P*<0.001, *P*<0.01), respectively), PICP (*P*<0.001), and higher PICP/PIIINP ratio (*P*<0.01) as compared to the patients with cirrhosis only (*Table 2*). The serum IL-6, IL-1β, TGF-β_1, PIIINP, and TIMP-1 levels, and activity of enzymes originated from myocardial tissue (*e.g.* CK-MB) in serum were similar between groups (*Table 2*). There was a small overlap between the levels of proinflammatory markers and values of PICP/PIIINP ratio in the patients with CCM and that of these indicators in the group of HCV patients with cirrhosis only. Although serum TIMP-1 levels were lower in the patients with CCM than in the cirrhosis only, this indicator was not a good discriminator because of the considerable overlap among groups. Meanwhile, there was no overlap between the

Table 1. Baseline characteristics of HCV patients with cirrhosis according to the presence cirrhotic cardiomyopathy (CCM).

	All patients	Non-CCM group	CCM group	*P* value
(n)	82	35	52	
Male:Female ratio (n)	38:45	14:17	24:28	0.75
Age (years)	62 (49–69)	58 (49–66)	64 (50–69)	0.56
Child-Pugh score	7.4±3.2	6.9±2.3	7.8±1.9	0.28
Child-Pugh grade; A/B/C (n)	18/33/32	3/18/10	15/15/22	0.1327
Model for end stage liver disease (MELD) score	10.9±8.1	10.2±7.3	12.9±6.1	<0.001
Ascites n (%)	44 (53.7)	12 (39)	32 (61)	<0.01
Varices n (%)	27 (32.9)	10 (28)	17 (33)	0.72
Encephalopathy n (%)	14 (17.1)	4 (12)	10 (19)	0.85
Biochemical parameters				
Albumin (g/L)	32 (24–39)	32 (25–39)	29 (24–34)	0.73
Bilirubin (mg/dL)	1.8 (1.3–3.1)	1.7 (1.3–3.1)	2.2 (1.7–3.1)	0.68
Aspartate transaminase (IU/L)	70 (32–163)	93 (47–163)	52(32–83)	<0.01
Alanine transaminase (IU/L)	55 (30–100)	65 (47–100)	51 (30.5–92.5)	0.14
Platelet count ($\times 10^9$/L)	200 (95–250)	208 (159–250)	187 (95–235)	0.41
International normalized ratio (INR) (0.8–1.1)	1.6 (0.8–2.9)	1.2 (0.8–1.3)	2.2 (1.7–2.9)	0.19
White blood cells ($\times 10^9$/L)	10.1 (2.9–14.5)	6.8 (3.0–10.5)	11.4 (2.9–14.5)	<0.01
Hemoglobin (mmol/L)	7.4±1.5	7.6±1.25	7.3±1.46	0.18
Creatinine (mg/dL)	1.15 (0.8–2.4)	1.02 (0.8–1.6)	1.5 (0.8–2.4)	0.12
Echocardiographic data				
Left ventricular ejection fraction (%)	56±9.8	58.0±6.3	60.6±7.14	0.35
E/e' ratio	9.5±0.95	6.9±0.57	12.8±0.75	<0.0001
Septal e' (cm/s)	7.3±1.85	8.86±1.41	6.14±1.43	<0.01
Tricuspid regurgitation maximum velocity (TR) (m/s)	2.5±0.31	1.99±0.14	2.98±0.14	<0.001
Left atrial volume index (LAVI) (mL/m^2)	25.9±2.95	23.6±3.88	29.4±4.46	<0.001
E/A ratio	1.2±0.52	0.66±0.22	1.73±0.26	<0.01
Left ventricular diastolic dysfunction:				
Grade I n (%)	22 (26.8)	–	22 (42.3%)	–
Grade II n (%)	19 (23.2)	–	19 (36.5%)	–
Grade III n (%)	11 (13.4)	–	11 (21.2%)	–

Continuous variables are expressed as median (interquartile range; IQR) and categorical variables as number (percentage).
e', septal early diastolic mitral annular velocity; E/A, early to late diastolic transmitral flow velocity; E/e', early diastolic transmitral flow to early diastolic mitral annular velocity.

levels of ARG and PICP in the CCM group and that of ARG and PICP in the group of cirrhotic patients without CCM.

