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Original article

Long-term prediction of hepatocellular carcinoma using serum autotaxin levels after antiviral therapy for hepatitis C

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ABSTRACT

Introduction and objectives: Continuous monitoring for hepatocellular carcinoma is necessary following treatment with direct-acting antivirals in patients with hepatitis C virus infection. We investigated whether the long-term follow-up of serum autotaxin levels could predict the development of hepatocellular carcinoma.
Patients and Methods: This prospective observational study enrolled adult patients with chronic hepatitis C virus infection who presented to the study center from January 2016 to March 2021. Among the patients who achieved a sustained viral response, the relationship between the development of hepatocellular carcinoma and serum autotaxin levels was assessed before treatment with direct-acting antivirals; at the end of therapy; at 12 and 24 weeks; and at 12, 24, 36, and 48 months after treatment.
Results: Data were analyzed for 139 patients. Thirteen patients developed hepatocellular carcinoma 48 months after treatment. The cut-off serum autotaxin values that predicted hepatocellular carcinoma after 24 weeks were 1.22 (men) and 1.92 (women) mg/L. The area under the curve for serum autotaxin was 0.83 (95% confidence interval [CI]:0.71−0.95) in men and 0.90 (95% CI: 0.82−0.99) in women. The positive predictive value of serum autotaxin was 0.208 (95% CI: 0.139−0.248), and the negative predictive value was 0.971 (95% CI: 0.939−0.990). The cumulative incidence of hepatocellular carcinoma was significantly higher when serum autotaxin levels were above the cut-off value after 24 weeks ($p < 0.0001$).
Conclusions: Serum autotaxin is a candidate biomarker for predicting hepatocellular carcinoma during the long-term follow-up of patients with a sustained viral response following treatment with direct-acting antivirals.
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1. Introduction

In chronic hepatitis C virus (CHC) infection, despite achieving sustained virological responses (SVRs) after treatment with direct-acting antivirals (DAAs), the risk of hepatocellular carcinoma (HCC) persists [1−3]. Recent research has indicated that autotaxin (ATX) is an effective marker of liver fibrosis [4] and has an important enzymatic function in converting lysophosphatidylcholine to lysophosphatidic acid (LPA) which is involved in various physiological processes such as cell migration, neurogenesis, angiogenesis, smooth muscle contraction, platelet aggregation, and wound healing [5]. ATX is present in the serum and is specifically metabolized by hepatic sinusoidal endothelial cells [6]. The ability to metabolize ATX is reduced in patients with liver fibrosis, leading to an increase in the concentration of serum ATX [4, 7]. Previous studies have demonstrated that serum ATX is a useful marker to determine the stage of fibrosis in patients with chronic hepatitis B and C [8, 9]. In addition, the concentration of serum ATX is correlated with liver hardness on transient elastography (TE) and is a good substitute for liver biopsy for the assessment of fibrosis [10]. While researchers have recently suggested that ATX can be used to predict the development of HCC [11−13], ATX is a relatively new marker, and the cut-off value for predicting HCC has not been fully analyzed. Although a few long-term studies have followed

Abbreviations: ATX, autotaxin; CHC, chronic hepatitis C virus; DAAs, direct-acting antivirals; HCC, hepatocellular carcinoma; LPA, lysophosphatidic acid; SVRs, achieving sustained virological responses; TE, transient elastography; WFA+/-M2BP, *Wisteria floribunda* agglutinin-positive mac-2-binding protein

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the development of HCC using serum *Wisteria floribunda* agglutinin-positive mac-2-binding protein (WFA+/-M2BP) [14], none have measured and analyzed serum ATX levels in this context. Therefore, the present study aimed to determine whether serum ATX levels predict HCC in patients with CHC who have achieved a SVR after DAA treatment.

2. Methods

This single-center, prospective observational study was conducted from January 1, 2016, to March 31, 2021 at the Kitasato University Medical Center. The study protocol was approved by the Ethics Committee of the Kitasato University Medical Center on December 25, 2015 (approval number: 27-10). The study is in conformance with the principles of the 1975 Declaration of Helsinki.

3. Study participants

Adult patients (aged ≥20 years) with CHC were enrolled. The exclusion criteria were as follows: patients with Child−Pugh class B or worse liver failure; use of hepatotoxic medicine or any agent that affects cytokines; need for hemodialysis; and complications such as HCC, primary biliary cirrhosis, primary sclerosing cholangitis, pancreatitis, uncontrolled thyroid deficiencies, or severe renal failure. Patients who failed to achieve SVRs and those lost to follow-up within 24 weeks post-DAA treatment were also excluded from the analysis.

The patient enrollment process is shown in Fig. 1. All the patients provided written informed consent to participate in the study.

4. Fibrosis scoring

To estimate fibrosis scores, liver stiffness was measured using TE (FibroScan; Echosens, Paris, France). Fibrosis grades were determined from the liver stiffness cut-off values [15]. The fibrosis levels were categorized as follows, where E denotes the liver stiffness score: E ≤ 7.4 kPa as stage 0-1 (F1); 7.4 kPa < E ≤ 10.3 kPa as stage 2 (F2); 10.3 kPa < E ≤ 14.9 kPa as stage 3 (F3); and E >14.9 kPa as stage 4 (F4). Fibrosis scoring was performed only before and after 24 weeks of DAA treatment.

5. Measurement of serum concentrations of ATX and WFA +/-M2BP

Serum levels of ATX were measured using a two-site enzyme immunoassay and an automated immunoassay analyzer (Tosoh Corporation, Tokyo, Japan). Immunoassay kits were used to measure the serum concentrations of WFA+/-M2BP (HISCL, Sysmex Corporation, Hyogo, Japan). Additional surrogate blood indices of liver fibrosis assessed at enrollment included the fibrosis (Fib)-4 index, which was calculated as follows: (age [years] × aspartate aminotransferase [AST] [IU/L] / (platelet count [10^9/L] × alanine aminotransferase [ALT] [IU/L]$^{1/2}$) [16]. Serum WFA+/-M2BP levels were used as comparative controls for HCC prediction. In patients with SVRs, serum levels of ATX and WFA+/-M2BP were measured immediately before treatment (pre-treatment); immediately after the end of treatment (post-treatment); and at 12 weeks (post-12 w), 24 weeks (post-24 w), 12 months (post-12 mo), 24 months (post-24 mo), and 36 months (post-36 mo) after DAA therapy. Patients with less than 48 months (post-48 mo) of follow-up were included in the study up to the maximum follow-up period.

