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ORIGINAL ARTICLE

High Fib4 index in patients with suspected NASH is associated with elevation of chymase-dependent angiotensin II-forming activity in circulating mononuclear leucocytes

Keisuke Okamura[1] · Tetsu Okuda[1] · Yosuke Takamiya[1] · Kazuyuki Shirai[1] · Hidenori Urata[1]

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Abstract
Fatal hepatic disease is closely related to non-alcoholic fatty liver disease, especially non-alcoholic steatohepatitis (NASH). NASH is associated with cardiovascular events because it develops on the background of lifestyle-related diseases. Chymase-dependent angiotensin II-forming activity (dAIIFA) in circulating mononuclear leucocytes (CML) is a marker of local angiotensin II production and inflammation. This study investigated the association between CML chymase dAIIFA and NASH. Cardiovascular outpatients were recruited and the Fib4 index (F4I) was calculated. Patients with an F4I > 2.67 were classified into the high F4I group and these patients were strongly suspected to have NASH, while patients with an F4I < 1.30 were classified into the low F4I group. Patient background factors were compared between these groups. CML chymase dAIIFA was measured by ELISA using Nma/Dnp-modified angiotensin I. Among 499 patients, 16% were classified into the high F4I group. Compared with the low F4I group, the high F4I group had a significantly higher age, pancytopenia, more frequent diabetes mellitus, lower diastolic blood pressure, lower estimated glomerular filtration rate, higher brain natriuretic peptide, lower plasma aldosterone concentration, higher total AIIFA, higher CML chymase dAIIFA, and higher pulse wave velocity. Contrary to expectations, the body mass index, triglycerides, and low-density lipoprotein cholesterol were relatively low in the high F4I group. Many cardiovascular outpatients have a high F4I and can probably be categorized as NASH. The high F4I patients had few features of metabolic syndrome and were suspected to have elevated tissue chymase dAIIFA contributing to inflammation in the liver as well as in cardiovascular organs.

Keywords Non-alcoholic steatohepatitis · Chymase · Angiotensin II-forming activity · Fib4 index · Fatty liver

Introduction

Non-alcoholic fatty liver disease (NAFLD) is fatty liver not caused by excessive alcohol intake and is a typical form of liver disease in patients with metabolic syndrome (MetS). Risk factors for NAFLD include weight gain and an increased supply of triglycerides and free fatty acids, along with decreased metabolism of these fats [1]. Along with an increase of obesity, approximately 30% of Japanese people currently have NAFLD [2] and its incidence has been increasing rapidly [3, 4]. NAFLD can be classified into non-alcoholic steatohepatitis (NASH), which may progress to liver cirrhosis (LC) and hepatocellular carcinoma with a high risk of liver-related mortality (LRM), and non-alcohol dependent fatty liver (NAFL) with a low risk of LRM [5]. Among NAFLD patients, those with NASH have the most advanced fibrosis and show elevation of fibrosis markers along with a decreased platelet count. The prevalence of NASH is estimated to be around 3–5% [6, 7]. Cohort studies have shown that NAFLD, especially NASH, is associated with a high incidence of cardiovascular disease (CVD) and an increased mortality risk [8, 9]. Thus, NASH may be strongly associated with CVD. Hypertension (HT) is also related to CVD and is an independent risk factor for NAFLD [10]. Angiotensin II binds to angiotensin II (AII) receptors on hepatic stellate cell and activates α-smooth muscle actin, leading to inflammation and fibrosis that may result in the development of NASH [11]. Moreover, steatohepatitis is associated with elevated expression of AII, while

✉ Keisuke Okamura
okamurakmd@cis.fukuoka-u.ac.jp

[1] Department of Cardiovascular Diseases, Fukuoka University Chikushi Hospital, 1-1-1, Zokumyoin, Chikushino, Fukuoka 818-8502, Japan

angiotensin-converting enzyme (ACE) inhibitors (ACE-I) and angiotensin receptor blockers (ARB) are reported to improve hepatic inflammation and fibrosis [12–15]. Thus, it is strongly suggested that a relationship exists between the renin-angiotensin system (RAS) and NAFLD.

In addition to ACE, various serine proteinases such as kallikrein, cathepsin G, and chymase are involved in the AII-forming pathways of the RAS [16–20]. It has been reported that chymase may be related to elevation of the blood pressure (BP) and to CVD, including left ventricular hypertrophy, arteriosclerosis, and myocardial infarction (MI) [21–31]. Interestingly, hepatic chymase levels are increased in LC patients [32] and chymase seems to be related to NASH, while it was reported that treatment with a chymase inhibitor suppressed NASH [33]. Liver biopsy is required for diagnosis of NASH and chymase cannot be measured easily in clinical practice because of intrinsic chymase inhibitors in plasma [34], so there have been no large-scale investigations of the relationship between NASH and chymase. We have measured chymase-dependent angiotensin-forming activity (AIIFA) in circulating mononuclear leukocytes (CML) and have reported on the relationship between tissue chymase activity and BP [35]. Our studies have suggested that chymase-dependent AIIFA in CML (CML chymase dAIIFA) is a marker of tissue AII production and inflammation. Accordingly, it is likely that CML chymase dAIIFA would be elevated in patients with NASH. Various liver fibrosis scores have been developed to screen patients for NASH, and the FIB4 index (F4I) has been demonstrated to be useful among these scoring systems [36, 37]. Accordingly, we investigated whether the F4I was related to CML AIIFA and various other parameters in cardiovascular outpatients (CV outpatients).