Associations between the selected markers of fibrosis, extracellular matrix, ventricular remodeling, and amphiregulin with left ventricle functional parameters in HCV patients with cirrhosis

In cirrhotic patients with LVDD, the ARG was found to have a positive correlation with E/A ratio (P<0.01). Significant negative correlations were identified between ARG and E/e' ratio, ventricular septal e', and LAVI (P<0.01, P<0.01, and P<0.001, respectively) (*Table 3*). Furthermore, E/A, which defines the severity of diastolic dysfunction, was significantly correlated with NT-proBNP (rho=0.72, P<0.001), hs-TnT (rho=0.64, P<0.01), MELD score (rho=0.50, P<0.001), TNF-α (rho=0.36, P=0.024), PICP (rho=0.31, P=0.05), and was inversely correlated with albumin (rho= –0.35, P=0.03). There were a significant correlation between ARG levels and NT-proBNP (P<0.001) and hs-TnT levels (P<0.001) (*Table 3*; *Fig. 2A* and *2B*, respectively). Additionally, serum ARG levels were significantly correlated with TGF-β_1 (P<0.01), PICP (P<0.001)

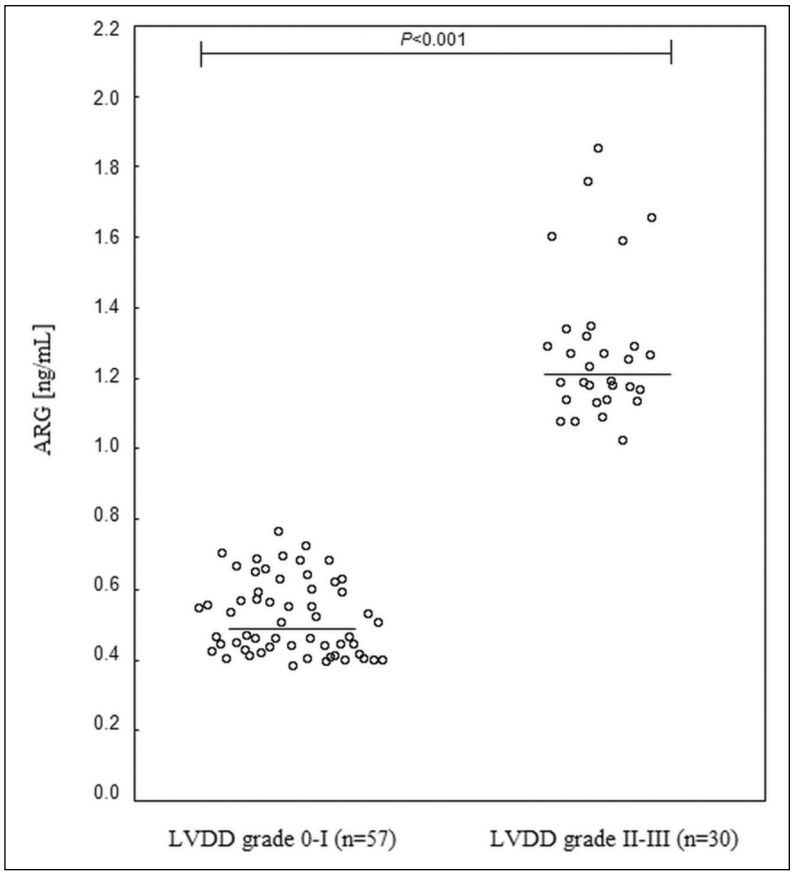

Fig. 1. Serum levels of amphiregulin in HCV patients with cirrhosis according to the presence of left ventricular diastolic dysfunction (LVDD). Horizontal bars represent medians of the concentrations.

(*Table 3*; *Fig. 2C* and *2D*, respectively) and PICP/PIIINP ratio (*Table 3*; *P*=0.045). In the study group, no significant correlations were also observed between ARG level and biochemical markers of liver injury (not reported in detail). These results suggest that ARG has a strong association with cardiac (diastolic) dysfunction and support the hypothesis that ARG enhances cardiac fibrosis.

Multiple logistic regression analysis of factors associated with the presence of cirrhotic cardiomyopathy

Based on stepwise multiple logistic regression analysis of factors (MELD score, albumin, IL-6, PICP, PICP/PIIINP ratio, TGF-β_1, hs-TnT, NT-pro-BNP, and LV functional parameters including the E/e'ratio, LAVI, and E/A ratio), serum ARG level (odds ratio (OR)=1.01, 95% confidence interval (CI)=0.76–1.34, $P < 0.001$), PICP level (OR=1.14, 95% CI=1.03–1.24, *P*<0.01), hs-TnT level (OR=1.11, 95% CI=0.94–1.13, *P*<0.01), and NT-pro-BNP level (OR=1.64, 95% CI=1.24–1.98, *P*<0.001) were found to be independent predictors of CCM (*Table 4*).