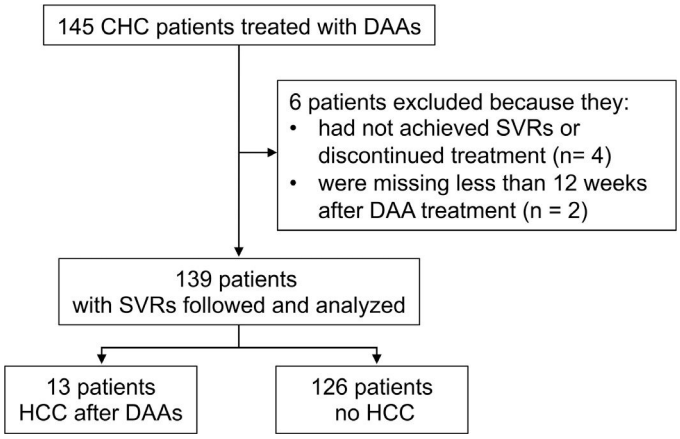

Fig. 1. Flow chart demonstrating the inclusion of patients with hepatitis C virus infection. *CHC*: chronic hepatitis C virus; *DAAs*, direct-acting antiviral agents; *HCC*, hepatocellular carcinoma; *SVRs*, sustained virological responses.

6. HCC Prediction

Cut-off values for the occurrence of HCC were calculated based on the serum concentrations of ATX and WFA+/-M2BP at post-24 w. The patients were divided into two groups: those whose serum concentrations were above the cut-off value and those whose concentrations were below it. The risk of developing HCC up to 48 months after DAA therapy was compared between the groups. The cumulative incidence of HCC according to serum levels of ATX and WFA+M2BP after DAA treatment was analyzed using the Kaplan−Meier method. The risk of HCC was calculated using a Cox proportional hazards model based on the occurrence of HCC and the months until the occurrence of HCC. HCC was diagnosed based on imaging, such as computed tomography (CT) or magnetic resonance imaging (MRI), and/or liver biopsy. When HCC was diagnosed, the observation was terminated.

7. Statistical analyses

Statistical analyses were conducted using Stata 16.0 Statistics for Windows (Stata Corp LLC, Texas, USA). Categorical variables are reported as frequencies and percentages.

Continuous variables were compared between the groups using the Mann−Whitney U-test. Changes in values in relation to treatment were analyzed using the Friedman test. The sensitivities and specificities of the serum fibrosis markers and fibrosis staging were calculated and assessed using the receiver operating characteristic (ROC) curves. The diagnostic performance of the scoring system was assessed by analyzing the ROC curves. An area under the ROC curve (AUC) close to 1.0 was considered to reflect a high level of diagnostic accuracy. The cumulative incidence of HCC was analyzed using the log-rank test for differences in the cut-off values of serum levels of ATX and WFA+/-M2BP.

Hazard ratios (HRs) for the risk of HCC were analyzed using Cox proportional hazards models (HR and 95% CI) adjusted for age [<65 years/≥65 years/], sex [male/female], ALT (<2 × the upper limit of normal [ULN] / ≥2 ULN), fibrosis stage [<3/≥3] at post-24 w, ATX [<cut-off value/≥cut-off value], WFA+/-M2BP [<cut-off value/≥cut-off value], and Fib-4 index [<cut-off value/≥cut-off value] at pre-treatment, post-12 w, post-24 w, and post-12 mo. First, we extracted factors with HR p-values of 0.2 or less in the univariate analysis; then, we performed a multivariate analysis using the factors identified by the univariate analysis. Fibrosis stage, ATX, WFA+/-M2BP, and Fib-4 index were analyzed independently using the Cox proportional hazards model, as they all had covariate factors reflecting cirrhosis and fibrosis staging as well as strong linearity. Statistical significance was set at $p < 0.05$.

Table 1
Clinical characteristics of CHC patients with HCC and non-HCC.