Methods

Study population

The subjects were CV outpatients attending Fukuoka University Chikushi Hospital (Fukuoka, Japan) from July 2007 to October 2008 who underwent measurement of the office blood pressure (OBP), routine blood tests, endocrine tests, echocardiography, and measurement of the brachial-ankle pulse wave velocity (baPWV), as well as assays for CML total AIIFA, CML chymase dAIIFA, and CML cathepsin G dAIIFA. NAFLD was diagnosed in patients who satisfied the following 3 conditions: fatty liver, no history of alcohol intake, and no underlying disease that could cause liver disorders [5]. It is very difficult to accurately estimate the intake of alcohol in clinical practice [38], so the current study excluded patients receiving treatment for alcohol-dependent liver disease and patients under treatment for other liver disorders.

Scoring of NASH

Pathological examination of a liver biopsy specimen is necessary for diagnosis NASH, but it is unrealistic to conduct liver biopsy in all CV outpatients. Findings that suggest NASH include an older age, obesity, diabetes mellitus (DM), high aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio (AAR), low platelet count [39], and elevation of liver fibrosis markers [5]. Various scoring systems based on these parameters have been considered useful for screening of NASH. The NAFIC score consists of 3 parameters (ferritin, fasting insulin, and Type IV collagen 7S) and is useful for screening NASH [40]. However, these 3 parameters are not measured routinely among CV outpatients, so it is not suitable as screening for NASH. Other scoring systems include the NAFLD fibrosis score [41], but it has a low positive PPV [40] and NASH tends to be overlooked [8]. In addition, systems that include the BMI such as the BARD score are reported to be less reliable for Japanese people with a low BMI [42]. Among the various scoring systems, F4I has been reported as the liver fibrosis system with the most valid AUROC [36, 37]. Therefore, we used the F4I scoring system for this study because it is currently considered to be the most reliable.

Calculation of the Fib4 index

F4I is a noninvasive test that allows the accurate prediction of hepatic fibrosis and is calculated as follows: [age (year) × AST (U/L)]/[(platelet count (PLT)(10^9/L)) × (ALT (U/L))$^{1/2}$] [43]. An F4I of over 2.67 means that NASH is strongly suspected and these patients were classified into the high F4I group, while patients with an F4I less than 1.30 were classified into the low F4I group and patients between these values formed the intermediate F4I group [36]. According to the NAFLD diagnostic algorithm, a low F4I value means that NASH is unlikely, while a high F4I value means that NASH is likely and liver biopsy should be considered.

Assay of CML AIIFA

Since chymase inhibitors exist in plasma [34], chymase activity was measured using an AIIFA assay system for CML isolated from peripheral blood [35]. In this assay, Nma-AII production is measured using Nma/Dnp-modified angiotensin I, which emits fluorescence when the Phe8–His9 bond of AI is cleaved. Nma-AII generation in CML was measured by comparison with the standard curve. The AIIFA after addition of a cathepsin G inhibitor (2 nM aprotinin) was defined as cathepsin G dAIIFA. Using a chymase

inhibitor (E-548; Teijin Pharma, 2 nM) that inhibits both chymase and cathepsin G, chymase dAIIFA was obtained by subtracting cathepsin G dAIIFA from AIIFA after addition of the chymase inhibitor.

We have reported that chymase-dependent angiotensin II-forming activity (AIIFA) in circulating poly- as well as mononuclear leukocytes [44]. Major (~99%) AIIFA in polynuclear leukocyte was cathepsin G, whereas AIIFA in mononuclear leukocyte was evenly balanced among chymase, cathepsin G and angiotensin-converting enzyme (ACE). This was the reason why we decided to utilize circulating mononuclear leukocyte in subsequent clinical experiments. Furthermore, mononuclear leukocyte was subfractionated and chymase activity was found to be in lymphocytes and monocytes. In several human tissue such as heart, artery, lung, alimentary tract, gene, and protein expression of only three enzymes (chymase, cathepsin G and ACE) were identified as Ang II-forming enzymes in the isolated human tissue [28, 45]. AIIFA by cathepsin G has been identified by a complete inhibition with aprotinin because aprotinin does not inhibit chymase and ACE activity at all. Chymase inhibitor used in our study inhibits both chymase and cathepsin G. Therefore, chymase-like activity represented in the manuscript was determined by a subtraction of AIIFA in the presence of chymase inhibitor by AIIFA in the presence of aprotinin. On the other hand, ACE-dependent AIIFA was identified by captopril inhibitable AIIFA. Since there is no completely specific chymase inhibitor inhibiting only chymase activity, our present method is only way to analyze chymase activity in human tissue in vitro.

