

## Article

# 12-Hydroxyheptadecatrienoic Acid Predicts Hepatocellular Carcinoma Development During Nucleos(t)ide Analogue Therapy

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## Simple Summary

Even with long-term antiviral therapy for chronic hepatitis B, some patients still develop liver cancer. We investigated whether polyunsaturated fatty acid metabolites—bioactive lipids involved in inflammation and other processes—could predict this risk before antiviral treatment begins. We measured 158 of these metabolites in pre-treatment blood samples from 195 patients starting nucleos(t)ide analogue therapy and followed them. Low levels of 12-hydroxyheptadecatrienoic acid (12-HHT) were strongly linked to liver cancer development. Patients with low 12-HHT had a 4.28-fold higher risk of liver cancer development than those with higher levels. Prediction improved further when 12-HHT was combined with the fibrosis-4 (FIB-4) index, a routine measure of liver scarring. Over 10 years of follow-up, about two-thirds of patients with both high FIB-4 and low 12-HHT developed liver cancer compared with about 1% of those with low FIB-4 and high 12-HHT. If confirmed, this marker could support personalised surveillance and help target prevention during long-term antiviral therapy.

## Abstract

**Background/Objectives:** Alterations in polyunsaturated fatty acid (PUFA) metabolites have been linked to the development of hepatocellular carcinoma (HCC). However, the association between PUFA metabolites and HCC development during nucleos(t)ide analogue (NUC) therapy in patients with chronic hepatitis B virus infection remains unclear. **Methods:** This study enrolled 195 NUC-naïve patients who received NUC therapy. Associations between metabolic factors—especially PUFA metabolites—and HCC development during NUC therapy were evaluated. Baseline serum concentrations of 158 PUFA metabolites were quantified using targeted lipidomic analysis. **Results:** Nineteen patients developed HCC during the follow-up period. The cumulative incidences of HCC at 5 and 10 years were 7.7% and 12.4%, respectively. Variable importance in projection analysis identified 12-hydroxyheptadecatrienoic acid (12-HHT) as the top-ranked metabolite differentiating patients with and without HCC development. Furthermore, 14 metabolites were significantly associated with HCC development based on the log-rank test with 12-HHT being the most significant predictor. The cumulative incidences of HCC at 5 and 10 years were 13.7% and 24.7%, respectively, in patients with 12-HHT concentration  $\leq 3.82$  ng/mL compared with 3.3% at both time points in those with 12-HHT concentration  $> 3.82$  ng/mL ( $p < 0.001$ ).



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In multivariate analysis, low 12-HHT concentration ( $\leq 3.82$  ng/mL;  $p = 0.027$ ; hazard ratio [HR], 4.28; 95% confidence interval [CI], 1.18–15.55) and a fibrosis-4 index  $\geq 4.08$  ( $p = 0.005$ ; HR, 5.19; 95% CI, 1.64–16.41) were significantly associated with HCC development during NUC therapy. **Conclusions:** Pre-treatment 12-HHT represents a novel predictive biomarker for HCC development during NUC therapy.

**Keywords:** biomarker; chronic hepatitis B; fibrosis-4 index; hepatocellular carcinoma; lipidomic analysis; oxylipins; polyunsaturated fatty acids; viral hepatitis

## 1. Introduction

Hepatitis B virus (HBV) infection affects approximately 254 million people worldwide and remains a leading cause of cirrhosis and hepatocellular carcinoma (HCC), resulting in an estimated 1.1 million deaths in 2022 [1].

Currently, nucleos(t)ide analogues (NUCs) including entecavir and tenofovir are widely prescribed for patients with chronic HBV infection [2]. NUC therapy for chronic HBV infection has been shown to suppress viral replication and reduce the risk of HCC development [3,4]. However, some patients develop HCC despite receiving effective NUC therapy. Therefore, identifying risk factors for HCC before initiating NUC therapy is clinically important in patients with chronic HBV infection. Risk factors for HCC development have been broadly classified into host, viral, and environmental categories [5–10]. Among environmental factors, metabolic conditions such as obesity and diabetes mellitus have been identified as risk factors for HCC development in patients with chronic HBV infection [3,11,12].

Notably, alterations in lipid metabolites have been closely linked to HCC development among metabolic factors [13]. Previous studies have demonstrated alterations in several lipid metabolites in patients with HBV-related HCC. Du et al. reported decreased levels of several lysophosphatidylcholine species and elevated levels of lysophosphatidic acid species in patients with chronic HBV infection who developed early-stage HCC [14]. Similarly, Abel et al. reported that alterations in membrane cholesterol, phospholipids, and fatty acid profiles are likely to play important roles in HCC progression [15].

Polyunsaturated fatty acids (PUFAs) are important components of cell membranes primarily derived endogenously from phospholipids and play critical roles in various biological processes including both pro- and anti-inflammatory effects [16]. PUFAs are metabolised through cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) pathways to produce a broad range of bioactive lipid mediators—prostaglandins, leukotrienes, and epoxyeicosatrienoic acids, respectively [17]. In HCC, alterations in PUFA metabolites have been identified as potential therapeutic targets [18]. Furthermore, lipidomic analyses have identified alterations in patients with HBV-related HCC, suggesting their potential utility as biomarkers [19,20].

However, the association between PUFA metabolites and HCC development during NUC therapy in patients with chronic HBV infection remains unclear. Therefore, we assessed the associations between metabolic factors, especially PUFA metabolites, and HCC development during NUC therapy.

## 2. Materials and Methods

### 2.1. Patients

A total of 195 patients with chronic HBV infection who initiated NUC therapy between September 2006 and July 2023 at Osaka Metropolitan University Hospital. For

whom stored serum samples collected before the initiation of therapy were available were included in this retrospective study. Patients who were NUC-naïve and had chronic HBV infection—defined as testing positive for hepatitis B surface antigen (HBsAg) and HBV DNA for at least 6 months before initiating therapy—were treated with entecavir, tenofovir alafenamide, or tenofovir disoproxil fumarate. The inclusion criteria were persistent elevation of serum alanine aminotransferase (ALT) ( $\geq 31$  U/L) and HBV DNA levels ( $\geq 4.0$  log copies/mL; equivalent to 3.3 log IU/mL) or advanced fibrosis even when ALT levels were within the normal range in accordance with published guidelines [2,21,22]; absence of clinical signs of HCC before initiating NUC therapy; and no evidence of co-infection with hepatitis C virus, human immunodeficiency virus, or other identifiable causes of chronic liver disease.

This study was conducted in accordance with the principles of the 2013 Declaration of Helsinki. Written informed consent was obtained from all patients before initiation of NUC therapy. The study protocol was approved by the Ethics Committee of Osaka Metropolitan University Hospital (approval numbers 1646, 3260, and 4361).

## 2.2. Study Design

Among the 195 patients, 157 were treated with entecavir, 13 with tenofovir alafenamide, and 25 with tenofovir disoproxil fumarate for more than one year. Entecavir (Baraclude; Bristol-Myers, Tokyo, Japan) was administered orally at a daily dose of 0.5 mg. Tenofovir disoproxil fumarate (Vemlidy; Gilead Sciences, Tokyo, Japan) was administered orally at a daily dose of 25 mg. Tenofovir alafenamide (Tenozet; GlaxoSmithKline, Tokyo, Japan) was administered orally at a daily dose of 300 mg.