	Non-HCC		HCC		Total	
Number of patients	126		13		139	
	Median	(IQR)	Median	(IQR)	Median	(IQR)
Age, years	70	(61-77)	74	(67-76)	70	(62-77)
Male/Female, n	55/71		6/7		61/78	
BMI, kg/m^2	22.5	(20.4-24.2)	22	(21.1-23.9)	22.5	(20.5-24.2)
HCV genotype (1/2/unknown), n	10/3/0		101/22/3		111/25/3	
HCV-RNA, log IU/mL	6.3	(5.8-6.7)	6.1	(5.6-6.3)	6.25	(5.8-6.6)
T-Bil, mg/L	0.8	(0.7-1.0)	1.2	(0.8-1.3)	0.8	(0.7-1.1)
AST, IU/L	35	(27-50)	44	(38-58)	36	(28-50)
ALT, IU/L	31	(21-49)	41	(29-44)	31	(22-48)
Alb, g/L	4.1	(3.9-4.3)	3.5	(3.5-3.9)	4.1	(3.9-4.3)
Plt, 10^9/L	153	(111-187)	113	(80-160)	152	(108-187
Pre-treat ATX, male, mg/L	1.07	(0.83-1.58)	1.63	(1.10-2.27)	1.12	(0.9-1.63)
female, mg/L	1.75	(1.28-2.31)	2.60	(2.28-2.73)	1.79	(1.31-2.38)
Post-24w ATX, male, mg/L	1.02	(0.82-1.34)**	1.41	(1.31-1.55)*	1.06	(0.87-1.41)**
female, mg/L	1.58	(1.26-1.94)**	2.47	(1.90-2.63)ns	1.64	(1.31-1.98)**
Pre-treat WFA(+)-M2BP, male, COI	2.01	(1.04-3.33)	4.06	(3.60-6.55)	2.06	(1.13-3.89)
female, COI	1.86	(1.17-3.26)	4.01	(3.63-6.01)	2.12	(1.21-3.63)
Post-24w WFA(+)-M2BP, male, COI	0.96	(0.67-1.80)**	2.77	(2.29-2.84)*	1.07	(0.70-1.94)**
female, COI	1.32	(0.91-2.01)**	3.63	(2.86-4.11)ns	1.38	(0.92-2.44)**
Pre-treat E, kPa	7.7	(5.3-12.1)	14	(9.9-16.6)	8	(5.4-13.3)
Post-24w E, kPa	7.1	(5.3-10.6)**	12.05	(9.1-19.1)ns	7.6	(5.5-11.0)**
Pre-treat Fib-4 index	3.1	(2.0-4.5)	5.4	(3.4-8.3)	3.2	(2.1-4.9)
Post-24w Fib-4 index	2.7	(1.8-4.0)**	3.5	(2.1-6.0)*	2.7	1.9-4.1)**
Fibrosis Stage 1−2, n, %	43	(34.1)	10	(76.9)	86	(61.8)
Stage 3−4, n, %	83	(65.9)	3	(23.1)	53	(38.1)
Follow-up period after DAA, month	45	(30-48)	24	(18-36)	42	(30-48)

Comparison of serum levels of ATX, WFA+/-M2BP, E score, and Fib-4 index was analyzed between pre-treatment (pre-treat) and 24 weeks after DAA (post-24w) using the Wilcoxon signed-rank test, *; $p < 0.05$, **; $p < 0.01$, ns; no significant difference. BMI, body mass index; HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Plt, platelet; Alb, albumin; T-Bil, total bilirubin; ATX, autotaxin; *WFA+/-M2BP, Wisteria floribunda* agglutinin-positive mac-2-binding protein; COI, cut-off index; E, elastography of liver stiffness.

8. Results

8.1. Patient characteristics

In total, 6 of 145 patients with CHC enrolled in the study were excluded (Fig. 1). Therefore, data from 139 patients were included in the analysis. Table 1 shows the characteristics of the included patients. Among them, 61 (43.9%) were men and 78 (56.1%) were women, with a mean age of 70 years (interquartile range [IQR]: 62−77). The patients had the following HCV genotypes: 1 (n = 111), 2 (n = 25), and unknown (n = 3), with HCV-RNA levels >1.2 log IU/mL before treatment. HCC was found in 13 patients (9.3%) up to 48 months after DAA treatment. The number of patients at each point of follow-up decreased as follows: 139 (100%) at pre-treatment, 136 (97.8%) at post-12 mo, 120 (86.3%) at post-24 mo, 85 (61.1%) at post-36 mo, and 79 (56.8%) at post-48 mo.

All 139 patients who achieved SVR received antiviral therapy, including ombitasvir/paritaprevir/ritonavir (n = 53), glecaprevir/pibrentasvir (n = 28), sofosbuvir/ledipasvir (n = 23), sofosbuvir/ribavirin (n = 21), daclatasvir/asunaprevir (n = 8), ombitasvir/paritaprevir/ritonavir combined with ribavirin (n = 3), elbasvir/grazoprevir (n = 1), pegylated interferon and ribavirin combined with vaniprevir (n = 1), and simeprevir (n = 1).

8.2. Concentrations of serum fibrosis marker

The mean serum ATX concentration was significantly lower at post-24 w than at pre-treatment in both men and women. In addition, the WFA+/-M2BP and Fib4-index values decreased relative to the pre-treatment values at post-24 w (Table 1). Changes in the concentration of each marker from pre-treatment to post-36 mo are shown in Fig. 2. Serum ATX levels in patients with F1-2 did not change significantly after treatment when compared with pre-treatment; however, in patients with F3-4, serum ATX levels decreased significantly at post-12 mo in men and at post-24 w in women (Fig. 2a−d). Serum WFA+/-M2BP levels were significantly lower at post-12 mo than at pre-treatment in both men and women with F1-2. In addition, serum WFA+/-M2BP levels were significantly lower at post-24 w than at post-12 mo in both men and women with F3-4 (Fig. 2e−h).

The decreases in the concentrations of ATX and WFA+/-M2BP at each measurement point compared with the pre-treatment value are shown in Fig. 3. Among the patients with F1-2, the level of ATX at post-24 w was 94.2% (95% CI: 84.0−98.2) in men and 87.2% (95% CI: 82.6−95.6) in women compared with the pre-treatment levels. Among those with F3-4, the level of ATX was 82.8% (95% CI: 78.7−88.1) in men and 82.5% (95% CI: 71.7−91.6) in women. Among patients with F1-2, the level of WFA+/-M2BP at post-24 w was 61.7% (95% CI: 44.5−75.0) in men and 73.7% (95% CI: 61.9−79.0) in women compared with the pre-treatment levels. Among those with F3-4, it was 56.1% (95% CI: 39.8−65.0) in men and 65.13% (95% CI: 54.2−75.6) in women.