Statistical analysis

Statistical analysis was performed at Fukuoka University using the IBM SPSS Statistics 23 package. For parameters with a normal distribution, the significance of differences was assessed with the *t* test, which was followed by Levene's test if homogeneity of variance was confirmed or by Welch's test if variance was not homogeneous. The Mann–Whitney test was also used for data with a normal distribution. For comparison among 3 groups, one-way ANOVA was employed if the data had a normal distribution, while the Kruskal–Wallis test was conducted when the distribution was not normal. Correlations were investigated by calculation of Spearman's rank correlation coefficients. Numerical values were expressed as the mean (standard deviation: SD), median (interquartile range: IQR) or ratio < percentage > . In all analyses, $P < 0.05$ was considered significant. Using the F4I value as a dependent variable, stepwise regression analysis was conducted to identify variables that influenced this index.

The protocol for this study was approved by the institutional review board of Fukuoka University Chikushi Hospital (approval no. R07-005 and R18-043), and written informed consent was given by the patients for collection of blood samples.

Results

The demographic profile of the patients is shown in Table 1. There were 79 patients in the high F4I group, accounting for 16% of all patients. Compared to the low F4I group, the high F4I group was significantly older, with a lower body mass index (BMI) and more DM, while smoking was less frequent. Many patients in the high F4I group were using ARBs. The systolic blood pressure (BP) did not differ significantly between the low F4I group and the high F4I group. On the other hand, diastolic BP (DBP) was lower in the high F4I group, although there was no difference of pulse rate. On comparison among the 3 groups, only DBP showed a significant difference (Fig. 1). Compared to the low F4I group, the high F4I group showed pancytopenia and lower levels of albumin (Alb), estimated glomerular filtration rate (eGFR), low-density lipoprotein cholesterol (LDL-C), triglycerides

Table 1 Baseline characteristics of the low F4I group and high F4I group

	Total ($n=499$)			
	N, mean (ratio, SD, IQR)	Low F4I group ($n=122$)	High F4I group ($n=79$)	*p* value
Gender (male)	264 (53%)	71 (58%)	40 (51%)	NS
Age (years)	65.4 (11.1)	54.5 (9.5)	75.9 (7.8)	< 0.001
BMI (kg/m^2)	23.6 (3.3)	24.8 (3.5)	22.4 (3.3)	< 0.001
HT	449 (90%)	111 (91%)	72 (91%)	NS
DM	100 (20%)	20 (16%)	25 (32%)	< 0.05
DL	259 (52%)	65 (53%)	34 (43%)	NS
CHD	120 (24%)	21 (17%)	21 (27%)	NS
Smoking	65 (13%)	24 (20%)	5 (6%)	< 0.005
ARB	309 (62%)	62 (51%)	55 (70%)	< 0.01
ACE-I	40 (8%)	11 (9%)	7 (9%)	NS
CCB	254 (51%)	60 (49%)	43 (54%)	NS
Diuretics	110 (22%)	20 (16%)	18 (23%)	NS
Sympathetic	55 (11%)	10 (8%)	10 (13%)	NS
Vasodilators	65 (13%)	9 (7%)	7 (9%)	NS
Spironolactone	5 (1%)	0 (0%)	3 (4%)	0.08

The high F4I group was significantly older and had more diabetes than the low F4I group, while it had a lower BMI and lower smoking rate. With regard to pharmacotherapy, use of ARB was high in the high F4I group

n number of patients, *SD* standard deviation, *IQR* interquartile range, *F4I* fib4 index, *BMI* body mass index, *HT* hypertension, *DM* diabetes mellitus, *DL* dyslipidemia, *CHD* coronary heart disease, *ARB* angiotensin receptor blocker, *ACEI* angiotensin-converting enzyme inhibitor, *CCB* calcium channel blocker, *NS* not significant

