Quantitative Evaluation of Hepatic Integrin Avβ3 Expression by Positron-emission Tomography Imaging Using 18F-FPP-RGD2 in Rats With Non-alcoholic Steatohepatitis

Shuichi Hiroyama (✉ shuichi.hiroyama@shionogi.co.jp)
Shionogi & Co., Ltd.

Takemi Rokugawa
Shionogi & Co., Ltd.

Miwa Ito
Shionogi & Co., Ltd.

Hitoshi Iimori
Shionogi & Co., Ltd.

Ippei Morita
Shionogi & Co., Ltd.

Hitoshi Iimori
Shionogi & Co., Ltd.

Kohji Abe
Shionogi & Co., Ltd.

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Abstract

Background Integrin αvβ3, which are expressed by activated hepatic stellate cells in non-alcoholic steatohepatitis (NASH), play an important role in the fibrosis. Recently, we reported that an RGD peptide positron emission tomography (PET) probe is useful as a predictor of hepatic fibrosis. Kinetic analysis of the RGD PET probe has been performed in tumours, but not in hepatic fibrosis. Therefore, we aimed to quantify hepatic integrin αvβ3 in a model of NASH by kinetic analysis using 18F-FPP-RGD2, an integrin αvβ3 PET probe.

Methods 18F-FPP-RGD2 PET/CT scans were performed in control and NASH rats. Tissue kinetic analyses were performed using a one-tissue, two-compartment (1T2C) and a two-tissue, three-compartment (2T3C) model using an image-derived input function (IDIF) for the left ventricle. We then conducted correlation analysis between standard uptake values (SUVs) or volume of distribution (VT), evaluated using compartment kinetic analysis, and integrin αv or β3 protein expression.

Results Biochemical and histological evaluation confirmed the development of NASH rats. Integrin αvβ3 protein expression and hepatic SUV were higher in NASH- than normal rats. The hepatic activity of 18F-FPP-RGD2 peaked rapidly after administration and then gradually decreased, whereas left ventricular activity rapidly disappeared. The 2T3C model was found to be preferable for 18F-FPP-RGD2 kinetic analysis in the liver. The VT (IDIF) for 18F-FPP-RGD2, calculated using the 2T3C model, was significantly higher in NASH- than normal rats and correlated strongly with hepatic integrin αv and β3 protein expression. The strengths of these correlations were similar to those between SUV60–90 min and hepatic integrin αv or β3 protein expression.

Conclusions We have demonstrated that the VT (IDIF) of 18F-FPP-RGD2, calculated using kinetic modelling, positively correlates with integrin αv and β3 protein in the liver of NASH rats. These findings suggest that hepatic VT (IDIF) provides a quantitative assessment of integrin αvβ3 protein in liver.

Background

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease [1,2], and the more severe form of this condition, non-alcoholic steatohepatitis (NASH), is a serious disease [3]. Approximately half of NASH patients develop liver fibrosis, which is the major predictor of subsequent liver cirrhosis, hepatocellular carcinoma and transplantation [4]. Consequently, early detection of fibrosis, before its progression, is important for the prevention and appropriate treatment of these pathologies. To date, liver biopsy has been regarded as the gold standard method of diagnosing and staging liver fibrosis [5,6]. However, liver biopsy is invasive and is associated with several risks, including high sampling variability, because of the small size of the tissue samples obtained, inter-observer variability, pain and complications due to the procedure itself [7,8]. A number of non-invasive methods of staging fibrosis and determining the treatment response have been reported in recent years [9–13], but none of these have become well established as diagnostic techniques. The most successful non-invasive approaches in clinical practice are ultrasound- and magnetic resonance-based elastography, which assess liver stiffness [12,13]. Consequently, they do not provide information regarding the molecular pathology of liver fibrosis [14,15]. Therefore, the development and validation of a non-invasive biomarker for the diagnosis and staging of liver fibrosis would be valuable.
The pathology of NASH and the mechanism of progression of liver fibrosis have been well documented [16]. The activation of hepatic stellate cells (HSCs) is one of the most important events in liver fibrogenesis [17]. Activated HSCs play an important role in fibrogenesis by secreting proteins such as integrin \( \alpha_v\beta_3 \) that play a critical role in the transformation of HSCs to myofibroblasts, which express \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA). This leads to excessive production of extracellular matrix (ECM) proteins, such as collagens type-1 and type-3 [18]. Therefore, the expression of integrin \( \alpha_v\beta_3 \) may have potential as a marker of fibrosis in the liver.