Clinical, biochemical, and HBV serological assessments were performed at intervals of one to three months during follow-up. Cirrhosis was diagnosed by histological examination (F4 stage) according to the METAVIR scoring system [23] supported by imaging findings from ultrasonography, computed tomography (CT), or magnetic resonance imaging (MRI), and by the presence of portal hypertension defined by clinical features such as oesophageal or gastric varices or ascites. Steatotic liver disease (SLD) was diagnosed by trained sonographers based on ultrasonographic findings including a bright liver, hepatorenal echo contrast, deep attenuation, and vessel blurring [24].

## 2.3. Hepatocellular Carcinoma Surveillance

The study endpoint was HCC development during NUC therapy. Patients who developed HCC within one year after initiating NUC therapy were excluded. All patients underwent ultrasonography or dynamic CT or MRI every 3–6 months for HCC surveillance. HCC was diagnosed by percutaneous needle biopsy or by characteristic imaging findings of arterial phase hyperenhancement and delayed washout on dynamic CT or MRI. Patients were followed up until the diagnosis of HCC was confirmed or until their last clinical visit before October 2024.

## 2.4. Laboratory Assays

Complete blood counts and serum measurements of aspartate aminotransferase (AST), ALT, gamma-glutamyl transferase, total bilirubin, and albumin levels were obtained using standard laboratory procedures. Serum  $\alpha$ -fetoprotein (AFP) concentrations were determined using a chemiluminescent enzyme immunoassay. The fibrosis-4 (FIB-4) index was calculated using Sterling's formula:  $\text{age (years)} \times \text{AST (IU/L)} / [\text{platelet count} (\times 10^9/\text{L}) \times \sqrt{\text{ALT (IU/L)}}]$ . Hepatitis B core-related antigen (HBcrAg) was quantified using a novel ultrasensitive assay, the "immunoassay for total antigen including complex via pre-treatment" (Fuji-Rebio, Tokyo, Japan) [9]. HBsAg was quantified using a chemiluminescent microparticle immunoassay (Architect HBsAg QT; Abbott Japan Corp., Tokyo, Japan).

HBV DNA was quantified by real-time polymerase chain reaction assay (COBAS TaqMan HBV Test, ver. 2.0; Roche Diagnostics K.K., Tokyo, Japan). HBV genotype was determined using an enzyme-linked immunosorbent assay employing monoclonal antibodies specific to epitopes in the preS2 region (Institute of Immunology, Tokyo, Japan).

### 2.5. Analysis of the Serum Lipidome

Serum samples (30 µL) were diluted with 300 µL of 0.1% formic acid (Fujifilm Wako, Osaka, Japan; 067-04531) in methanol (Fujifilm Wako; 138-14521) containing an internal standard mixture of 10 ng/mL prostaglandin (PG) E2-d4 (Cayman Chemical, Ann Arbor, MI, USA; 314010), 10 ng/mL leukotriene (LT) B4-d4 (Cayman Chemical; 320110), and 100 ng/mL arachidonic acid (AA)-d8 (Cayman Chemical; 390010). The mixture was then centrifuged at  $15,000\times g$  for 10 min to remove insoluble materials. The resulting supernatant was loaded onto Strata-X extraction cartridges (Phenomenex, Torrance, CA, USA; 8B-S100-AAK) for purification, evaporated using a centrifugal evaporator (SpeedVac, Thermo Fisher Scientific, Waltham, MA, USA), and reconstituted in 30 µL of methanol. Finally, 5 µL of each of the reconstituted samples was analysed using an LC/MS system (LCMS-8060, Shimadzu, Kyoto, Japan) comprising a Nexera™ X2 unit (Shimadzu) coupled to a mass spectrometer (MS). The concentrations of 158 polyunsaturated fatty acid metabolites were estimated using a triple quadrupole MS (Shimadzu) and the LC/MS/MS Method Package for Lipid Mediators Ver. 2 (Shimadzu). Peak deconvolution was performed using the Traverse MS software, version 1.2.9 (Reifycs Inc., Tokyo, Japan). In addition, 12-hydroxyheptadecatrienoic acid (12[S]-HHTre [12-HHT] [Cayman Chemical; 34590]) was quantified using a calibration curve.

### 2.6. Statistical Analysis

Statistical analyses were performed using R statistical software, version 4.2.3. Baseline characteristics between groups were compared using the  $\chi^2$  test for categorical variables and the Mann–Whitney U test for continuous variables. Receiver operator curves were generated for each variable to determine optimal cut-off values distinguishing patients with and without HCC during NUC therapy. Multivariate analyses were performed using the MetaboAnalyst 6.0 platform ([www.metaboanalyst.ca](http://www.metaboanalyst.ca) (accessed on 10 August 2025)). Metabolite peak areas were  $\log_2$ -transformed and auto-scaled for normalisation. Partial least squares discriminant analysis (PLS-DA) was conducted to visualise the separation between patients with and without HCC development. Variable importance in projection (VIP) scores were used to identify metabolites contributing to group separation. Kaplan–Meier analysis and the log-rank test were used to estimate and compare cumulative incidences of HCC development between the two groups. *p* values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate procedure. Cox proportional hazard models were applied to analyse factors associated with HCC development. Correlation significance was evaluated by Spearman’s rank analysis. All reported *p*-values were two-sided, with statistical significance set at *p*-value < 0.05.

## 3. Results

### 3.1. Baseline Characteristics of the Patients

Baseline patient characteristics are summarised in Table 1. The median age of the patients was 46.0 years (interquartile range [IQR], 39.0, 56.0). The cohort included 117 (60.0%) males, 31 (15.9%) with cirrhosis, 12 (6.2%) with diabetes, and 58 (29.7%) with SLD. A total of 167 patients (85.6%) had HBV genotype C. The median follow-up duration was 7.4 years (range: 1.0–17.9).

**Table 1.** Baseline characteristics of the patients.

| Variables                            | Total<br>(n = 195) | No HCC Development<br>(n = 176) | HCC Development<br>(n = 19) | p Value    |
|--------------------------------------|--------------------|---------------------------------|-----------------------------|------------|
| Age (yr)                             | 46.0 [39.0, 56.0]  | 44.5 [37.8, 56.0]               | 55.0 [48.0, 62.5]           | 0.002 **   |
| Sex: Male                            | 117 (60.0)         | 103 (58.5)                      | 14 (73.7)                   | 0.20       |
| BMI (kg/m <sup>2</sup> )             | 22.4 [20.8, 25.0]  | 22.4 [20.5, 24.9]               | 22.5 [21.9, 25.7]           | 0.23       |
| Cirrhosis                            | 31 (15.9)          | 21 (11.9)                       | 10 (52.6)                   | <0.001 *** |
| Alcohol intake                       | 27 (13.8)          | 23 (13.1)                       | 4 (21.1)                    | 0.34       |
| Diabetes                             | 12 (6.2)           | 9 (5.1)                         | 3 (15.8)                    | 0.079      |
| Steatotic liver disease              | 58 (29.7)          | 56 (31.8)                       | 2 (10.5)                    | 0.054      |
| Platelet count (10 <sup>3</sup> /μL) | 173 [134, 214]     | 181 [138, 216]                  | 115 [72, 151]               | <0.001 *** |
| FIB-4 index                          | 1.99 [1.33, 3.28]  | 1.90 [1.29, 2.99]               | 4.25 [2.37, 6.74]           | <0.001 *** |
| AST (U/L)                            | 66 [42, 122]       | 63 [42, 125]                    | 67 [45, 91]                 | 0.80       |
| ALT (U/L)                            | 87 [54, 201]       | 94 [54, 206]                    | 74 [52, 117]                | 0.26       |
| GGT (U/L)                            | 46 [26, 95]        | 45 [25, 92]                     | 57 [37, 112]                | 0.22       |
| Total bilirubin (mg/dL)              | 0.80 [0.60, 1.05]  | 0.80 [0.60, 1.00]               | 0.90 [0.70, 1.15]           | 0.36       |
| Albumin (g/dL)                       | 4.10 [3.80, 4.30]  | 4.10 [3.80, 4.40]               | 4.00 [3.80, 4.10]           | 0.122      |
| AFP (ng/mL)                          | 4.6 [2.9, 10]      | 4.5 [2.7, 8.2]                  | 10.9 [6.5, 29.9]            | <0.001 *** |
| HBsAg (log IU/mL)                    | 3.55 [3.08, 4.03]  | 3.64 [3.17, 4.06]               | 3.03 [2.86, 3.52]           | 0.001 **   |
| HBcrAg (log U/mL)                    | 5.90 [4.40, ≥7.1]  | 6.10 [4.40, ≥7.1]               | 5.30 [4.60, 6.05]           | 0.140      |
| HBV DNA (log copies/mL)              | 7.30 [6.00, 8.70]  | 7.40 [6.10, 8.80]               | 6.20 [5.35, 7.20]           | 0.010 *    |