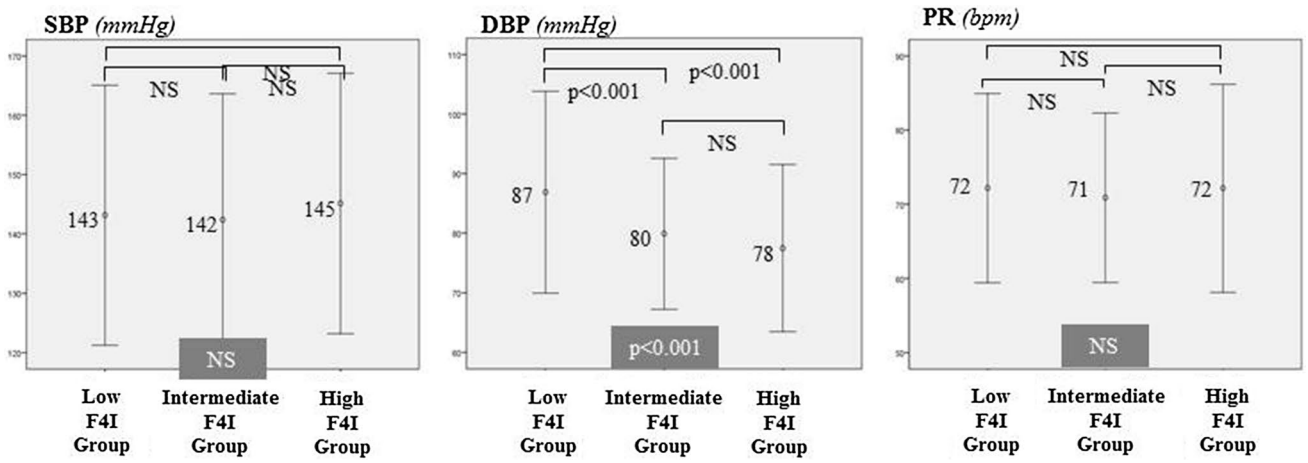

Fig. 1 Office blood pressure and pulse rate of the 3 F4I groups. SBP was not significantly different between the high F4I group and the low F4I group. DBP was lower in the high F4I group, but PR was not different. In the 3-group comparison, only DBP was significantly different. *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *PR* pulse rate, *bpm* beats per minute

Table 2 Laboratory data of the low F4I group and high F4I group

	Low F4I group ($n=122$)	High F4I group ($n=79$)	p value
WBC ($10^3/\mu L$)	6.1 (1.6)	5.3 (2.2)	<0.01
Hb (g/dl)	13.8 (1.5)	12.6 (1.8)	<0.001
PLT ($10^4/\mu L$)	23.8 (5.2)	13.6 (3.5)	<0.001
TP (g/dl)	7.2 (0.5)	7.1 (0.6)	NS
Alb (g/dl)	4.3 (0.4)	4.0 (0.5)	<0.005
T-bil (mg/dL)	0.9 (0.3)	1.0 (0.3)	NS
AST (U/L)	20 (16–24)	26 (22–33)	<0.001
ALT (U/L)	21 (15–33)	18 (12–28)	NS
LDH (U/L)	174 (36)	218 (80)	<0.001
CK (U/L)	91 (66–124)	108 (82–150)	<0.005
γ-GTP (U/L)	30 (18–44)	25 (14–42)	NS
eGFR (ml/min/1.73 m^2)	73 (16)	59 (23)	<0.001
UA (mg/dL)	5.5 (1.4)	5.7 (1.7)	NS
HDL-C (mg/dL)	58 (18)	61 (14)	NS
LDL-C (mg/dL)	119 (28)	106 (27)	<0.005
TG (mg/dL)	124 (88–179)	96 (73–140)	<0.01
Glu (mg/dL)	99 (92–107)	92 (99–116)	NS
Insulin (μU/mL)	6.7 (5.4–9.1)	6.1 (4.0–8.8)	NS
HOMA-IR	1.7 (1.3–2.3)	1.6 (1.0–3.1)	NS
HbA1c (%)	5.5 (0.7)	5.8 (1.5)	NS
BNP (pg/mL)	17 (9–24)	58 (30–121)	<0.001

The high F4I group showed pancytopenia compared to the low F4I group. Alb, eGFR, LDL-C, and TG were lower in the high F4I group. In contrast, AST, LDH, CK, and BNP were higher in the high F4I group.

WBC white blood cell count, *Hb* hemoglobin, *Plt* platelet count, *TP* total protein, *Alb* albumin, *T-bil* total bilirubin, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactate dehydrogenase, *CK* creatine kinase, *γ-GTP* γ-glutamyltransferase, *eGFR* estimated glomerular filtration rate, *UA* uric acid, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *TG* triglycerides, *Glu* glucose, *HOMA-IR* homeostasis model assessment of insulin resistance, *HbA1c* hemoglobin A1c (NGSP) , *BNP* brain natriuretic peptide, for other abbreviations, please refer to the previous table

Table 3 Endocrine findings in the low F4I group and high F4I group

	Low F4I group ($n=122$)	High F4I group ($n=79$)	p value
PRA (ng/ml/h)	0.95 (0.50–1.68)	1.35 (0.67–1.90)	NS
PAC (pg/mL)	76 (48–94)	47 (18–68)	<0.01
Adrenaline (pg/mL)	0.03 (0.01–0.06)	0.04 (0.02–18.0)	NS
Noradrenaline (pg/mL)	0.41 (0.28–0.94)	0.57 (0.41–113.5)	NS
Dopamine (pg/mL)	0.02 (0.02–0.06)	0.03 (0.02–7.5)	NS
Cortisol (μg/dL)	8.5 (7.4–12.2)	9.6 (6.7–12.0)	NS
TSH (μIU/mL)	1.2 (0.9–2.1)	1.6 (1.1–2.1)	NS
FT4 (ng/dL)	1.1 (1.0–1.2)	1.1 (1.0–1.2)	NS

PAC was lower in the high F4I group than in the low F4I group

PRA plasma renin activity, *PAC* plasma aldosterone concentration, *TSH* thyroid-stimulating hormone, *FT4* free thyroxine, for other abbreviations, please refer to the previous table

(TG), and plasma aldosterone concentration (PAC), while this group had higher levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase (CK), and brain natriuretic peptide (BNP) (Tables 2, 3).