Integrin \( \alpha_v\beta_3 \) positron emission tomography (PET) probes, such as \( ^{18} \)F-labelled RGD [19,20], have previously been used to detect liver fibrosis in animal models. However, evaluation of the kinetic profile of the binding of \( ^{18} \)F-FPP-RGD\(_2\) to integrin \( \alpha_v\beta_3 \) in the livers of animal models of NASH is a critical step. There have been previous kinetic analyses of RGD peptide PET probes, such as \( ^{18} \)F- and \( ^{68} \)Ga-labeled RGD [21,22], in animal models of angiogenesis and neoplasia, but not in models of liver fibrosis and NASH. Therefore, we aimed to conduct a kinetic analysis to calculate the parameter \( V_T \) in an animal model of NASH.

In the present study, we have investigated the relationship between the hepatic \( V_T \) of \( ^{18} \)F-FPP-RGD\(_2\), calculated using a kinetic analysis, or the standard uptake value between 60 and 90 min (SUV\(_{60-90\text{min}}\)) and integrin \( \alpha_v\beta_3 \) protein expression using PET imaging in a model of diet-induced NASH.

**Methods**

**Animals**

Male, 7-week-old RccHan\(^{\text{®}}\): WIST rats (Wistar Hannover Rcc rats) were obtained from Japan SLC, Inc. (Shizuoka, Japan). They were allowed free access to tap water and fed a normal diet (CE-2; CLEA, Tokyo, Japan) or a choline-deficient, low-methionine high-fat diet (CDHFD; no choline, 45% fat, 0.1% methionine and 1% cholesterol) prepared by Oriental Yeast Co., ltd. (Tokyo, Japan), for 3–4 or 9–10 weeks. The rats were housed at a controlled temperature and under a 12-h light-dark cycle (lights on at 07:00 h). The experiments were approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine (approval number: 29-030-002, 21st July 2017).

**Biochemical and histological analysis**

After PET/CT scanning, rats were euthanased by exsanguination under isoflurane anaesthesia. Plasma samples were collected and assayed for aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activity, and total cholesterol (TC), triglyceride (TG), glucose (GLU), total bile acid (TBA), albumin (ALB) and total bilirubin (T-BIL) concentrations by enzymatic methods using commercially available kits (Sekisui Medical, Tokyo, Japan) and a Hitachi 7170 autoanalyser (Hitachi, Tokyo, Japan), according to the manufacturer’s instructions.

Liver samples for protein analysis were quickly frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until use. The right hepatic lobes were fixed in 10% formalin, routinely processed, and embedded in paraffin. Four-micrometre-thick paraffin sections were prepared, and these were stained with haematoxylin and eosin (H&E) and Sirius red. Steatosis, inflammation, and ballooning were graded for severity on H&E-stained sections. Steatosis and inflammation were scored from 0 to 3: normal = 0; minimal = 1; moderate = 2 and marked = 3. Ballooning was
scored from 0 to 2: normal = 0; minimal = 1 and marked = 2. NAFLD activity score (NAS) was then calculated as the sum of each of these scores. To assess hepatic fibrosis, Sirius red staining images were captured using a BZ-X700 microscope (Keyence Co., Osaka, Japan) and the Sirius red-positive area (%), corresponding to fibrosis, was measured using the BZ-X analysis application (Keyence Co.).

Protein analysis

Hepatic integrin αv and β3 subunit protein levels were determined using a JESS Automated Western Blotting system (ProteinSimple, San Jose, CA, USA). Liver tissue lysates were prepared in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific K.K., Tokyo, Japan), and lysates containing 0.2 mg/mL protein were separated using a 12–230 kDa Separation Module (ProteinSimple). Specific proteins were detected using mouse anti-integrin αv (1:50; BD Biosciences, San Jose, CA, USA; ab611012), rabbit anti-integrin β3 (1:50; Abcam, Toronto, ON, Canada; ab210515) and an Anti-Mouse Detection Module (ProteinSimple), according to the manufacturer’s instructions.

PET probe synthesis

18F-FPP-RGD2 was synthesized using a two-step method, as reported previously [20,23], to a specific activity of 273.3±60.7 GBq/μmol. The RGD dimeric peptide (PEG3-c[RGDyK]2) was purchased from Peptides International, Inc. (Louisville, KA, USA).