Comparison of amphiregulin with other indicators for the diagnosis of cirrhotic cardiomyopathy by areas under the receiver operating curves (AUROC)

The ROC curves for ARG, PICP, hs-TnT, and NT-proBNP values for predicting CCM are shown in *Fig. 3*. The optimal cut-off point of ARG for predicting CCM was 540 ng/mL, and its sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 75.0%, 58.0%, 53.3%, and 78.4%, respectively (*Table 5*). For estimating CCM, the AUROC for ARG level (AUROC=0.709) was greater to those for the PICP (AUROC=0.662, *P*>0.05). Compared to ARG, both NT-proBNP as well as hs-TnT levels were significantly better for the diagnosis CCM (AUROC=0.796, *P*<0.01; AUROC=0.753, *P*=0.02; respectively) (*Table 5*).

DISCUSSION

Cirrhotic cardiomyopathy is a disease characterized by myocardial dysfunction in response to physical stress, and the basic pathophysiological mechanism of CCM is ventricular remodeling, especially excessive myocardial fibrosis and the accumulation of extracellular matrix. Myocardial injury in the setting of CCM may be a cause, but may also predispose patients to subsequent progression of cardiac disease (14). The search for accurate diagnostic noninvasive cardiac markers is ongoing in the myocardial injury literature, and many of these may induce alteration in left ventricle, a critical component in the pathogenesis of CCM (2, 14). Our new finding is that this myocardial fibrosis is reflected to some extent by elevated circulating ARG, which also have diagnostic value.

There are no any reports that have studied the clinical correlates of ARG levels in patients with heart failure. It is well known that the diversity in the patients may reduce the reliability of the result. Therefore, cirrhotic patients were appropriately screened by survey and laboratory testing to exclude those with known or undiagnosed liver and cardiac conditions. However, CCM patients are with relatively low diversity in the clinical manifestation. The prevalence of CCM particularly tended to increase with significantly higher MELD score and levels of white blood cells (WBC), the only cellular marker of systemic inflammation. Several cohort studies suggest that the WBC counts

Table 2. Comparison between HCV patients classified according to the presence of cirrhotic cardiomyopathy (CCM).

	Non-CCM group	CCM group	*P* value
(n)	35	52	
Biomarkers of fibrosis			
Amphiregulin (ng/mL)	0.281 (0.239–0.370)	0.875 (0.439–1.96)	<0.001
Transforming growth factor-β_1 (ng/mL)	2.77 (1.87–3.67)	6.0 (4.12–31.5)	0.45
Procollagen type I carboxy-terminal propeptide soluble (PICP) (ng/mL)	6.2 (2.2–22.1)	11.8 (4.6–27.6)	<0.001
Amino-terminal propeptide of type III procollagen (PIIINP) (ng/mL)	0.331 (0.232–0.386)	0.209 (0.109–0.266)	0.35
PICP/PIIINP ratio	17.4 (13.5–23.8)	30.8 (25.9–42.5)	<0.01
Tissue inhibitor of matrix proteinase-1 (TIMP-1) (ng/mL)	214.6 (163.3–245.5)	163.8 (115.9–209.5)	0.43
Biomarkers of cardiac dysfunction			
Creatine kinase (CK) (IU/L)	63.2 (24.8–99.7)	65.1 (37.9–92.3)	0.32
CK-MB (IU/L)	1.28 (0.7–1.92)	1.69 (0.9–4.66)	0.44
N-terminal pro B-type natriuretic peptide (NT proBNP) (ng/mL)	31.5 (18.5–35.8)	66.8 (29.3–88.4)	<0.0001
High-sensitivity cardiac troponin-T (hs-TnT) (ng/mL)	2.06 (0.95–3.54)	6.3 (1.6–8.0)	<0.001
Biomarkers of inflammation			
Tumor necrosis factor-alpha (TNF-α) (pg/mL)	19.8 (16.7–36.5)	38.8 (34.5–49.4)	<0.001
Interleukin-1β (pg/mL)	16.4 (15.3–18.7)	17.2 (14.4–24.0)	0.35
Interleukin-6 (pg/mL)	7.8 (5.11–8.4)	9.9 (6.6–12.9)	0.48
High-sensitivity C-reactive protein (hs-CRP) (mg/L)	3.8 (3.1–7.4))	6.2 (4.9–10.5)	0.35
sCD163 (ng/mL)	1.37 (0.51–2.42)	1.77 (1.05–5.45)	<0.01