The low F4I group and high F4I group showed no significant differences of echocardiography findings. An index of arteriosclerosis (baPWV) was higher in the high F4I group (Table 4). When plasma renin activity (PRA), PAC, and AIIFA were compared among 3 groups (low F4I group, high F4I group, and intermediate F4I group), there were significant differences of PAC and CML total AIIFA. PAC was lower in the high F4I group compared to the low F4I group, while both CML total AIIFA and CML chymase dAIIFA were higher in the high F4I group (Fig. 2).

Three-group comparison also revealed significant differences of BMI, baPWV, and BNP. BMI was lower in the high F4I group than in the low F4I group, while baPWV and BNP were elevated in the high F4I group (Fig. 3). The F4I value showed a negative correlation with DBP, while it showed a positive correlation with CML total AIIFA and CML chymase dAIIFA ($r=-0.233$, $p<0.001$; $r=0.093$, $p<0.05$; and $r=0.105$, $p<0.05$, respectively). When stepwise regression analysis was done using F4I as a dependent variable, with SBP, DBP, PR, total AIIFA, chymase dAIIFA, cathG dAIIFA and baPWV as independent variables, only baPWV was demonstrated to be a significant determinant of F4I (β 0.234, odds ratio:6.563, 95% CI 4.169–8.957, $p<0.001$).

Discussion

The major findings of the present study were as follows: (1) among 499 CV outpatients, 16% were categorized into the high F4I group. (2) compared with the low F4I group, the high F4I group was significantly older and had a tendency for pancytopenia, as well as more frequent DM and a lower DBP, lower eGFR, higher BNP, lower PAC, higher total AIIFA, higher CML chymase dAIIFA, and higher PWV. (3) Contrary to our expectations, BMI, TG, and LDL-C were relatively low in the high F4I group. (4) Regression analysis demonstrated that baPWV was an independent determinant of F4I.

Table 4 Echocardiography findings and PWV in the low F4I group and high F4I group

	Low F4I group ($n=122$)	High F4I group ($n=79$)	p value
AOD (mm)	32.1 (4.1)	32.7 (4.2)	NS
LAD (mm)	37.2 (5.7)	38.2 (7.5)	NS
LVDd (mm)	49.7 (5.8)	48.2 (5.1)	NS
IVST (mm)	8.7 (1.8)	8.8 (1.8)	NS
LVPWT (mm)	8.6 (1.5)	8.7 (1.6)	NS
EF (%)	66.0 (8.4)	65.6 (8.2)	NS
E/e	8.0 (0.0–11.0)	6.6 (0.0–14.8)	NS
baPWV (cm/s)	1549 (1383–1727)	1897 (1676–2253)	<0.001

Echocardiography findings were not significantly different between the high F4I group the low F4I group. However, baPWV (an index of arteriosclerosis) was higher in the high F4I group

UCG echocardiography, *baPWV* brachial-ankle pulse wave velocity, *AOD* aortic dimension, *LAD* left atrial dimension, *LVDd* left ventricular end-diastolic diameter, *IVST* interventricular septal thickness, *LVPWT* left ventricular posterior wall thickness, *EF* ejection fraction, for other abbreviations, please refer to the previous tables and figures