8.3. Predictability of HCC

Fig. 4 shows the results of the ROC analysis of carcinogenicity using ATX and WFA+/-M2BP in men and women before treatment and at post-12 w, post-24 w, and post-12 mo. The cut-off values for ATX at each post-treatment time point were lower than those at pre-treatment, although they were almost the same at post-12 w and post-24 w (Fig. 4b−c, f−g). In contrast, for WFA+/-M2BP, the cut-off values continued to decrease compared with the pre-treatment values (Fig. 4j−k, n−o). The cut-off values for ATX were 1.22 mg/L and 1.92 mg/L at post-24 w in men and women, respectively; while the AUC values were 0.83 (95% CI: 0.71−0.95) and 0.90 (95% CI: 0.82−0.99) in men and women, respectively (Fig. 4c, g). The cut-off values

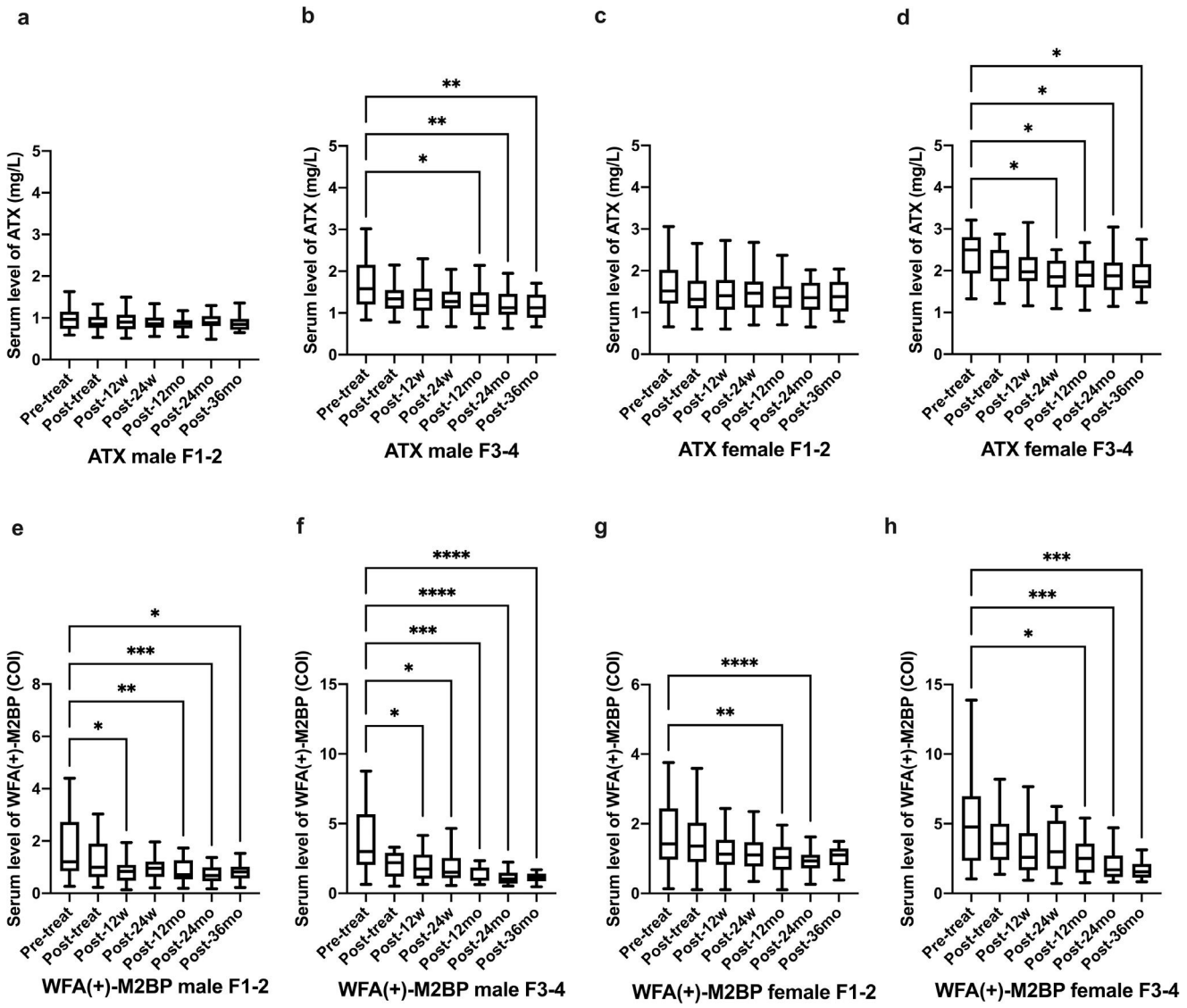

Fig. 2. Changes in serum levels of ATX and WFA+/-M2BP from pre-treatment to 36 months after DAA.

(a) ATX in male patients with F1-2. (b) ATX in male patients with F3-4. (c) ATX in female patients with F1-2. (d) ATX in female patients with F3-4. (e) WFA+/-M2BP in male patients with F1-2. (f) WFA+/-M2BP in male patients with F3-4. (g) WFA+/-M2BP in female patients with F1-2. (h) WFA+/-M2BP in female patients with F3-4.

F: fibrosis stage at pre-treatment, *ATX*: autotaxin, *DAA*: direct-acting antiviral agent, WFA+/-M2BP, *Wisteria floribunda* agglutinin-positive Mac-2-binding protein. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.001$.

of WFA+/-M2BP were 1.63 and 1.53 for men and women, respectively, at post-24 w, with AUC values of 0.88 (95% CI: 0.79−0.98) and 0.85 (95% CI: 0.74−0.97) in men and women, respectively (Fig. 4 k, o). Since the highest AUC value was observed at post-24 w, the cut-off value at post-24 w was used for subsequent analyses.

Fig. 5 shows the incidence of HCC among the observation periods using the cut-off value observed at post-24 w. The overall incidence of carcinogenesis was 9.3% (13/139) (Fig. 5a). Among 38 patients with an ATX level above the cut-off value, HCC was observed in 26.3% (10/38) over the entire period, while the rate was 3.0% (3/101) in patients with an ATX level below the cut-off value (Fig. 5b). The positive predictive value (PPV) of ATX for HCC was 0.208 (95% CI: 0.139 −0.248), while the negative predictive value (NPV) was 0.971 (95% CI: 0.939−0.990). In contrast, HCC was observed in 6.1% (7/114) of patients whose WFA+/-M2BP values were above the cut-off and 24.0% (6/25) of patients whose values were below it (Fig. 5c). The PPV of WFA+/-M2BP was 0.058 (95% CI: 0.033−0.082), while the NPV was 0.806 (95% CI: 0.709−0.899). The cumulative incidence of HCC was significantly higher than the respective cut-off values for ATX and WFA+/-M2BP ($p < 0.0001$ and $p = 0.0003$, respectively).