Variables are expressed as median (interquartile range; IQR).

as a potential predictor of cardiovascular diseases mortality (17). These studies have not definitively assessed the relationship between subnormal WBC counts and healthy states, therefore leaving the relationship unclear. And it is also known that an acute decompensation is often caused by precipitants which induce a cascade of pathomechanistic processes, but in approximately 40–50% of patients with the acute decompensation have systemic inflammation without any identifiable precipitating triggers (18). The mechanism of systemic inflammation suggests that metabolites produced by the gut microbiome may affect the systemic compartment and trigger systemic inflammation (19). This may, at least in part, serve as an explanation for the higher values of WBC counts in our CCM patients. Another result of the present study is that levels of ARG were negatively correlated with albumin levels, but not correlated with low hemoglobin levels in CCM patients. Patients with decompensated cirrhosis almost always have hypoalbuminemia, which may also be associated with malnutrition in these subject (20). Hypoalbuminemia may also be age-related change and caused by comorbidities that may be unrecognized in our study CCM group. Moreover, the serum levels of albumin are influenced by systemic congestion known as a cause of activation of endothelium (21), which in response to a local inflammatory stimulus may cause systemic in addition to local release of ARG (22). Therefore it is theoretically possible that systemic congestion may be a factor contributing to the elevated serum ARG in patients with heart failure. The liver function in our study was not an important cofactor of raised serum ARG, in the patients with CCM whose aminotransferases were within the normal range. On the other hand, as mentioned above only one factor tended to be related to cirrhosis only (non-CCM group): high values of AST activity. Gastrointestinal bleeding may precipitate hepatic decompensation or further deteriorate liver function. This occurs in 15% to 20% of cases within 6 weeks from bleeding (23). In this view, it is highly likely that in a small subgroup of bleeders the liver deterioration was preceded by an elevation of the serum AST activity due to liver hypoxic damage occurring just after the hemorrhage. However, the lack of proper data on episodes of variceal bleeding or the use of beta-blockers in cirrhotic patients, for variceal bleeding prevention is a limitation of our research.

Table 3. The amphiregulin (ARG) correlations with markers of fibrosis, ventricular remodeling, inflammation, and left ventricle functional parameters in patients with cirrhosis.

	Rho	***P* value**
Model for end stage liver disease score (MELD)	0.285	0.039
Albumin (g/L)	–0.21	<0.01
Tumor necrosis factor-α (pg/mL)	0.19	0.132
Interleukin-1β (pg/mL)	0.02	0.861
Interleukin-6 (pg/mL)	0.25	0.042
Procollagen type I carboxy-terminal propeptide soluble (PICP) (ng/mL)	0.50	0.001
PICP/PIIINP ratio	0.26	0.045
Transforming growth factor-β_1 (ng/mL)	0.27	<0.01
High-sensitivity cardiac troponin-T (hs-TnT) (ng/mL)	0.55	<0.001
N-terminal pro B-type natriuretic peptide (NT pro-BNP) (ng/mL)	0.65	<0.001
Left ventricular ejection fraction (LVEF) (%)	0.022	0.861
E/e' ratio	–0.35	<0.01
Tricuspid regurgitation maximum velocity (TR) (m/s)	0.149	0.331
Septal e' (cm/s)	–0.305	<0.01
Left atrial volume index (LAVI) (mL/m^2)	–0.435	<0.001
E/A ratio	0.316	<0.01
Left ventricle mass index (g/m^2)	0.043	0.728

Data were analyzed by Spearman's rank correlation coefficient test.
e', septal early diastolic mitral annular velocity; E/A, early to late diastolic transmitral flow velocity;