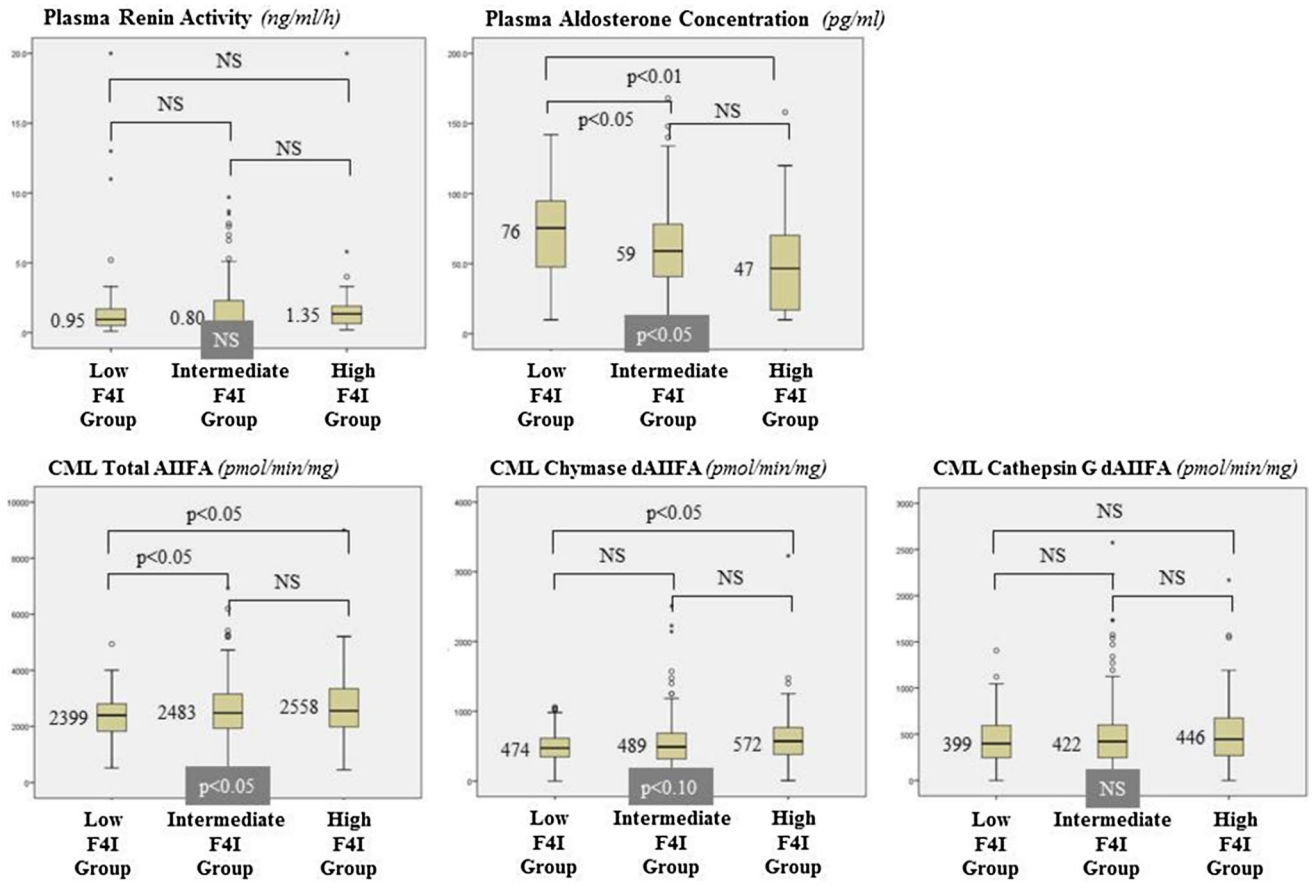

Fig. 2 PRA, PAC, and AIIFA in the 3 F4I groups. When PRA, PAC, and AIIFA were compared among the 3 groups, PAC and CML total AIIFA showed significant differences. PAC was lower in the high F4I group than in the low F4I group, while CML total AIIFA and high CML chymase dAIIFA were elevated in the high F4I group. *CML* circulating mononuclear leukocytes, *AIIFA* angiotensin II-forming enzyme activity, *dAIIFA* dependent AIIFA, For other abbreviations, please refer to the previous tables and figure

It was a surprising finding of this study that 16% of CV outpatients were in the high F4I group, meaning that a diagnosis of NASH was likely. As with patients who have hepatitis virus infection in Japan, most of these patients do not undergo detailed liver function tests and are not followed up carefully.

Similar to a previous report [46], F4I and age were closely correlated (0.67, $p<0.001$), which suggests that aging leads to progression of liver fibrosis. The age had considerable correlation with baPWV ($r=0.486$, $p<0.001$) and BNP ($r=0.484$, $p<0.001$). Since baPWV, BNP, and F4I increased with aging, respectively, these might be mutually related each other.

In Japan, NAFLD is reported to be more common among men than women [2]. However, the proportion of men and women was similar in the high F4I group, possibly because this study targeted patients who already had cardiovascular disorders.

DBP was lower in the high F4I group than in the low F4I group, but total AIIFA and CML chymase dAIIFA were higher in the high F4I group. Moreover, the F4I value was negatively correlated with DBP, while it was positively correlated with CML total AIIFA and CML chymase dAIIFA. The NAFLD fibrosis score is similar to F4I, and it was also correlated with DBP, CML total AIIFA, and CML chymase dAIIFA ($r=-0.268$, $p<0.001$; $r=0.132$, $p<0.005$; and $r=0.100$, $p<0.05$, respectively). In the present study, patients with suspected NASH had a low DBP, contradicting a report that NAFLD is an independent risk factor for hypertension [10]. We found that DBP and PAC were both lower in the high F4I group, possibly due to extensive use of RAS inhibitors. We attempted to conduct an analysis of patients without antihypertensive therapy, but there was only 1 such patient in the high F4I group. In fact, most NAFLD patients receive antihypertensive drugs in clinical practice and analysis restricted to untreated patients appears to be unrealistic. We also performed analysis after excluding patients receiving oral RAS inhibitors. Results in the low F4I group ($n=55$) and the high F4I group ($n=21$) showed no significant differences of SBP, DBP, total AIIFA, chymase