Changes in the false-positive rate (1-specificity) and false-negative rate (1-sensitivity) at post-12 mo, post-24 mo, and post-36 mo were analyzed using the cut-off value observed at post-24 w to assess the long-term predictability of HCC. There were no differences in the false-positive rates (Fig. 5d); however, the false-negative rate of ATX was maintained above 0.8 from post-12 mo to post-36 mo, while that of WFA+/-M2BP decreased over time (Fig. 5e).

8.4. Cox proportional hazards analysis of HCC occurrence

When the ATX level was above the post-24 w cut-off value, the HR for HCC determined via the multivariate analysis was 13.09 (95% CI: 2.86−59.94, $p = 0.001$), and age ≥65 years was not a significant factor (Table 2). In the univariate analysis for WFA+/-M2BP, values above the cut-off indicated an HCC risk with a HR of 6.39 (95% CI: 2.13 −19.10, $p = 0.001$). HRs for fibrosis stage ≥3 and the Fib-4 index were

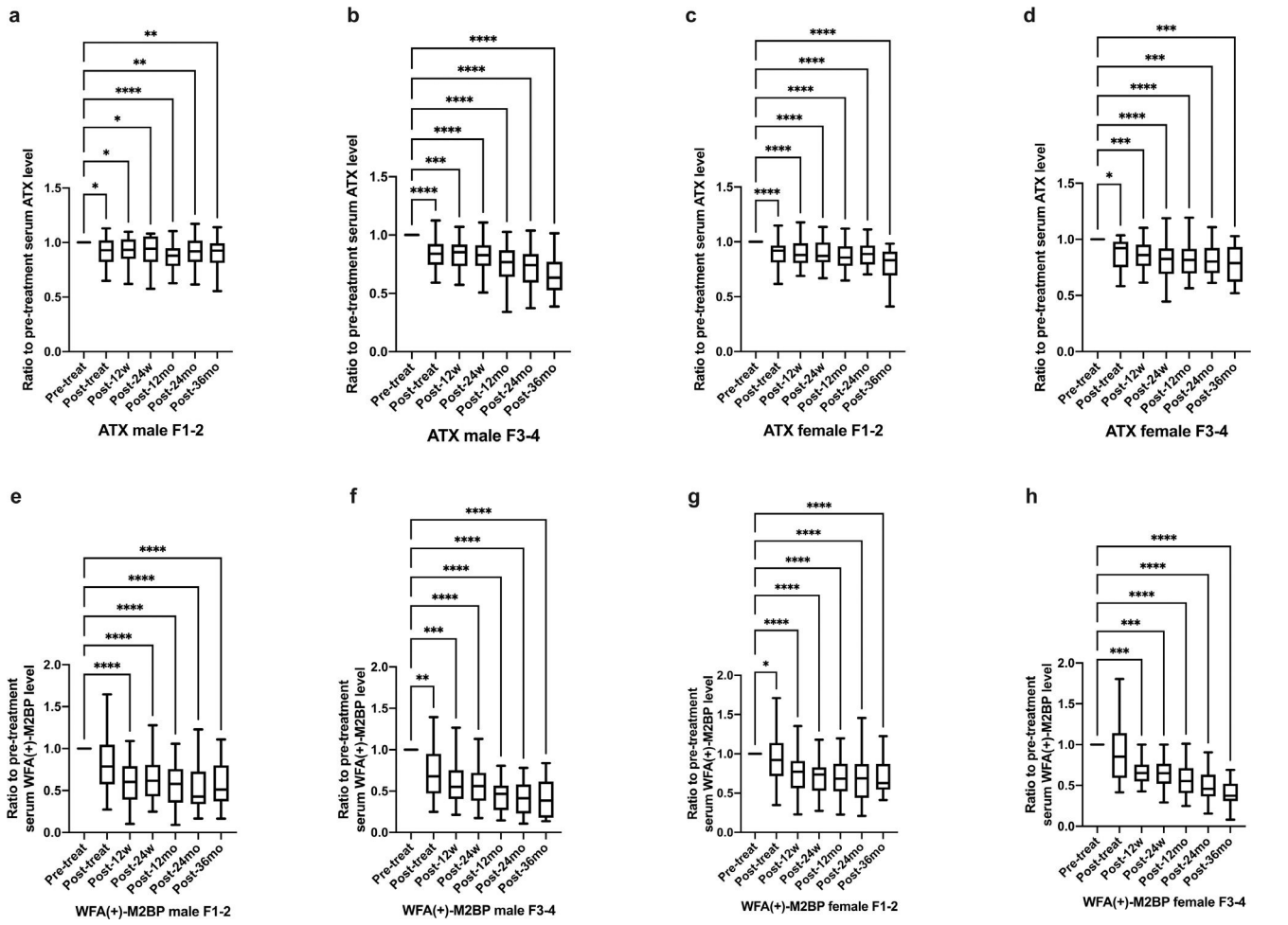

Fig. 3. The ratio of serum levels of ATX and WFA+/-M2BP at each time point when compared with pre-treatment levels. (a) Serum ATX levels in male patients with F1-2. (b) ATX in male patients with F3-4. (c) ATX in female patients with F1-2. (d) ATX in female patients with F3-4. (e) WFA+/-M2BP in male patients with F1-2. (f) WFA+/-M2BP in male patients with F3-4. (g) WFA+/-M2BP in female patients with F1-2. (h) WFA+/-M2BP in female patients with F3-4.