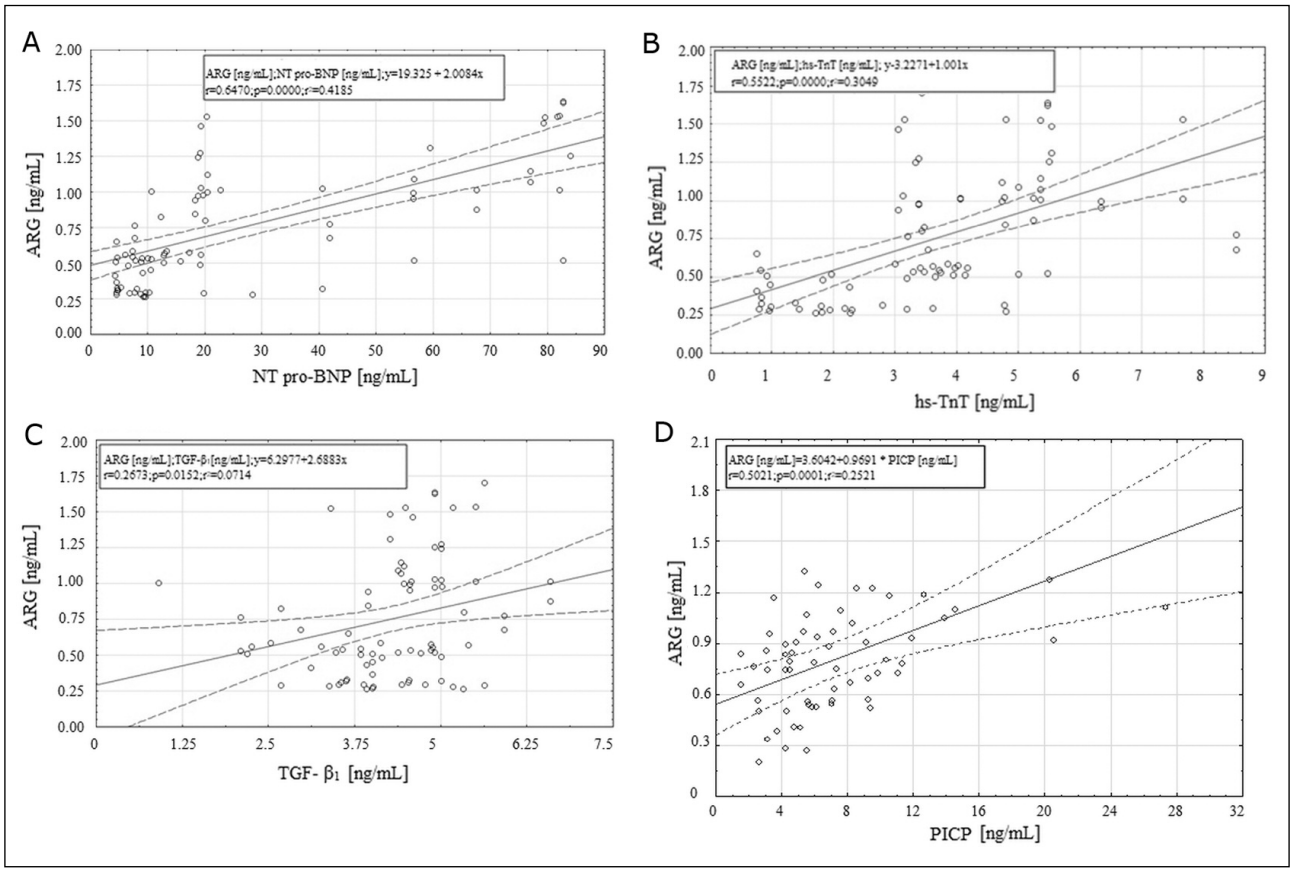

Fig. 2. Correlation between ARG levels and (A) NT-proBNP, (B) hs-TnT, (C) TGF-β_1, and (D) PICP.
Data were analyzed by Spearman's rank correlation coefficient test.
Abbreviations: ARG, amphiregulin; hs-TnT, high-sensitivity cardiac troponin-T; NT-proBNP, N-terminal pro B-type natriuretic peptide; PICP, procollagen type I carboxy-terminal propeptide; TGF-β_1, transforming growth factor β_1.

Table 4. Multiple logistic regression analysis of factors associated with the presence of cirrhotic cardiomyopathy.

	Odds ratio (OR)	95% CI	*P* value
Amphiregulin (ng/mL)	1.01	0.76–1.34	0.001
High-sensitivity cardiac troponin-T (hs-TnT) (ng/mL)	1.11	0.94–1.13	0.01
N-terminal pro B-type natriuretic peptide (NT pro-BNP) (ng/mL)	1.64	1.24–1.98	0.001
Procollagen type I carboxy-terminal propeptide soluble (PICP) (ng/mL)	1.14	1.03–1.24	0.01

Odds ratio and 95% Confidence Interval (CI) of variables independently related with the presence of cirrhotic cardiomyopathy.