Analysis of the metabolites of 18F-FPP-RGD2 using thin-layer chromatography (TLC)

Metabolite analysis of plasma and liver samples from six normal diet-fed rats was conducted as described previously [24]. Briefly, ~20 MBq 18F-FPP-RGD2 was administered via a tail vein, then blood and liver samples were collected 30 and 90 min later under isoflurane anaesthesia. Blood was drawn from the abdominal vena cava, then the rats were exsanguinated, and their livers were collected and quickly homogenized on ice. Plasma was prepared by 1 min of centrifugation at 4°C and 20,817 × g. The plasma and liver samples were deproteinised by precipitation with acetonitrile, then centrifuged at 20,817 × g and 4°C for 5 min, and the supernatants were applied to RP-18 TLC plates (Merck KGaA, Darmstadt, Germany). The plates were developed at room temperature using 10% ammonium acetate/methanol (50:50) as the mobile phase, then dried and used to expose an imaging plate (Fuji Film Corp., Tokyo, Japan) for 30 min. With reference to the Rf value of an 18F-FPP-RGD2 standard, the distribution of radioactivity for 18F-FPP-RGD2 on the imaging plates was determined by digital PSL autoradiography using a Typhoon FLA 7000 imaging analyser (GE Healthcare, Uppsala, Sweden), and the data were analysed using Multi-Gauge imaging analysis software (Fuji Film Corp.).

Dynamic PET imaging

Dynamic PET/CT imaging was performed using a Triumph LabPET-12 PET/CT (TriFoil Imaging Inc., Chatsworth, CA, USA) and a PET camera with an intrinsic axial resolution of 1.38 mm FWHM (full width at half maximum) [25]. Under 2% isoflurane anaesthesia, a tail vein was catheterised for intravenous injection of the PET probe and the rats were placed on a heated pad on the scanner bed. Normal diet- and CDHFD-fed rats were imaged 3–4 or 9–10 weeks after starting the diets. At the start of the PET scan, 18F-FPP-RGD2 (15.5±2.2 MBq) was administered intravenously at a constant rate of 0.5 mL/30 s via a syringe pump (Legato 210; KD Scientific Inc., Holliston, MA,
USA). PET scanning was performed in dynamic scan mode for 90 min, then CT scanning was performed to acquire anatomical information and correct the PET images for attenuation.

For the analysis of the arterial input function (AIF) in the normal diet-fed rats, a femoral artery was also catheterised for blood collection under 2% isoflurane anaesthesia and the cannula was flushed with heparinized saline. Arterial blood sampling was conducted 10 times over the following time periods: 0–10, 10–20, 20–30, 30–40, 40–50, 50–60, 90–100, 300–310, 900–910, 1,800–1,810, 3,600–3,610, and 5,390–5,400 s after $^{18}$F-FPP-RGD$_2$ administration. The volume of blood removed during each time period was ~50 µL, making the total volume ~500 µL, which was ~5% of the total blood volume. Plasma was prepared by 1 min of centrifugation at 4°C and 20,817 × $g$. The radioactivity of the plasma was measured using a gamma counter (2480 Wizard$^2$, PerkinElmer, Inc., Waltham, MA, USA), and was expressed as counts per minute/mL (cpm/mL). Each radioactive count was corrected for decay since the start of the gamma counting and was converted into an SUV.

**Image processing and kinetic analysis**

CT images were reconstructed using the filtered back-projection method (512 slices), and PET images were reconstructed into 25 frames of increasing length (6 × 10 s, 4 × 60 s, 11 × 300 s and 3 × 600 s) using the three-dimensional maximum-likelihood expectation maximization (3D-MLEM) algorithm and CT-based attenuation correction. To obtain time-activity curves (TACs) for kinetic analysis, the left ventricle (33 mm$^3$) and liver (1,280 mm$^3$) volumes of interest (VOIs) were manually defined for each animal on their CT images using PMOD PET data analysis software (v3.905, PMOD Technologies Ltd., Zurich, Switzerland). A spherical VOI on the left ventricle was used to obtain an image-derived plasma input function. TACs for the left ventricle and the liver were constructed by normalizing decay-corrected time-activity measurements to the injected doses of $^{18}$F-FPP-RGD$_2$ and are expressed as mean standardized uptake values (SUVs), where SUV = radioactivity concentration (MBq/cm$^3$) × body mass (g)/amount of radioactivity injected (MBq)/1,000.

Kinetic modelling to determine the total distribution volume ($V_T$) of $^{18}$F-FPP-RGD$_2$ was performed by fitting the TAC using a one-tissue, two-compartment (1T2C) and a two-tissue, three-compartment (2T3C) model using the PMOD software [26]. The Akaike information criterion (AIC) and curve fitting were used to determine the most appropriate compartment model for $^{18}$F-FPP-RGD$_2$. When we compared the $V_T$ calculated using the AIF and the $V_T$ calculated using the image-derived input function (IDIF), the latter provided a stronger linear correlation (Spearman's rank correlation $r_S = 0.943, P < 0.05$) in the present study; therefore, IDIF was used in the present study. Models with irreversible binding were not considered because of the reversibility of the binding of this probe indicated by tissue TACs.