F: fibrosis stage at pre-treatment, *ATX*: autotaxin, WFA+/-M2BP*: Wisteria floribunda* agglutinin-positive Mac-2-binding protein. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.001$.

10.05 (95% CI: 1.27−79.43, $p = 0.029$) and 1.79 (95% CI: 0.57−5.57, $p = 0.183$), respectively.

9. Discussion

Early detection of HCC in the follow-up period after DAA treatment is critical for improving the prognosis in patients with hepatitis C, as are studies that track and compare serum levels of ATX and WFA+/-M2BP after long-term treatment for HCV. A high expression of ATX is associated with various cancers such as HCC, breast [17, 18], pancreatic [19], colorectal [20], and lung cancer [21]. In addition, ATX has been suggested to have a functional interaction with vascular endothelial growth factor receptors 2 and 3 (VEGFR-2 and VEGFR-3), which regulate the activation of cells in blood vessels and lymphatic vessels during vascular development and may promote carcinogenesis [22–24]. We believe that ATX is not only a biomarker for liver fibrosis but may also be useful in detecting cancers with excessive angiogenesis. ATX may act as a docking molecule for LPA and may be involved in metastasis via binding to adhesion molecules such as integrins on the cell surface [25–27]. Therefore, high ATX levels may be a precursor to carcinogenesis; elucidating the direct relationship between ATX and carcinogenesis may help to clarify this.

In this study, we investigated changes in the levels of ATX and WFA+/-M2BP before and after DAA therapy using the ROC analysis to determine their association with the occurrence of HCC. The largest AUC value relative to the pre-treatment value was observed at post-24 w, followed by post-12 w and post-12 mo. Therefore, we considered the serum ATX level at post-24 w to be an indicator of the incidence of HCC. Our findings indicated that when ATX levels at post-24 w were ≥1.22 (men) and ≥1.92 mg/mL (women), the risk of developing HCC until post-36 mo increased approximately 13-fold. This suggests that if high ATX levels are maintained after HCV treatment, the risk of developing HCC remains high despite the elimination of HCV.

In general, inflammation caused by HCV results in higher ATX and WFA+/-M2BP levels, which decrease after treatment [28, 29]. In this study, the ATX and WFA+/-M2BP values were highly variable immediately after DAA treatment, although they converged after the end of the treatment. Therefore, it may be best to wait for marker values to converge with the inflammation caused by HCC before attempting to predict the risk of HCC. In patients with F1-2, we observed that ATX levels remained constant from post-24 w to post-36 mo. This suggests that the metabolism of ATX in hepatic sinusoidal endothelial cells (which had been suppressed by inflammation) improved following DAA treatment, indicating stabilization and maintenance of sinusoidal endothelial cells and the surrounding hepatocytes.

However, the levels of WFA+/-M2BP decreased over time, suggesting that they require a long period to stabilize during the recovery process after HCV elimination. Therefore, when using WFA +/-M2BP as a marker of HCC, the time elapsed since DAA treatment