Values are reported as n (%) or median [IQR]. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . AFP,  $\alpha$ -fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FIB-4 index, fibrosis-4 index; GGT, gamma-glutamyl transferase; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IQR, interquartile range.

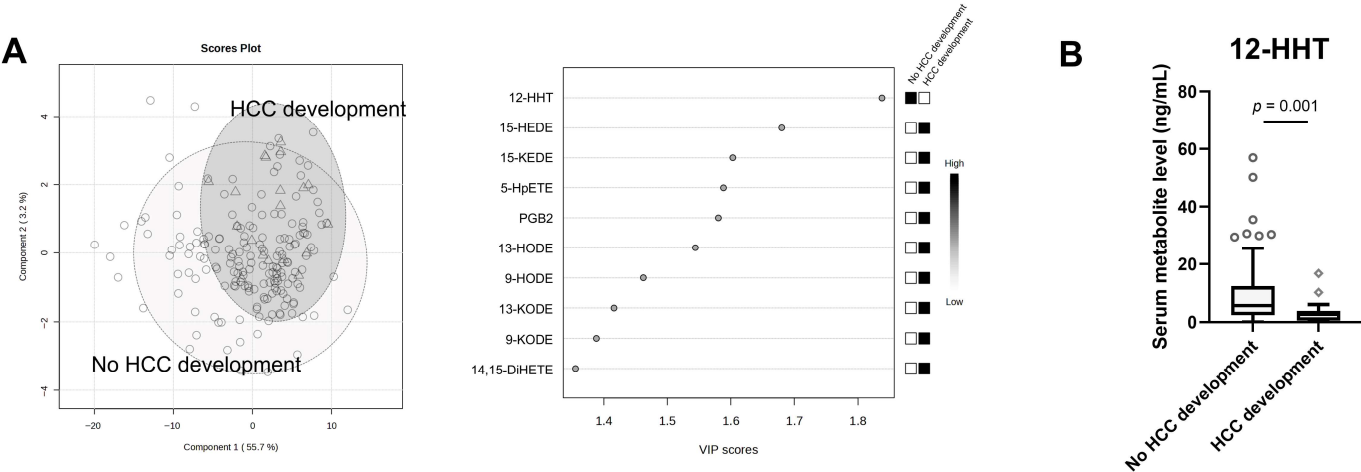
### 3.2. Cumulative Rates of HCC Development According to Clinical Factors at Baseline

During follow-up, 19 patients developed HCC, with a median duration, 4.3 years (range, 1.1–12.4). The cumulative incidence of HCC at 5 and 10 years were 7.7% and 12.4%, respectively (Figure A1A). Based on log-rank testing, age  $\geq 47$  years ( $p < 0.001$ ), cirrhosis ( $p < 0.001$ ), platelet count  $\leq 141 \times 10^3/\mu\text{L}$  ( $p < 0.001$ ), FIB-4 index  $\geq 4.08$  ( $p < 0.001$ ), AFP  $\geq 6.4$  ng/mL ( $p < 0.001$ ), HBsAg  $\leq 3.37$  log IU/mL ( $p = 0.002$ ), HBV-DNA  $\leq 6.9$  log copies/mL ( $p = 0.015$ ), and SLD ( $p = 0.049$ ) at baseline were significantly associated with HCC development (Figure A1B–I).

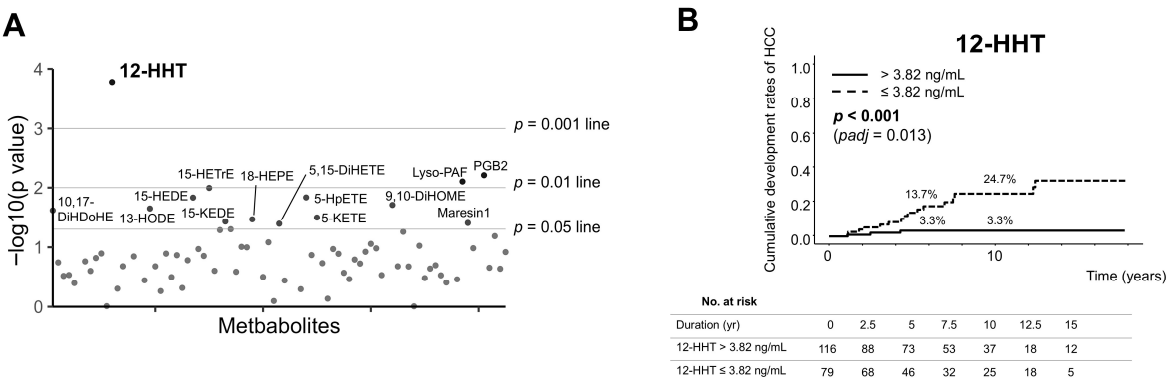
### 3.3. Metabolites at Baseline Associated with Hepatocellular Carcinoma Development During NUC Therapy

Among 158 PUFA metabolites measured, 76 were detected in serum and included in the analysis. Score plots based on metabolite profiles were generated separately for patients with and without HCC development. The VIP analysis identified 12-HHT as the highest-ranked metabolite (VIP score = 1.84), differentiating patients with and without HCC development (Figure 1A). Serum 12-HHT concentrations were significantly lower in patients with HCC development ( $p = 0.001$ ) (Figure 1B).

According to the log-rank test, 14 metabolites were significantly associated with HCC development with 12-HHT showing the smallest  $p$ -value (Figures 2A and A2). We additionally calculated false discovery rate (FDR)-adjusted  $p$  values using the Benjamini–Hochberg procedure ( $q$  values) and only 12-HHT remained statistically significant ( $q = 0.013$ ). The cumulative rates of HCC development at 5 and 10 years were 13.7% and 24.7%, respectively, among patients with 12-HHT levels  $\leq 3.82$  ng/mL, and 3.3% at both time points among those with 12-HHT levels  $> 3.82$  ng/mL ( $p < 0.001$ ) (Figure 2B).