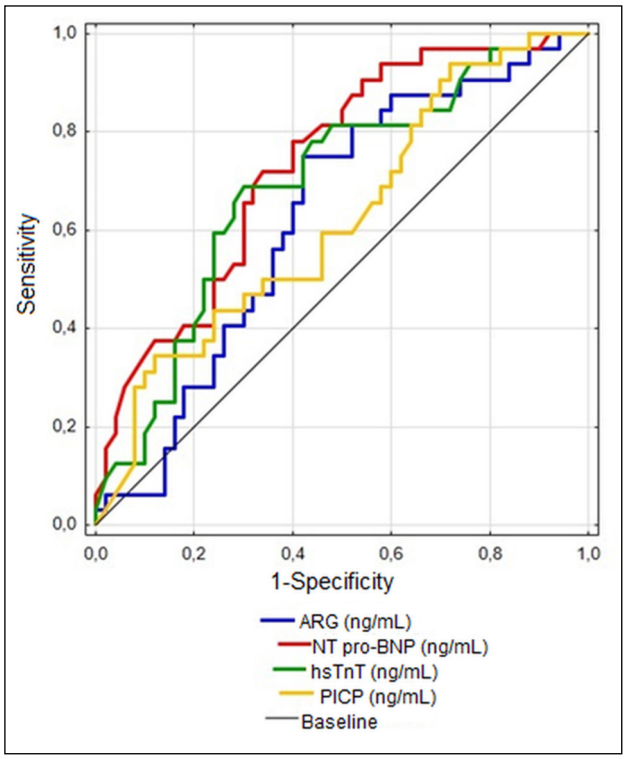

Fig. 3. Comparison of amphiregulin with other indicators, such as PICP, hs-TnT and NT -pro-BNP for the diagnosis of CCM by areas under the receiver operating curves (AUROCs).
Abbreviations: ARG, amphiregulin; hs-TnT, high-sensitivity cardiac troponin-T; NT-proBNP, N-terminal pro B-type natriuretic peptide; PICP, procollagen type I carboxy-terminal propeptide; TGF-β_1, transforming growth factor β_1.

Clinical examinations and echocardiography assessments confirmed that the stretch-induced production of NT-proBNP from fibroblasts modulates extracellular protein turnover and fibrosis, and NT-proBNP concentrations in plasma significantly correlate with markers of diastolic function (24-27). Elevated hs-TnT levels could be a reliable indicator of cardiomyocyte injury and ventricular remodeling occurring in different etiologic origins of cardiac injury (28, 29). And it is also known that the ability of the appropriate stimulus to drive transformation of fibroblasts indicated that these cells were primed for fibrosis and were susceptible to clinically relevant fibrotic triggers, such as ARG (22, 30). Our preliminary results are the first to show closer correlation between ARG and NT-proBNP, hs-TnT along with the LVDD in HCV patients with cirrhosis. Accordingly, there are reasons to that the cardiac fibrosis, a main process of ventricular remodeling, is partly mediated by ARG activation and increased extracellular matrix turnover by NT-proBNP. In this context, the cirrhotic patients may have an elevated ARG level, although a rise in the serum NT-proBNP or hs-TnT concentration seems to be a more consist believe feature that distinguishes cirrhotic patients who experience CCM symptoms from those who do not. Thus, especially in HCV patients with decompensated cirrhosis, measurement of ARG, NT-proBNP, and hs-TnT concentrations could help to identify patients with high baseline concentrations and possibly at greater risk for cardiac side effects.