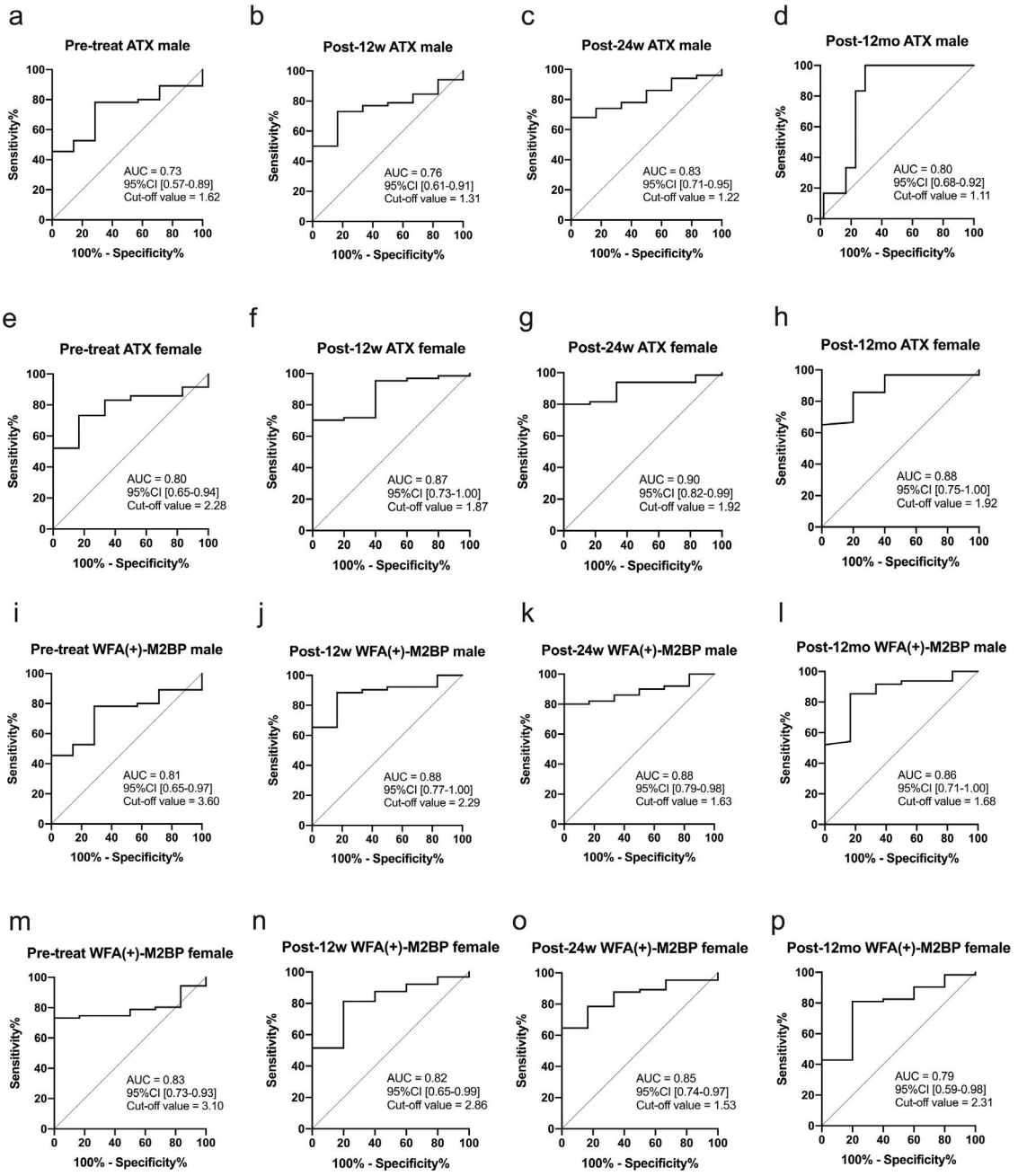

Fig. 4. The AUC and cut-off values of ATX and WFA+/-M2BP from pre-treatment to 12 months after DAA therapy in men and women, as determined by the ROC analysis. (a-h) ROC analysis of ATX. (i-p) ROC analysis of WFA+/-M2BP.

AUC, area under the curve; *DAA*, direct-acting antiviral agent; *ROC*, receiver operating characteristic; *ATX*, autotaxin; WFA+/-M2BP, *Wisteria floribunda* agglutinin-positive Mac-2-binding protein.

should be considered. Moreover, the sustained decline in the levels of WFA+/-M2BP after DAA treatment with liver inflammation may affect the predictability of HCC. The long-lasting decrease in the levels of WFA+/-M2BP may require that the cut-off value for HCC be reviewed at regular intervals. Specifically, several cut-off values for WFA +/-M2BP may be required depending on the time elapsed since DAA treatment for each patient. However, previous studies have reported a strong correlation between the predictability of HCC and WFA +/-M2BP levels [30–32]. Moreover, Takemura et al. reported that WFA+/-M2BP performed better than ATX, although the difference in the AUC value post-SVR was marginal (ATX: 0.76 vs. WFA+/-M2BP: 0.81) [33]. These findings indicate that ATX is non-inferior to WFA +/-M2BP for the prediction of HCC and can be selected in clinical practice as needed.

Serum ATX levels were relatively higher in women than in men, although the mechanism underlying this difference remains unclear. Adipocytes contain a large amount of ATX, and adipose tissue occupies a larger volume in women than in men [34]; however, previous studies have reported that there is no correlation between ATX levels and BMI [35].

The present study has some limitations. First, a liver biopsy was not performed to assess liver fibrosis because most patients were older adults and a liver biopsy was not included in the protocol. Instead, the staging of liver fibrosis was based on TE, because the findings have been shown to correlate with those of liver biopsy. Second, given the length of the observation period, it was not possible to follow up all the patients until the end of the study. Third, this was a single-center study, which resulted in a small sample size. It is also