**Statistical analysis**

Comparisons of the two groups of rats were made using unpaired Student's $t$-tests with Welch's correction. Spearman's rank correlation was used to evaluate the relationships between two variables (integrin $\alpha_v$ or $\beta_3$ protein expression and SUV$_{60–90\ min}$, $V_T$(IDIF), $V_T$(IDIF) and $V_T$(AIF)). Statistical analyses were performed using GraphPad Prism (version 6) statistical software (GraphPad Software, San Diego, CA). $P < 0.05$ was considered to indicate statistical significance (two-tailed).
Results

Biochemical and histological evaluation

Body and liver mass, liver/body mass ratio and biochemical parameters are shown in Table 1. The body masses of the CDHFD-fed rats were significantly lower than those of the normal diet-fed rats after 3–4 and 9–10 weeks, whereas the liver masses and liver/body mass ratios were significantly higher. The AST and TBA of the 9–10-week CDHFD-fed group were significantly higher than those of the normal diet-fed group, and the ALT, ALP and T-BIL of both the 3–4- and 9–10-week CDHFD-fed groups were significantly higher than those of the normal diet-fed groups. The ALB of the 9–10-week CDHFD-fed group was significantly lower than that of the normal diet-fed group, and the TG and GLU of both the 3–4- and 9–10-week CDHFD-fed groups were significantly lower than those of the normal diet-fed groups.

As shown in Table 2, histopathological analysis demonstrated that steatosis and inflammation were induced by 3–4 and 9–10 weeks of CDHFD consumption (steatosis score: 3–4 weeks, \( P < 0.01 \) and 9–10 weeks, \( P < 0.01 \); inflammation score: 3–4 weeks, \( P < 0.01 \) and 9–10 weeks, \( P < 0.01 \), and ballooning was induced by 9–10 weeks of CDHFD consumption (\( P < 0.01 \)). In addition, the inflammation (\( P < 0.01 \)) and ballooning (\( P < 0.05 \)) scores were higher in 9–10-week CDHFD-fed rats than in 3–4-week CDHFD-fed rats. The NAFLD activity score and histopathological NASH diagnostic criteria in both 3–4- and 9–10-week CDHFD-fed groups were significantly higher than those in the respective normal diet-fed groups (\( P < 0.01 \)), and the scores in the 9–10-week CDHFD-fed group were higher than those in the 3–4-week CDHFD-fed group (\( P < 0.01 \)). The area of fibrosis in the 9–10-week CDHFD-fed group was significantly higher than those in the normal diet-fed (\( P < 0.05 \)) and 3–4-week CDHFD-fed (\( P < 0.05 \)) groups, whereas the area of fibrosis in the 3–4-week CDHFD-fed group was not significantly larger than that in the 3–4-week normal diet-fed group.

Hepatic integrin \( \alpha_v \) and \( \beta_3 \) expression

As shown in Figure 2, the integrin \( \alpha_v \) and \( \beta_3 \) protein expression in the CDHFD-fed groups was significantly higher than that in the normal diet-fed groups (3–4 weeks: \( \alpha_v, P < 0.01; \beta_3, P < 0.001 \); 9–10 weeks: \( \alpha_v, P < 0.05; \beta_3, P < 0.05 \)). Moreover, the integrin \( \alpha_v \) and \( \beta_3 \) protein expression in the 9–10-week CDHFD-fed group tended to be higher than that in the 3–4-week CDHFD-fed group.

Metabolic stability of \( ^{18}F\text{-FPP-RGD}_2 \)

The metabolic stability of \( ^{18}F\text{-FPP-RGD}_2 \) in the plasma and liver were evaluated by TLC autoradiography in normal diet-fed animals. Supplemental table 1 shows the mean non-metabolised percentages of \( ^{18}F\text{-FPP-RGD}_2 \) in the plasma and liver 30 and 90 min after \( ^{18}F\text{-FPP-RGD}_2 \) was intravenously administered. The mean non-metabolised percentage in all of the rats was >96% and there were no significant radioactive signals on the imaging plates corresponding to metabolites (Supplemental figure 1), suggesting that \( ^{18}F\text{-FPP-RGD}_2 \) is not readily metabolised.