Collagen is an important component of the extracellular component of myocardium, and alterations in its composition and metabolism lead to myocardial fibrosis (31). To date, only PICP (formed during the extracellular conversion of type I procollagen to fibrillar collagen I) and PIIINP (formed during the extracellular conversion of type III procollagen to fibrillar collagen III) have evidence indicating a meaningful relationship between circulating concentrations and other parameters of heart disease (4). Theoretically, serum collagen-derived peptides mirror the rate of collagen deposition, not the amount of collagen deposition. However, the levels of these markers can be modified by confounding factors and should be interpreted with caution. The presence of noncardiac comorbidities affecting collagen metabolism may also affect circulating levels of these molecules (4). In our study, although PICP levels and PICP/PIIINP ratio were significantly increased in the CCM patients, no correlation between these markers of collagen and a molecular marker (*i.e.* TGF-β_1) were found (data not shown). These and other results indicate that there is an excessive collagen deposition and a shift in collagen type proportion, predominantly a reduction in collagen III, result in increased cardiac stiffness in CCM patients (34). Some studies have also demonstrated an association between specific fibrosis markers and LVDD in a range of cardiovascular conditions (32, 33). The present study has shown that serum PICP levels were considerably correlated with ARG along with the LVDD, and the TGF-β_1 levels significantly correlated with ARG levels, but not with LVDD in CCM patients. Increasing data suggest that TGF-β_1 is crucial molecule stimulating cardiac fibrosis, and ARG may contribute to the pathogenesis of TGF-β_1-mediated cardiac fibrosis (11). Since serum levels of TGF-β_1 are not equivalent to the expression of TGF-β_1 in the myocardium (35), the expression levels of ARG in CCM are also worthy of investigation. In present study, the conclusions were only based on the one-time measurement of circulating ARG and specific fibrosis markers. Serial measurements of these parameters would be more useful to further explore the dynamic correlations among them. However, this study supports the hypothesis that circulating ARG plays a role in the development of myocardial fibrosis in the cirrhotic patients.

Table 5. Comparison of amphiregulin with other indicators for the diagnosis of cirrhotic cardiomyopathy by areas under the receiver operating curves (AUROC).

	Cutoff value (ng/mL)	**AUROC** (95% CI)	**Sensitivity** (%)	**Specificity** (%)	**PPV** (%)	**NPV** (%)	**ACC** (%)	***P* value**
ARG	0.540	0.709 (0.507–0.751)	75.0	58.0	53.3	78.4	64.0	Ref.
PICP	3.53	0.662 (0.498–0.745)	38.1	92.0	69.2	66.7	51.0	> 0.05
hs-TnT	2.56	0.753 (0.576–0.810)	59.4	76.0	61.3	74.5	68.2	0.02
NT-pro-BNP	47.02	0.796 (0.628–0.844)	44.4	90.0	68.8	68.2	70.0	< 0.01

The optimal cut-off value was calculated from the ROC analysis for ARG, PICP, hs-TnT and NT-pro-BNP subsequently the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the markers was calculated.
ARG, amphiregulin; hs-TnT, high-sensitivity cardiac troponin-T; NT-proBNP, N-terminal pro B-type natriuretic peptide; PICP, procollagen type I carboxy-terminal propeptide soluble.

Elevated IL-6 and TNF-α secretions from macrophages, T and B lymphocytes during chronic inflammation contribute to the aggravation of cardiac fibrosis. In accordance with established hypotheses of systemic inflammatory conditions and myocardial inflammatory pathways in liver cirrhosis (36), our findings of slight association of inflammation (IL-6) with ARG indicate the presence of proinflammatory activities of ARG in CCM. Moreover, studies showed the possible association between LVDD and inflammation reflected by serum TNF-α in HCV patients with decompensated cirrhosis (37). Meanwhile, very recent study first addressed the existence of cardiac TRIF/EGFR signaling, which mediated the pathophysiological role of ARG in cardiac inflammation and fibrosis (12). Since TNF-α and ARG are associated with LVDD, it is likely to effected the progress of myocardial injury in HCV patients with decompensated cirrhosis (presence of ascites (54%)). Extensive research in CCM patients have shown that LVDD severity (regardless of criteria used) is related to the worsening of liver function but its presence is not correlated with the etiology liver disease (38). However, patients with the nonalcoholic steatohepatitis (NASH) (inflammatory subtype of nonalcoholic fatty liver disease; NAFLD) more likely to have CCM rather than in the HCV patients (39). Given the diastolic dysfunction association with metabolic syndrome, this finding may reflect a baseline diastolic dysfunction that is exacerbated by the cirrhotic state as opposed to CCM in the HCV patients (*i.e*. with less likelihood of co-existing risk for cardiomyopathy). Whether CCM is uniquely increased in NAFLD/NASH cirrhosis is not clear, but some of the components of CCM overlap with NAFLD-related changes in the heart and this makes it even more confusing. The association between EGFR ligands (*e.g*. ARG) and CCM in NAFLD patients has not been established as of yet; however, circulating ARG in cirrhotic patients is expected to be influenced by cardiometabolic risk factors (obesity, and type 2 diabetes) and various factors resulted by liver cirrhosis (40). Although obesity is a key predisposing factor in NAFLD/NASH, genetic predispositions also exist for the development of fibrosis in the setting of this disease (*e.g*., in the *ABCB4* and *PNPLA3* genes) (41). On the other hand, there is now increasing evidence supporting the claim that EGF receptors mediate the pathogenesis of hyperlipidemia/obesity-related cardiac diseases (42). In cirrhotic cardiomyopathy, the fibrotic remodeling may occurs in the liver and heart of cirrhotic patients simultaneously. Since the relationship between the ARG and histological fibrosis in cirrhotic patients has not been explored, both ARG measurement and histological severity of liver fibrosis are worthy of investigation. In this context, an accurate, non-invasive investigation like laparoscopic evaluation of the liver surface, which allows macroscopical assessment structural changes, is preferable (43). It is conceivable (and indeed likely) that triggering ARG/EGFR signaling pathways is dependent on the nature of the myocardial injury, but additional research would be required to clarify this issue in patients with CCM according to the severity and etiologies of underlying liver disease.