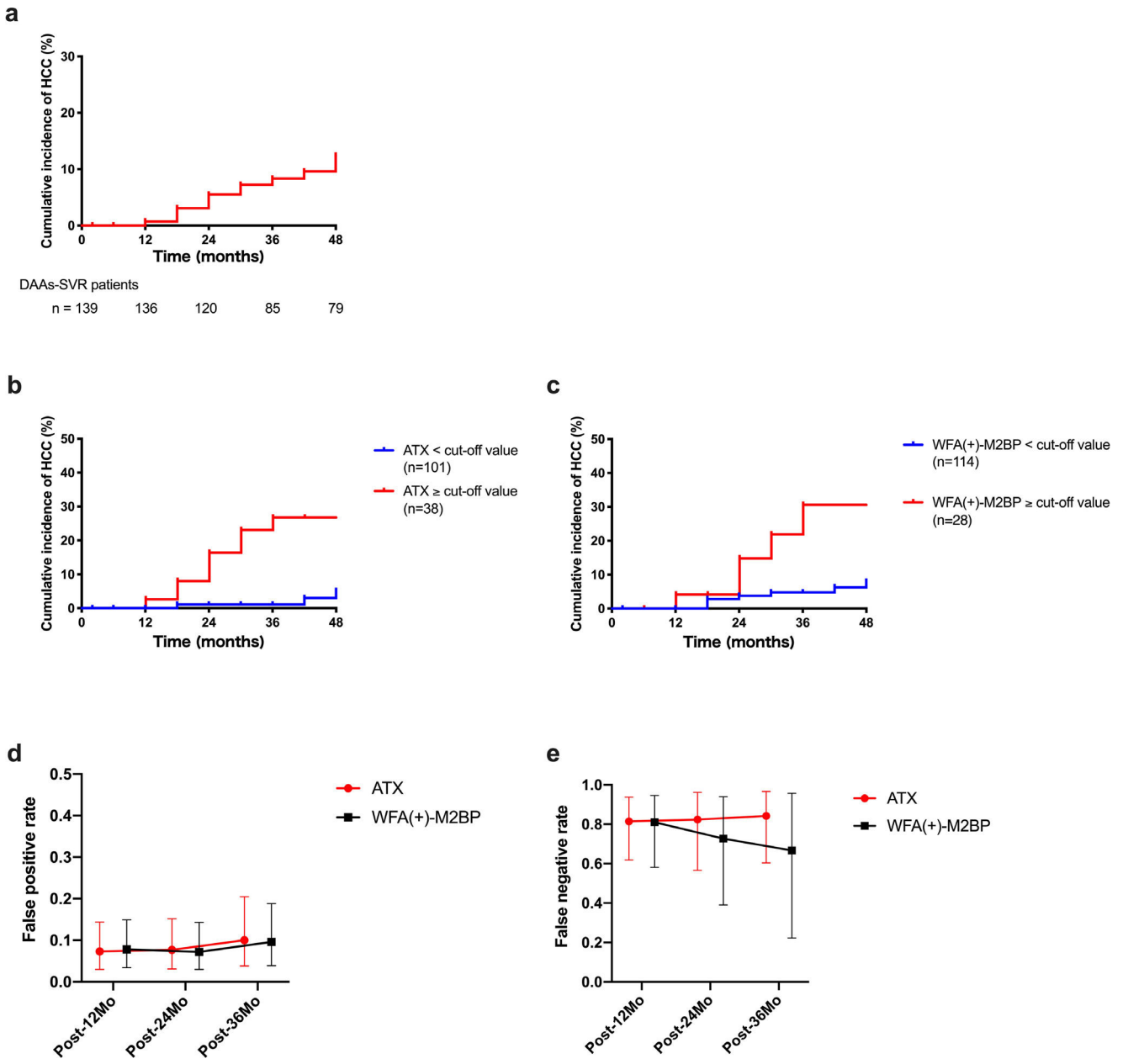

Fig. 5. The cumulative incidence of hepatocellular carcinoma (HCC) from the end of treatment to 48 months after DAA. The incidence of HCC from the end of DAA to 48 months was analyzed using each cut-off value of ATX and WFA+/-M2BP at 24 weeks after DAA. The cumulative incidence of HCC based on the levels of ATX or WFA+/-M2BP after DAA treatment was analyzed using the Kaplan–Meier method. (a) All the patients with CHC and the number of patients followed-up at each point as shown at the bottom, (b) the cut-off value of ATX after 24 weeks of DAA, (c) the cut-off value of WFA+/-M2BP after 24 weeks of DAA, (d) false-positive rate (1- specificity) for ATX and WFA+/-M2BP, (e) false-negative rate (1-sensitivity) for ATX and WFA+/-M2BP.

ATX, autotaxin; WFA+/-M2BP, *Wisteria floribunda* agglutinin-positive Mac-2-binding protein; *SVRs*, sustained virological responses; *DAAs*, direct-acting antiviral agents; *CHC*, chronic hepatitis C.

important to note that this study did not include a control group of patients with HCV who did not undergo DAA treatment. The reason for this is that DAA treatment is more likely to result in a SVR, in addition to the availability of antiviral drugs that can be administered to pan-genotypes and in non-compensated cirrhosis. Furthermore, there are ethical concerns regarding the long-term observation of patients with untreated HCV. Future studies should also focus on including difficult-to-treat cases for which DAA treatment has not been administered or is contraindicated (e.g., dialysis, serious complications, or other tumor-related complications) among the untreated controls. Lastly, we did not investigate HCC-related deaths, as it would have been difficult to determine whether the death was caused by liver failure due to advanced cirrhosis or HCC. The sample size was also insufficient for clarifying the relationship between HCC-related deaths and markers of liver fibrosis.

In conclusion, the present results indicate that ATX may be a useful marker in the long-term prediction of HCC development after DAA treatment in patients with CHC. However, it is difficult to accurately detect HCC using markers of liver fibrosis alone. Therefore, future studies should examine the predictive power of HCC alone and in combination with other factors over multiple and longer periods.

Table 2
Factors affecting the incidence HCC analyzed using Cox proportional hazards model.

		Univariate analysis			Multivariate analysis		
		HR	95%CI	p value	HR	95%CI	p value
Age	<65 years	1					
	≥65 years	5.01	[0.65—38.55]	0.12	4.27	[0.55-33.22]	0.165
Sex	Male	1					
	Female	0.73	[0.25—2.19]	0.58			
ALT in post-24w	< 2 × ULN	1					
	≥ 2 × ULN	0.92	[0.12—7.16]	0.933			
Fibrosis stage in pre-treat	< 3	1					
	≥ 3	5.50	[1.51—20.03]	0.01			
Fibrosis stage in post-24w	< 3	1					
	≥ 3	10.05	[1.27—79.43]	0.029			
ATX in pre-treat	< cut-off value	1					
	≥ cut-off value	3.78	[1.23—11.61]	0.02			
ATX in post-12w	< cut-off value	1					
	≥ cut-off value	4.46	[1.35—14.64]	0.014			
ATX in post-24w	< cut-off value	1					
	≥ cut-off	13.17	[2.88—60.23]	0.001	13.09	[2.86-59.94]	0.001
ATX in post-12mo	< cut-off value	1					
	≥ cut-off	7.00	[2.04—24.01]	0.002			
WFA(+)-M2BP pre-treat	< cut-off value	1					
	≥ cut-off value	4.75	[1.55—14.56]	0.006			
WFA(+)-M2BP in post-12w	< cut-off value	1					
	≥ cut-off value	4.27	[1.43—12.74]	0.009			
WFA(+)-M2BP in post-24w	< cut-off value	1					
	≥ cut-off value	6.39	[2.13—19.10]	0.001			
WFA(+)-M2BP in post-12mo	< cut-off value	1					
	≥ cut-off value	5.90	[1.62—21.79]	0.007			
Fib4-index in pre-treat	< 3	1					
	≥ 3	3.42	[0.94—12.44]	0.062			
Fib4-index in post-12w	< 3	1					
	≥ 3	2.24	[0.68—7.34]	0.183			
Fib4-index in post-24w	< 3	1					
	≥ 3	1.79	[0.57—5.57]	0.31			
Fib4-index in post-12mo	< 3	1					
	≥ 3	2.71	[0.79—9.28]	0.111			

The cut-off values of ATX and WFA+/-M2BP in the univariate analysis at each point are shown in Fig. 4. Multivariate analysis was performed for ATX levels post-24w with the highest HR and age ≥65 years. Fibrosis stage, WFA+/-M2BP, and Fib-4 index were excluded from the multivariate analysis because of their high collinearity.
HR: hazard ratio, 95% CI: 95% confidence interval, ULN: upper limit of normal.

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Availability of data: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

Satoshi Shimamoto and Koji Igarashi are employees of Tosoh Corporation. The other authors declare that they have no conflicts of interest.

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