**Figure 1.** (A) PLS-DA score plots (left) and metabolites with the highest VIP scores (right) to distinguish patients with and without HCC development during NUC therapy. Component 1 and 2 represent the first and second latent components of the PLS-DA model. The percentages on each axis indicate the proportion of variance in the metabolite data explained by each component. Each data point represents an individual patient sample. Different point shapes are used only to distinguish between patient groups. (B) Serum 12-HHT levels at baseline between patients with and without HCC development. 12-HHT, 12-hydroxyheptadecatrienoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; HCC, hepatocellular carcinoma; HEDE, hydroxy-eicosadienoic acid; HODE, hydroxy-octadecadienoic acid; HpETE, hydroperoxy-eicosatetraenoic acid; KEDE, 15-oxo-11Z,13E-eicosadienoic acid; KODE, keto-octadecadienoic acid; NUC, nucleos(t)ide analogue; PG, prostaglandin; PLS-DA, partial least squares discriminant analysis; VIP, variable importance in projection.



**Figure 2.** (A) Statistical results of the log-rank test for HCC development during NUC therapy based on binary groups defined by ROC-derived cut-off values from MS measurements. The y-axis represents the  $p$ -value ( $-\log_{10}$  scale).  $p$  value was calculated by log-rank test. (B) Cumulative rates of HCC development according to serum quantitative 12-HHT levels. Adjusted  $p$  values were calculated using the Benjamini–Hochberg false discovery rate (FDR) procedure. “No. at risk” indicates the number of patients at risk of HCC development at each time point. 12-HHT, 12-hydroxyheptadecatrienoic acid; DiHDoHE, dihydroxy-docosahexaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHOME, dihydroxyoctadecenoic acid; HCC, hepatocellular carcinoma; HEDE, hydroxy-eicosadienoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETRe, hydroxy-eicosatrienoic acid; HODE, hydroxy-octadecadienoic acid; HpETE, hydroperoxy-eicosatetraenoic acid; KEDE, oxoeicosadienoic acid; KETE, oxoeicosatetraenoic acid; Lyso-PAF, lyso-platelet activating factor; NUC, nucleos(t)ide analogue; PGB2, prostaglandin B2; ROC receiver operating characteristic.

### 3.4. Correlations Between PUFA Metabolites and Age, FIB-4 Index, or $\alpha$ -Fetoprotein Levels at Baseline

12-HHT and 5-hydroxyeicosatetraenoic acid (HETE) showed negative correlation with age, whereas docosahexaenoic acid (DHA) showed positive correlation (Figure A3A). 12-HHT, PGD2, and 5-ketoeicosatetraenoic acid (5-KETE) showed negative correlation with the FIB-4 index, whereas 9,10-dihydroxyoctadecenoic acid (9, 10-DiHOME), 12,13-DiHOME, 8,9-dihydroxyeicosatrienoic acid (8,9-DHET), and 11,12-DHET showed positive correlation (Figure A3B). 5-KETE showed negative correlation with AFP, whereas 8,9-DHET, 9,10-DiHOME, 14,15-DHET, and 11,12-DHET showed positive correlation (Figure A3C).

### 3.5. Factors at Baseline Predicting Hepatocellular Carcinoma Development During NUC Therapy

Univariate analysis identified several baseline factors predicting HCC development during NUC therapy including 12-HHT  $\leq 3.82$  ng/mL ( $p = 0.001$ ; hazard ratio [HR], 7.51; 95% confidence interval [CI], 2.19–25.82), age  $\geq 47$  years ( $p = 0.005$ ; HR, 8.24; 95% CI, 1.90–35.70), cirrhosis ( $p < 0.001$ ; HR, 7.03; 95% CI, 2.84–17.38), platelet count  $\leq 141 \times 10^3/\mu\text{L}$  ( $p < 0.001$ ; HR, 5.88; 95% CI, 2.11–16.33), FIB-4 index  $\geq 4.08$  ( $p < 0.001$ ; HR, 10.41; 95% CI, 4.07–26.60), AFP level  $\geq 6.4$  ng/mL ( $p = 0.001$ ; HR, 6.10; 95% CI, 2.02–18.38), HBsAg  $\leq 3.37$  log IU/mL ( $p = 0.005$ ; HR, 4.01; 95% CI, 1.52–10.55), and HBV DNA  $\leq 6.9$  log copies/mL ( $p = 0.022$ ; HR, 3.29; 95% CI, 1.19–9.14).

Multivariate analysis revealed that 12-HHT  $\leq 3.82$  ng/mL ( $p = 0.027$ ; HR, 4.28; 95% CI, 1.18–15.55) and FIB-4 index  $\geq 4.08$  ( $p = 0.005$ ; HR, 5.19; 95% CI, 1.64–16.41) were independent factors significantly associated with HCC development during NUC therapy (Table 2). Other multivariable analyses adjusted for 12-HHT and AFP are presented in Table A1.

**Table 2.** Factors at baseline predicting the development of HCC during NUC therapy.

| Variables       | Category                           | Univariate Analysis |              | Multivariate Analysis |           |
|-----------------|------------------------------------|---------------------|--------------|-----------------------|-----------|
|                 |                                    | HR (95% CI)         | $p$ Value    | HR (95% CI)           | $p$ Value |
| 12-HHT          | $\leq 3.82$ ng/mL                  | 7.51 (2.19–25.82)   | 0.001 **     | 4.28 (1.18–15.55)     | 0.027 *   |
| Age             | $\geq 47$ years                    | 8.24 (1.90–35.70)   | 0.005 **     |                       |           |
| Sex             | Male                               | 1.69 (0.61–4.69)    | 0.32         |                       |           |
| Liver fibrosis  | Cirrhosis                          | 7.03 (2.84–17.38)   | $<0.001$ *** | 1.84 (0.60–5.65)      | 0.28      |
| Diabetes        | Diabetes                           | 2.68 (0.78–9.25)    | 0.118        |                       |           |
| Alcohol intake  | Drinker                            | 1.53 (0.51–4.60)    | 0.45         |                       |           |
| SLD             | (+)                                | 0.26 (0.06–1.11)    | 0.068        |                       |           |
| Platelet count  | $\leq 141 \times 10^3/\mu\text{L}$ | 5.88 (2.11–16.33)   | $<0.001$ *** |                       |           |
| FIB-4 index     | $\geq 4.08$                        | 10.41 (4.07–26.60)  | $<0.001$ *** | 5.19 (1.64–16.41)     | 0.005 **  |
| AST             | $\geq 80$ U/L                      | 1.19 (0.48–2.97)    | 0.70         |                       |           |
| ALT             | $\leq 81$ U/L                      | 2.04 (0.80–5.20)    | 0.133        |                       |           |
| GGT             | $\geq 55$ U/L                      | 1.81 (0.73–4.51)    | 0.20         |                       |           |
| Total bilirubin | $\geq 1.0$ mg/dL                   | 2.05 (0.83–5.06)    | 0.110        |                       |           |
| Albumin         | $\leq 4.0$ g/L                     | 2.41 (0.91–6.34)    | 0.075        |                       |           |
| AFP             | $\geq 6.4$ ng/mL                   | 6.10 (2.02–18.38)   | 0.001 **     |                       |           |
| HBsAg           | $\leq 3.37$ log IU/mL              | 4.01 (1.52–10.55)   | 0.005 **     |                       |           |
| HBV-DNA         | $\leq 6.9$ log copies/mL           | 3.29 (1.19–9.14)    | 0.022 *      |                       |           |
| HBcrAg          | $\leq 5.9$ log U/mL                | 2.51 (0.90–6.97)    | 0.077        |                       |           |

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . 12-HHT, 12-hydroxyheptadecatrienoic acid; AFP,  $\alpha$ -fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; FIB-4 index, fibrosis-4 index; GGT, gamma-glutamyl transferase; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio; NUC, nucleos(t)ide analogue; SLD, steatotic liver disease.