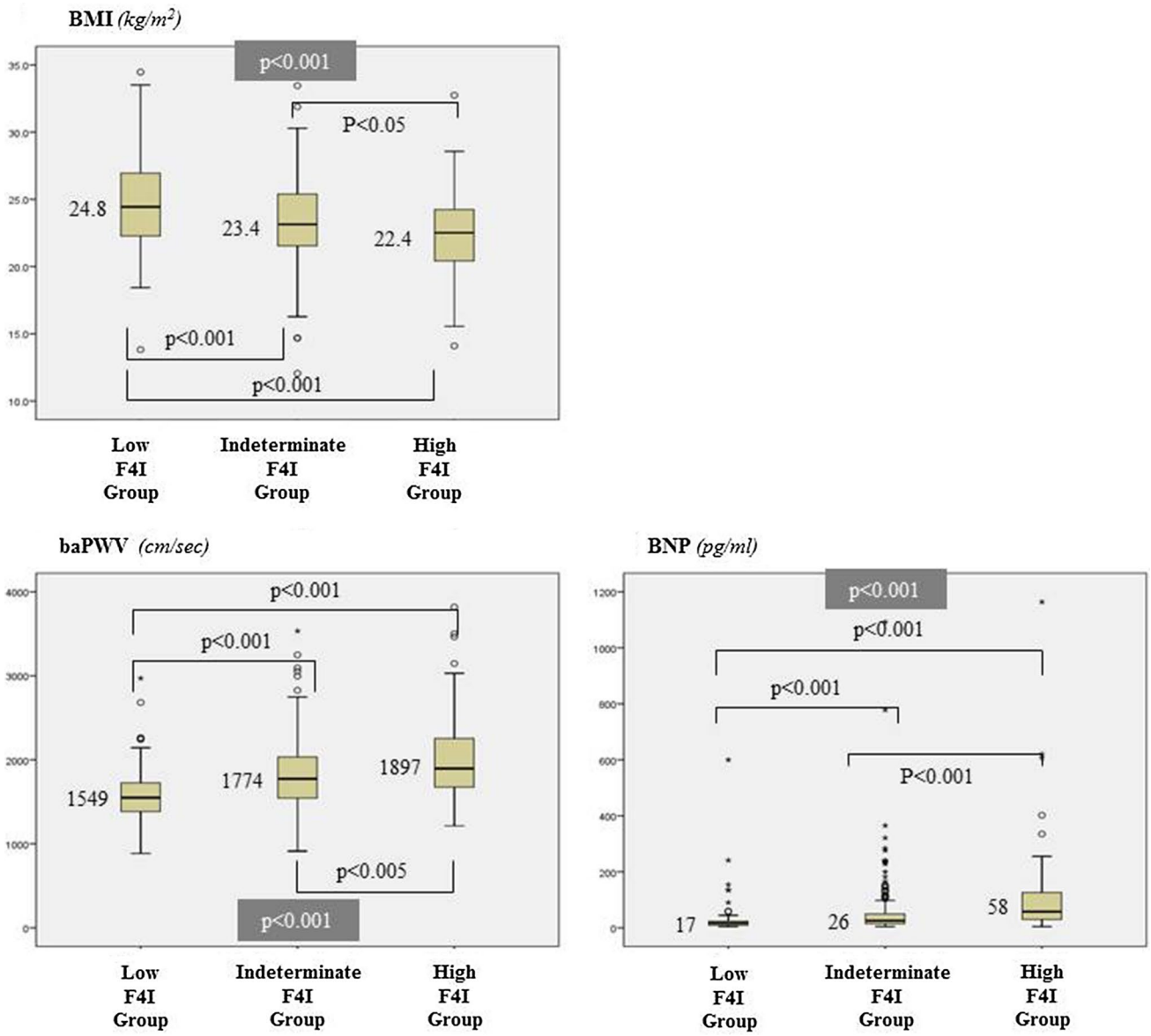

Fig. 3 BMI, baPWV and BNP among 3 F4I groups. For BMI, baPWV and BNP, there were significant differences among the 3 groups. The high F4I group was low in BMI and high in baPWV and BNP than the low F4I group; For abbreviations, please refer to previous tables and figures

dAIIFA, PRA, or PAC (data not shown). While it is difficult to draw conclusions, it may be worth re-evaluating the relationship between NASH and BP. The decrease of PAC in the high F4I group could be considered to suggest RAS inhibition. However, despite the low DBP of this group, tissue AIIFA such as CML total AIIFA and CML chymase dAIIFA were all increased, so the hepatic RAS may be activated in NASH patients.

Chymase induces steatosis, inflammation, and fibrosis of the liver via AII, matrix metalloproteinase-9 (MMP9), and transforming growth factor-β (TGFβ), which have been shown to be activated in the livers of NASH patients and animal models [32, 33, 47–50]. Previous report revealed that increased CML chymase dAIIFA associated with high BP, congestive heart failure, heart remodeling, and myocardial dysfunction [29, 35, 51]. For example, CML chymase dAIIFA was increased as well as in the myocardium after AMI, and that the level of chymase-dependent AIIFA might reflect the severity of infarction. It is possible that the influence of metabolic factors and congestive hemodynamics induces hepatic microvascular inflammation and oxidative stress which increase chymase activity, leading to release of leukocytes containing chymase from the bone marrow and an increase of CML chymase dAIIFA. In addition, our

previous basic experiment has revealed that radical oxidative stress (ROD) strongly induces chymase expression in cultured mastocytoma cell [52]. Therefore, various life style dysregulation induces enhanced ROD, which may upregulate chymase expression.