As the area under the curves (AUROCs) of PICP and indicators of ventricular remodeling (NT-proBNP, hs-TnT) increased by the progression of LV diastolic dysfunction, the AUROC of ARG increased accordingly as well. It worth to note that ARG shows better diagnostic accuracy than PICP however with overlapping confidence. In addition, the ARG was more sensitive, whereas both PICP and NT-proBNP were more specific in CCM diagnosis. Our observations highlight that an increased ARG levels in CCM signifies myocardial structural changes. This is of diagnostic value because current definition of CCM includes only functional, hemodynamic, and electrocardiographic alterations, all of which are expected as secondary phenomena following structural alterations. Most authors favor the opinion that the collagen-derived peptides (including PICP) are ready for routine clinical use as a screening or monitoring tool (44). Nonetheless, taking into account the ambiguous data on the diagnostic significance of PICP depending on the etiology of heart failure, comorbidities affecting collagen metabolism (4), the obtained results of our research require further investigation. Acknowledging the fact that circulating ARG is easily/reproducibly measurable and provides insight into the myocardial fibrosis, ARG may serve as a promising diagnostic marker of CCM. In addition, ARG levels are not significantly affected by impairment of renal function, which are important cofactors in cardiovascular patients, and are known to limit diagnostic performance of other markers such NT-proBNP. Increasing data suggest that the novel markers may have added value alone or on top of established biomarkers or clinical variables in heart failure diagnosis and risk prediction. There is still no widespread clinically available multibiomarker panel that is able to securely rule in heart failure, although the combinational use of established and novel markers was shown to increase the diagnostic accuracy of heart failure (45) and to improve risk stratification. In the future studies, serial measurements of circulating ARG levels should be mapped

against contemporaneous serial histology and magnetic resonance scanning in the clinic. In this way, it will be possible to determine the most important relationships between a single or serial marker (*i.e*. ARG) levels and the fibrosis progression rate and total accumulated cardiac fibrosis.

Our study should also be interpreted in the context of its limitations. First, the division of cirrhotic patients was based the diastolic dysfunction classification in accordance with the recent CCM criteria (14), resulting in relatively small subgroups, which may have impacted statistical relevance. However, the numbers of subjects in each group were enough to get a sufficient power for data analysis. Second, we excluded some diseases that may influence ARG levels; on the other hand, some diseases may be unrecognized in our study group. Thirdly, the conclusions were only based on the one-time measurement of circulating biomarkers. Serial measurements of these parameters would be more useful to further explore the dynamic correlations among them. Lastly, this study was not designed and powered to assess the ability of ARG to predict the CCM in patients with LVDD of indeterminate grade. The 'indeterminate grade' is attributed to the fact that standard echocardiographic measurements cannot determine the degree of LVDD in cirrhotic patients with only 2 of the 4 CCM criteria.

Together, the results of our study provide information that ARG can plays a potential role in aggravating left ventricular diastolic dysfunction, which is the earliest hallmark of CCM. Furthermore, we found that the efficiency of circulating ARG in diagnosing CCM was better than that of PICP, a validated fibrosis marker. However, further prospective studies with large sample size using a multibiomarker panel (including ARG), methods of multivariate statistics in patients with CCM, and various etiologies of cirrhosis are needed.

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Author's address: Assoc. Prof. Jolanta Zuwala-Jagiello, Department of Pharmaceutical Biochemistry, Wroclaw Medical University, 211 Borowska Street, 50-556 Wroclaw, Poland.
E-mail: jolanta.zuwala-jagiello@umw.edu.pl