### 3.6. Correlation Between 12-Hydroxyheptadecatrienoic Acid and Clinical Factors at Baseline

Baseline characteristics of patients with 12-HHT  $\leq 3.82$  ng/mL and those with 12-HHT  $> 3.82$  ng/mL are presented in Table 3. 12-HHT levels showed negative correlations with age (Spearman's correlation coefficient  $r = -0.34$ ,  $p < 0.001$ ), the FIB-4 index ( $r = -0.34$ ,  $p < 0.001$ ), and total bilirubin ( $r = -0.24$ ,  $p < 0.001$ ), and positive correlation with platelet count ( $r = 0.32$ ,  $p < 0.001$ ) and ALT ( $r = 0.21$ ,  $p = 0.003$ ) (Figure 3A). 12-HHT levels were significantly decreased in patients with cirrhosis ( $p < 0.001$ ) and those with SLD ( $p = 0.030$ ) (Figure 3B).

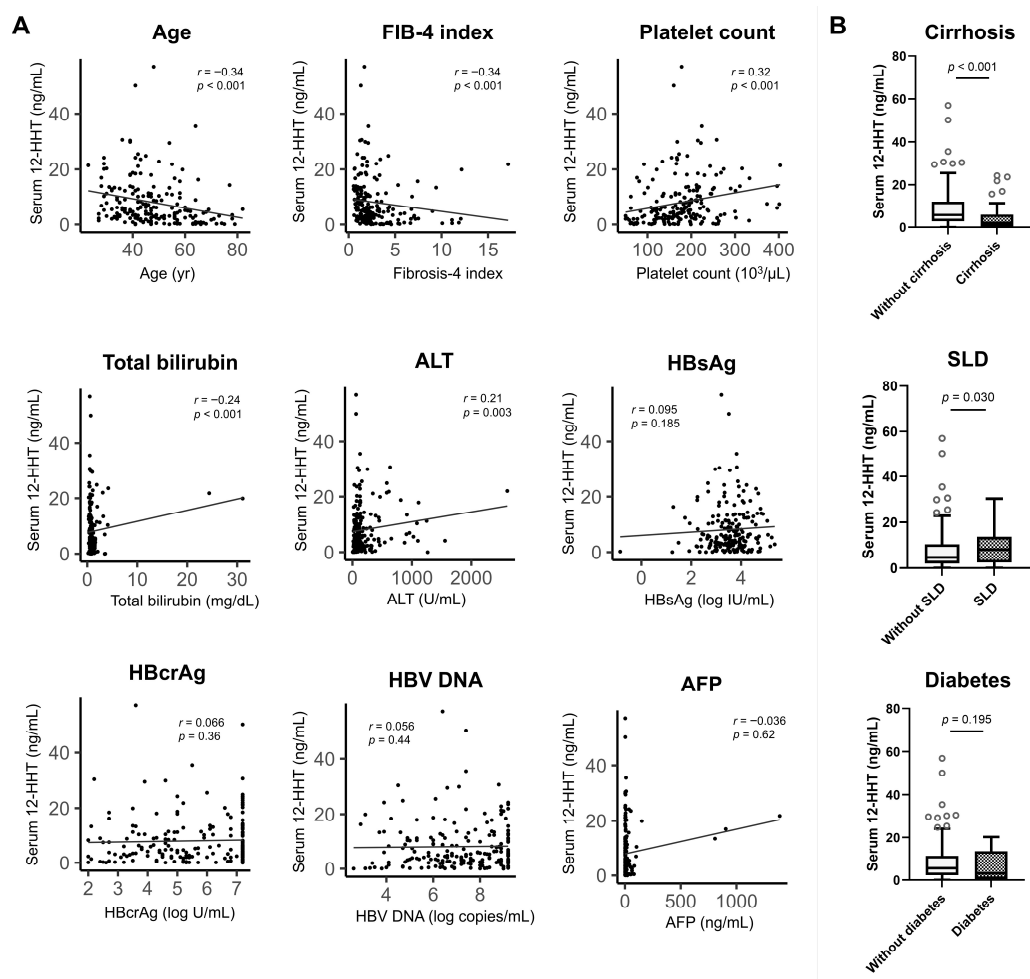
**Table 3.** Factors at baseline predicting the development of HCC during NUC therapy.

| Variables                            | Low 12-HHT<br>(n = 79)   | High 12-HHT<br>(n = 116) | p Value    |
|--------------------------------------|--------------------------|--------------------------|------------|
| Age (yr)                             | 52.0 [42.5, 63.0]        | 42.5 [37.0, 50.3]        | <0.001 *** |
| Sex: Male                            | 47 (59.5)                | 70 (60.3)                | 0.91       |
| BMI (kg/m <sup>2</sup> )             | 22.4 [20.5, 24.8]        | 22.4 [20.9, 25.1]        | 0.49       |
| Cirrhosis                            | 22 (27.8)                | 9 (7.8)                  | <0.001 *** |
| Alcohol intake                       | 11 (13.9)                | 16 (13.8)                | 0.98       |
| Diabetes                             | 8 (10.1)                 | 4 (3.4)                  | 0.057      |
| SLD                                  | 17 (21.5)                | 41 (35.3)                | 0.038 *    |
| Platelet count (10 <sup>3</sup> /μL) | 148 [111, 200]           | 188 [157, 220]           | <0.001 *** |
| FIB-4 index                          | 2.41 [1.78, 4.11]        | 1.75 [1.12, 2.84]        | <0.001 *** |
| AST (U/L)                            | 66 [41, 104]             | 65 [45, 130]             | 0.23       |
| ALT (U/L)                            | 72 [47, 158]             | 113 [60, 254]            | 0.017 *    |
| GGT (U/L)                            | 40 [24, 77]              | 53 [28, 99]              | 0.099      |
| Total bilirubin (mg/dL)              | 0.90 [0.70, 1.20]        | 0.70 [0.50, 1.00]        | 0.004 **   |
| Albumin (g/dL)                       | 4.00 [3.80, 4.30]        | 4.15 [3.90, 4.40]        | 0.151      |
| AFP (ng/mL)                          | 4.90 [2.90, 11.05]       | 4.55 [2.88, 8.57]        | 0.54       |
| HBsAg (log IU/mL)                    | 3.53 [3.00, 3.94]        | 3.68 [3.21, 4.07]        | 0.074      |
| HBcrAg (log U/mL)                    | 5.40 [4.30, $\geq 7.1$ ] | 6.10 [4.50, $\geq 7.1$ ] | 0.25       |
| HBV DNA (log copies/mL)              | 7.20 [5.85, 8.55]        | 7.40 [6.00, 8.72]        | 0.42       |