It was reported that angiotensin binds to AII receptors of hepatic stellate cells and activates these cells to promote the onset of NASH. It was also reported that ARB treatment decreases the production of reactive oxygen species (ROS), downregulates sterol regulatory element-binding protein (SREBP)-1c and fatty acid synthase (FAS) gene expression, and inhibits hepatic inflammation and fibrosis [13–15, 53–55]. Similarly, treatment with a chymase inhibitor was reported to decrease SREBP-1c production and FAS gene expression, as well reducing the levels of AII, ROS, MMP9, and TGFβ in the liver to improve hepatic steatosis. Thus, chymase inhibitor therapy may inhibit the onset and progression of NASH [33, 56, 57], and measurement of CML chymase dAIIFA may be useful when monitoring the effect of chymase inhibitor therapy for NASH [33].

NAFLD is the hepatic component of MetS due to insulin resistance induced by visceral obesity. Both cardiovascular events and mortality are increased by MetS [8, 9, 58]. In the present study, the high F4I group had a low BMI, BP, and markers of lipid and glucose metabolism, and such findings seem to be inconsistent with the assumption that NASH is caused by MetS. Although the waist circumference (an index of abdominal obesity) was not measured in this study, TG, HDL-C, BP, and blood glucose were not elevated in the high F4I group, which does not support the existence of MetS. While this may be the effect of pharmacotherapy, BMI is considered to be less influenced by pharmacotherapy and it showed a negative correlation with F4I (-0.215, $p<0.001$). It has been reported that NAFLD has a prevalence of 15%, even among non-obese patients [1], and our high F4I group was rather lean. This result is consistent with a previous study [38] and it is interesting to speculate that the high F4I group may not be related to MetS.

It was reported that dyslipidemia and NAFLD are closely related [3]. When we measured LDL and TG in 132 patients who were not on lipid-lowering drugs, both values were lower in the high F4I group than the low F4I group (data not shown).

Diabetes mellitus and NAFLD are closely related because of the interactions among hepatic insulin resistance, liver fibrosis, hyperglycemia due to increased shunting of blood as a result of elevated portal pressure, and increased systemic insulin resistance due to obesity and pancreatic β-cell dysfunction [7, 59–62]. In this study, diabetes was more frequent in the high F4I group than in the low F4I group, but there were no differences of glucose, HbA1c, insulin, or HOMA-IR. However, it was considered that treatment for diabetes might have decreased the markers of glucose metabolism in these patients. Accordingly, we analyzed 177 patients without oral antidiabetic drugs, but there were still no significant differences of the above markers between the low and high F4I groups (data not shown). Therefore, NASH may not be related to diabetes.

It is unlikely that only medications were responsible for the lack of MetS in the high F4I group. The high F4I group might already have had LC with decreased protein production and a poor nutritional state, which would influence the albumin level, blood pressure, lipid levels, and glucose metabolism. The isolated decrease of DBP in the high F4I group may indicate progression of arteriosclerosis. PWV is a good index of arteriosclerosis that is less influenced by treatment and it was elevated in the high F4I group, suggesting that arteriosclerosis may be advanced in NASH patients.

An important message from the current study is that the assumption that NASH is related to MetS may lead to the risk of overlooking NASH. It seems possible that MetS has already progressed to the end stage in patients with suspected NASH. Both liver-related mortality and CAD are increased by NASH. Accordingly, F4I should be calculated as part of routine screening for CV outpatients.

In addition to F4I, the combination of an index of liver fibrosis (Mac-2 binding protein glycosylation isomer) [63] and an index of hepatocyte ballooning (fucosylated haptoglobin) was reported to be an effective and noninvasive diagnostic method for NASH that may replace liver biopsy [64]. However, these are not routine tests in clinical practice. In addition, the CT ratio of the liver and spleen can be calculated to predict NASH and quantitation of liver fat by MRI is feasible [65], but these methods are also difficult to perform as routine tests. Ultrasound-based fibroscan can noninvasively quantitate the amount of fat in the liver [66], and is also useful for evaluation of NAFLD and the response to treatment [67]. For routine screening of CV outpatients, determination of F4I appears to be the best option at present, while the fibroscan may be used to replace liver biopsy.

Conclusion

In patients with a high F4I value who are suspected of NASH, this study suggested that as with cardiovascular organs, the elevation of tissue chymase dAIIFA is involved in inflammation and fibrosis in the liver. It seems there are potentially many NASH patients among CV outpatients, suggesting that cardiologists should take more interest in NAFLD and actively screen their patients by using F4I.

Study limitations

First, ACE was not measured in this study. Additionally, although home BP and ambulatory monitoring are important

for obtaining accurate data, we did not perform those tests in this study. Furthermore, we cannot exclude the possibility of undiagnosed liver disorders in the study population. Finally, F4I is a useful screening tool for NAFLD, but it is a surrogate marker and liver biopsy is needed for definitive diagnosis of NASH.

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Compliance with ethical standards

Conflict of interest This work was supported by JSPS KAKENHI (Grants No. 21590916 and No. 26461118).

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