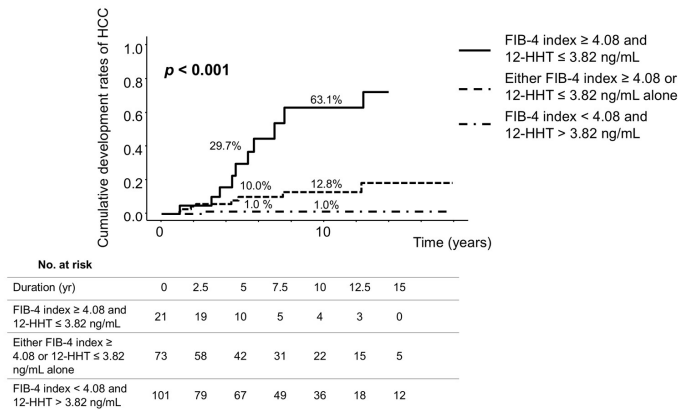
Values are reported as n (%) or median [IQR]. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . Low 12-HHT was defined as serum 12-HHT levels  $\leq 3.82$  ng/mL, and high 12-HHT as  $> 3.82$  ng/mL. 12-HHT, 12-hydroxyheptadecatrienoic acid; AFP,  $\alpha$ -fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FIB-4 index, fibrosis-4 index; GGT, gamma-glutamyl transferase; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IQR, interquartile range; SLD, steatotic liver disease.

### 3.7. Cumulative Rates of the Hepatocellular Carcinoma Development According to the Combination of FIB-4 Index and 12-HHT at Baseline After NUC Therapy

Patients were classified into three groups according to the baseline FIB-4 index and 12-HHT levels after NUC therapy using cut-off values of 4.08 and 3.82 ng/mL, respectively, to distinguish between patients with and without HCC during NUC therapy. The 5- and 10-year cumulative rates of HCC development were 29.7% and 63.1%, respectively, among patients with both FIB-4 index  $\geq 4.08$  and 12-HHT  $\leq 3.82$  ng/mL ( $n = 21$ ); 10.0% and 12.8%, respectively, among those with either FIB-4 index  $\geq 4.08$  or 12-HHT  $\leq 3.82$  ng/mL alone ( $n = 73$ ); and 1.0% at both time points among patients with FIB-4 index  $< 4.08$  and 12-HHT  $> 3.82$  ng/mL ( $n = 101$ ) ( $p < 0.001$ ) (Figure 4).



**Figure 3.** (A) Correlations between 12-HHT and clinical factors (age, FIB-4 index, platelet count, total bilirubin, ALT, HBsAg, HBcrAg, HBV DNA, and AFP). (B) Serum 12-HHT levels at baseline between patients with and without cirrhosis, SLD, or diabetes. 12-HHT, 12-hydroxyheptadecatrienoic acid; AFP,  $\alpha$ -fetoprotein; ALT, alanine aminotransferase; FIB-4 index, fibrosis-4 index; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; SLD, steatotic liver disease.



**Figure 4.** Cumulative rates of HCC development based on the combination of FIB-4 index and 12-HHT at baseline after NUC therapy. Patients were classified into three groups based on the FIB-4 index and 12-HHT levels at baseline after NUC therapy, using cut-off values of 4.08 and 3.82 ng/mL, respectively, to distinguish between patients with and without HCC during NUC therapy. “No. at risk” indicates the number of patients at risk of HCC development at each time point. 12-HHT, 12-hydroxyheptadecatrienoic acid; FIB-4 index, fibrosis-4 index; HCC, hepatocellular carcinoma; NUC, nucleos(t)ide analogue.

#### 4. Discussion

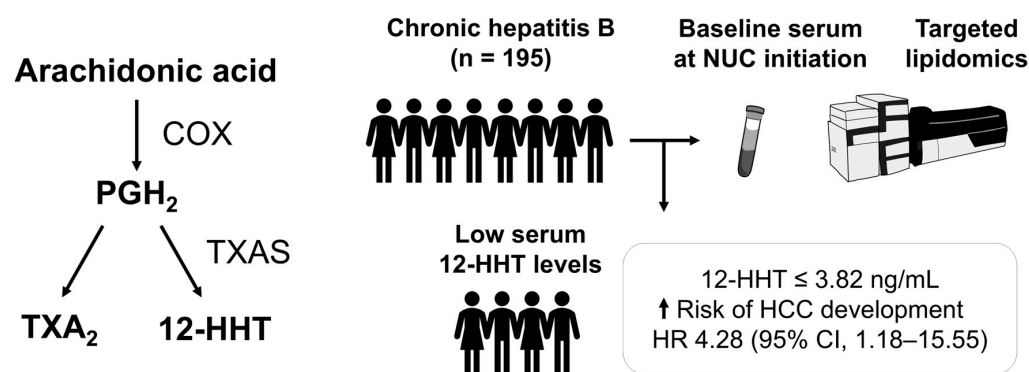
As far as we know, this study is the first report that evaluates the association between PUFA metabolites and HCC development during NUC therapy in patients with chronic HBV infection using targeted lipidomic analysis. In the present analysis, baseline serum PUFA metabolite levels were measured in patients receiving NUC therapy. Our findings indicate that pre-treatment levels of 12-HHT and FIB-4 index are useful predictors of HCC development during NUC therapy and that predictive accuracy improves when the two are combined.

Altered lipid metabolism represents one of the most prominent metabolic changes observed in cancer [25]. The eicosanoid pathway, which generates PUFA metabolites, is regarded as a key pathway associated with liver inflammation and carcinogenesis [18,26]. PUFA metabolites are derived from PUFAs including arachidonic acid, DHA, and eicosapentaenoic acid through COX, LOX, or CYP pathways [16]. Several of these metabolites have been implicated in carcinogenesis. For instance, PGE<sub>2</sub> has been shown to promote cancer progression [27,28]. The LOX family has also been suggested to contribute, at least in part, to HCC development [29–31].

In the context of clinical biomarker research, lipidomic analyses have been conducted to identify PUFA metabolites associated with HCC. Gong et al. performed a metabolomic analysis that included several PUFA metabolites and reported significantly higher serum levels of PGF<sub>2</sub>α, thromboxane (TX)B<sub>2</sub>, 5-HETE, and 15-HETE in patients with HCC compared with those with chronic HBV infection [19]. Similarly, Lu et al. demonstrated that serum levels of 9,10-DiHOME and 12,13-DiHOME were higher in patients with HBV-related HCC than in those with chronic hepatitis [20]. However, these studies neither adjusted for clinical confounders nor assessed the long-term risk of HCC development during NUC therapy. In this study, we conducted targeted lipidomic analysis in patients treated with NUC and evaluated the predictive value of PUFA metabolites for HCC development during NUC therapy using both univariate and multivariate analyses. We identified 14 metabolites that may serve as novel prognostic biomarkers for HCC with 12-HHT emerging as the strongest independent predictor of HCC development.

12-HHT is a 17-carbon hydroxy fatty acid biosynthesised either through enzymatic pathways including thromboxane synthase (TXAS) and COX, or via non-enzymatic processes [32]. TXAS catalyses the isomerisation of PGH<sub>2</sub> into 12-HHT and TXA<sub>2</sub> (Figure 5). Traditionally, 12-HHT was considered a byproduct of TXA<sub>2</sub> biosynthesis and its biological role remained unclear. However, several studies have demonstrated that 12-HHT acts as an endogenous agonist of leukotriene B<sub>4</sub> receptor 2 (BLT2) [33,34]. The 12-HHT–BLT2 axis has been implicated in wound healing [35] and evidence suggests that this interaction may also enhance intestinal barrier function [36–38]. Emerging evidence suggests that alterations in gut microbiota and intestinal barrier dysfunction are strongly associated with HCC development [39–41]. Reduced levels of 12-HHT in patients with HBV infection may therefore contribute to intestinal barrier impairment and promote HCC progression. Further studies are required to elucidate the mechanisms through which 12-HHT may exert tumour-suppressive effects.

The FIB-4 index is well established as a surrogate marker of liver fibrosis and a predictor of HCC development [42]. Several studies have shown that an elevated baseline FIB-4 index is strongly associated with HCC development in patients undergoing NUC therapy [43,44], findings that are consistent with our results. Furthermore, combining the FIB-4 index with other clinical and serological markers such as AFP or novel biomarkers enhances predictive performance. Notably, 12-HHT emerged as a strong independent predictor in this study and the combination of 12-HHT with the FIB-4 index further enhanced risk stratification during NUC therapy.



**Figure 5.** Low baseline serum 12-HHT ( $\leq 3.82 \text{ ng/mL}$ ) was associated with an increased risk of hepatocellular carcinoma development during nucleos(t)ide analogue therapy. 12-HHT, 12-hydroxyheptadecatrienoic acid; COX, cyclooxygenase; HCC, hepatocellular carcinoma; NUC, nucleos(t)ide analogue;  $\text{PGH}_2$ , prostaglandin  $\text{H}_2$ ;  $\text{TXA}_2$ , thromboxane  $\text{A}_2$ ; TXAS, thromboxane synthase.

The present study has some limitations. First, it was conducted at a single centre with a relatively small sample size. Second, the potential mechanisms by which 12-HHT may contribute to carcinogenesis were not investigated. Third, given the large number of metabolites analysed, the possibility of multiple testing cannot be excluded. This concern was addressed by applying PLS-DA, which integrates all variables into a single multivariate model and thereby reduces the risk of false-positive findings.

## 5. Conclusions

In summary, low baseline serum levels of 12-HHT are strongly associated with an increased risk of HCC in patients undergoing NUC therapy. Moreover, combining 12-HHT with the FIB-4 index markedly enhanced risk stratification. These findings suggest that pre-treatment 12-HHT is a novel predictive biomarker for HCC development in patients undergoing NUC therapy.

**Author Contributions:** Conceptualization, H.I. and R.K.; methodology, H.I., R.K. and T.M.; software, H.I.; formal analysis, H.I. and R.K.; investigation, K.I., H.I. and R.K.; resources, H.I., R.K., N.O., K.Y., K.K., E.K., A.H., H.F., M.E. and S.U.-K.; data curation, H.I. and R.K.; writing—original draft preparation, H.I.; writing—review and editing, R.K., T.M. and M.E.; visualisation, H.I.; supervision, R.K. and M.E.; project administration, H.I. and R.K.; funding acquisition, H.I. and M.E. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Osaka Metropolitan University Hospital (approval numbers. 1646, 5 November 2009; 3260, 1 December 2015; and 4361, 25 July 2019).

**Informed Consent Statement:** Written informed consent has been obtained from the patients to publish this paper.

**Data Availability Statement:** The datasets generated and analysed during the current study are not publicly available due to patient confidentiality and ethical restrictions but are available from the corresponding author on reasonable request and with appropriate institutional approval.

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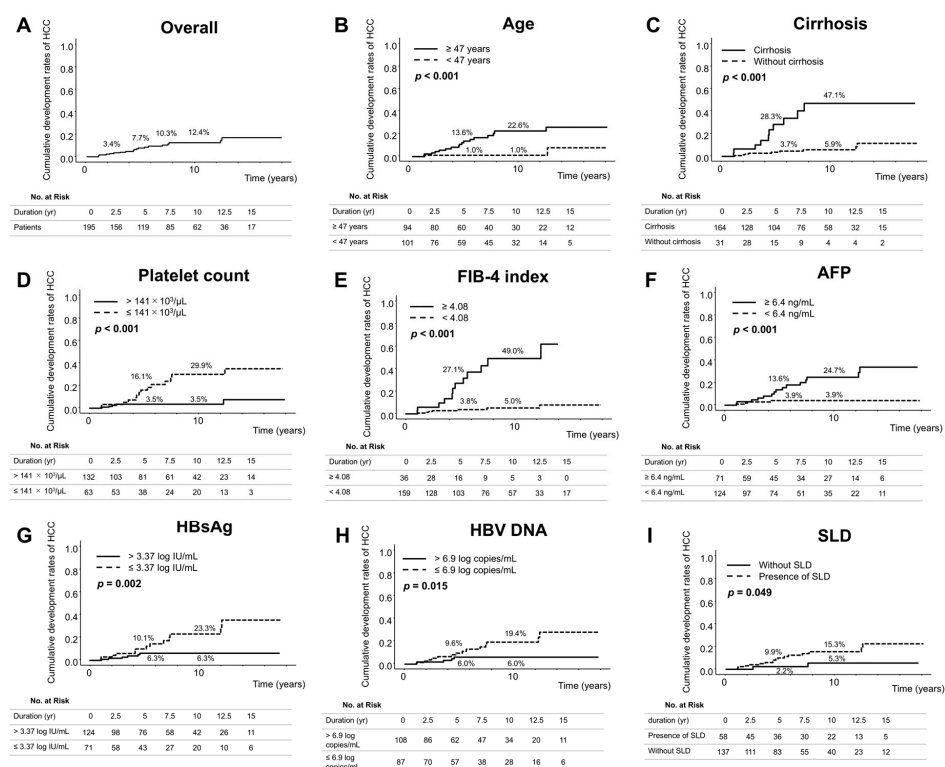
**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

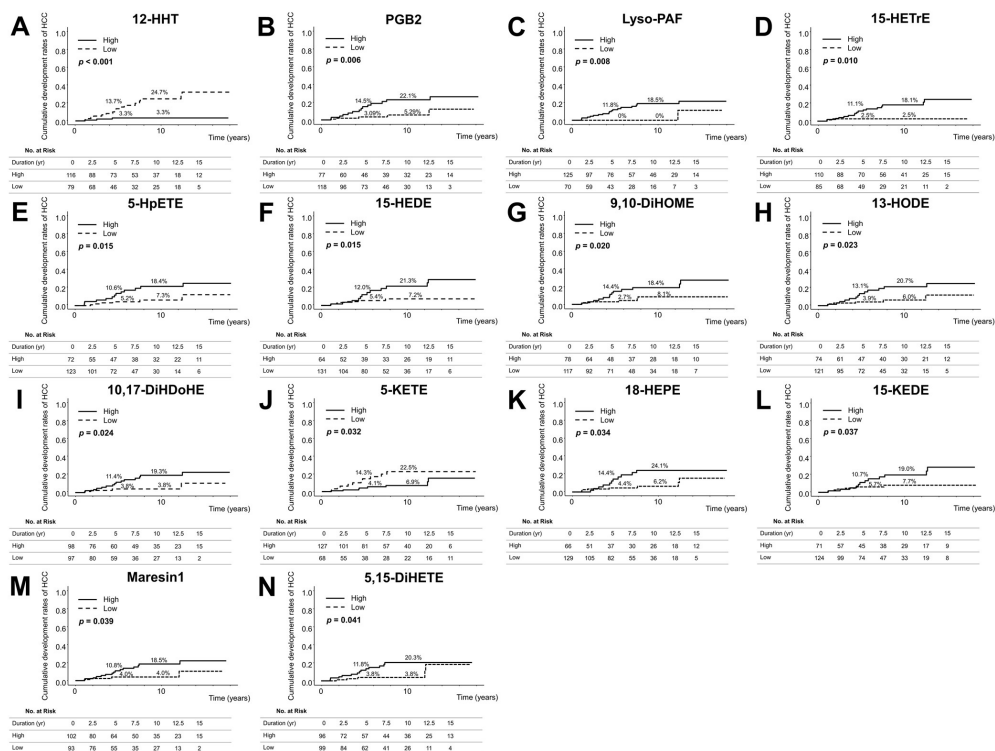
The following abbreviations are used in this manuscript:

|        |                                             |
|--------|---------------------------------------------|
| 12-HHT | 12-hydroxyheptadecatrienoic acid            |
| AA     | arachidonic acid                            |
| AFP    | $\alpha$ -fetoprotein                       |
| ALT    | alanine aminotransferase                    |
| AST    | aspartate aminotransferase                  |
| BLT2   | leukotriene B4 receptor 2                   |
| CI     | confidence interval                         |
| COX    | cyclooxygenase                              |
| CT     | computed tomography                         |
| CYP    | cytochrome P450                             |
| DHA    | docosahexaenoic acid                        |
| DHET   | dihydroxyeicosatrienoic acid                |
| DiHOME | dihydroxyoctadecenoic acid                  |
| FIB-4  | fibrosis-4                                  |
| HBcrAg | hepatitis B core-related antigen            |
| HBsAg  | hepatitis B surface antigen                 |
| HBV    | hepatitis B virus                           |
| HCC    | hepatocellular carcinoma                    |
| HETE   | hydroxyeicosatetraenoic acid                |
| HR     | hazard ratio                                |
| IQR    | interquartile range                         |
| KETE   | ketoecicosatetraenoic acid                  |
| LOX    | lipxygenase                                 |
| LT     | leukotriene                                 |
| MRI    | magnetic resonance imaging                  |
| MS     | mass spectrometry                           |
| NUC    | nucleos(t)ide analogue                      |
| PG     | prostaglandin                               |
| PLS-DA | partial least squares discriminant analysis |
| PUFA   | polyunsaturated fatty acid                  |
| SLD    | steatotic liver disease                     |
| TX     | thromboxane                                 |
| TXAS   | thromboxane synthase                        |
| VIP    | variable importance in projection           |

## Appendix A

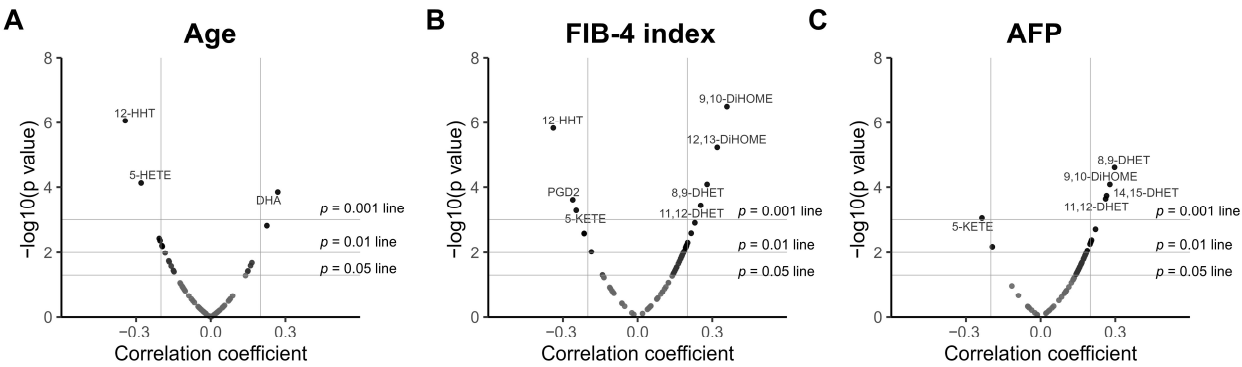


**Figure A1.** Cumulative rates of HCC development during NUC therapy in (A) all patients and according to (B) age, (C) cirrhosis, (D) platelet count, (E) FIB-4 index, (F) AFP, (G) HBsAg, (H) HBV DNA, and (I) SLD at baseline. AFP,  $\alpha$ -fetoprotein; FIB-4, fibrosis-4 index; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NUC, nucleos(t)ide analogue; SLD, steatotic liver disease.



**Figure A2.** Cumulative rates of HCC development during NUC therapy according to the serum (A) 12-HHT, (B) PGB2, (C) Lyso-PAF, (D) 15-HETrE, (E) 5-HpETE, (F) 15-HEDE, (G) 9,10-DiHOME, (H) 13-HODE, (I) 10,17-DiHDoHE, (J) 5-KETE, (K) 18-HEPE, (L) 15-KEDE, (M) Maresin1, and (N) 5,15-DiHETE.

(H) 13-HODE, (I) 10,17-DiHDoHE, (J) 5-KETE, (K) 18-HEPE, (L) 15-KEDE, (M) Maresin1, and (N) 5,15-DiHETE levels. Low and high groups were classified based on ROC-derived cut-off values from MS measurements and *p*-values were calculated using the log-rank test. 12-HHT, 12-hydroxyheptadecatrienoic acid; DiHDoHE, dihydroxy-docosahexaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHOME, dihydroxyoctadecenoic acid; HCC, hepatocellular carcinoma; HEDE, hydroxy-eicosadienoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETRe, hydroxy-eicosatrienoic acid; HODE, hydroxy-octadecadienoic acid; HpETE, hydroperoxy-eicosatetraenoic acid; KEDE, oxoeicosadienoic acid; KETE, oxoeicosatetraenoic acid; Lyso-PAF, lyso-platelet activating factor; NUC, nucleos(t)ide analogue; PG, prostaglandin; ROC, receiver operating characteristic.



**Figure A3.** Correlations between PUFA metabolites and (A) age, (B) FIB-4 index, and (C) AFP at baseline. Spearman’s correlation analysis was conducted to identify metabolites with particularly strong associations, and those with *p* < 0.001 and an absolute Spearman’s correlation coefficient (*|r|*) > 0.2 were selected. 12-HHT, 12-hydroxyheptadecatrienoic acid; AFP, α-fetoprotein; DHA, docosahexaenoic acid; DHET, dihydroxyeicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; FIB-4, fibrosis-4 index; HETE, hydroxyeicosatetraenoic acid; KETE, keto-eicosatetraenoic acid; PG, prostaglandin; PUFA, polyunsaturated fatty acid.

**Table A1.** Multivariable Cox proportional hazards models including 12-HHT and AFP for the development of HCC during NUC therapy.

| Multivariate Analysis |             |                   |                |
|-----------------------|-------------|-------------------|----------------|
| Variables             | Category    | HR (95% CI)       | <i>p</i> Value |
| 12-HHT                | ≤3.82 ng/mL | 7.08 (2.06–24.34) | 0.002 **       |
| AFP                   | ≥6.4 ng/mL  | 5.73 (1.90–17.27) | 0.002 **       |

\*\* *p* < 0.01. 12-HHT, 12-hydroxyheptadecatrienoic acid; AFP, α-fetoprotein; CI, confidence interval; HCC, hepatocellular carcinoma; HR, hazard ratio; NUC, nucleos(t)ide analogue.

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