Hepatic uptake of \( ^{18}F\text{-FPP-RGD}_2 \)
Left ventricular SUV peaked within 1 min of the administration of $^{18}$F-FPP-RGD$_2$, then was rapidly eliminated, such that approximately constant values were reached at 60–90 min in all the groups (Fig. 3a). Therefore, the mean hepatic SUV was calculated using the SUVs between 60 and 90 min after administration. Figures 3b and c show the hepatic TACs and SUVs, respectively, 60–90 min ($\text{SUV}_{60-90\text{ min}}$) after the administration of $^{18}$F-FPP-RGD$_2$. The $\text{SUV}_{60-90\text{ min}}$ of the 3–4- and 9–10-week CDHFD-fed groups were significantly higher than those of the normal diet-fed groups (3–4 weeks: $P < 0.001$; 9–10 weeks: $P < 0.001$, Fig. 3c). In addition, the $\text{SUV}_{60-90\text{ min}}$ of the 9–10-week CDHFD-fed group was significantly higher than that of the 3-4-week CDHFD-fed group ($P < 0.05$, Fig. 3c). The $\text{SUV}_{60-90\text{ min}}$ of the 3–4- and 9–10-week normal diet-fed groups were almost identical (Fig. 3c). Figure 3d shows representative colour-coded PET images of the livers of rats that had consumed normal diets or CDHFD for 3–4 or 9–10 weeks.

**Kinetic analysis**

Table 3 shows the rate constants ($K_1$, $k_2$, $k_3$, and $k_4$) and AICs obtained from the kinetic analysis using the 1T2C and 2T3C models in normal diet- and CDHFD-fed rats after 3–4 and 9–10 weeks. The AIC associated with the 2T3C model was lower than that associated with the 1T2C model for all the groups. As shown in Fig. 4a, the quality of the curve fitting also indicated that the 2T3C model was more appropriate than the 1T2C model for the kinetic analysis of $^{18}$F-FPP-RGD$_2$ in the liver. To confirm the accuracy of IDIF, we evaluated the relationships between $V_T$ obtained using IDIF and the $V_T$ obtained using AIF in normal diet-fed rats, with or without inhibition using unlabelled RGD (0.1–3 mg/kg). The $V_T$ (IDIF) strongly correlated with the $V_T$ (AIF) (Spearman's rank correlation, $r_s = 0.943$, $P < 0.05$, Fig. 4b). Figure 5 shows the $V_T$ (IDIF) for $^{18}$F-FPP-RGD$_2$ calculated using the 2T3C model. The $V_T$ (IDIF) was significantly higher in both the 3–4- and 9–10-week CDHFD-fed groups than in the normal diet-fed groups (3–4 weeks, $P < 0.05$; 9–10 weeks, $P < 0.01$, Fig. 5), and significantly increased with the duration of CDHFD feeding ($P < 0.01$, Fig. 5).

**Relationships between SUV$_{60-90\text{ min}}$ or $V_T$ and integrin protein expression**

Figure 6 shows the correlations between SUV$_{60-90\text{ min}}$ or $V_T$ (IDIF) and integrin $\alpha_v$ or $\beta_3$ protein expression. Both SUV$_{60-90\text{ min}}$ and $V_T$ (IDIF) strongly correlated with integrin $\alpha_v$ and $\beta_3$ protein expression (Spearman's rank correlation, SUV$_{60-90\text{ min}}$ vs. $\alpha_v$, $r_s = 0.680$, $P < 0.001$; SUV$_{60-90\text{ min}}$ vs. $\beta_3$, $r_s = 0.776$, $P < 0.0001$; $V_T$ vs. $\alpha_v$, $r_s = 0.717$, $P < 0.001$; $V_T$ vs. $\beta_3$, $r_s = 0.644$, $P < 0.01$).

**Discussion**

In the present study, we have evaluated the kinetics of $^{18}$F-FPP-RGD$_2$ in an animal model of NASH, as a means of quantifying hepatic integrin $\alpha_v\beta_3$ protein expression. We found strong correlations between hepatic $V_T$ (IDIF) evaluated using the 2T3C model, or SUV$_{60-90\text{ min}}$, and hepatic integrin $\alpha_v$ or $\beta_3$ protein expression. The present findings indicate that higher hepatic $V_T$ (IDIF) values for $^{18}$F-FPP-RGD$_2$ may be a predictor of fibrosis associated with NASH.

The CDHFD-fed rat is a suitable model of NASH because it demonstrates clinically-relevant onset and progression of hepatic fibrosis [27,28]. In the present study, histological and biochemical analysis confirmed the progression of NASH pathology, and the severity of the pathological changes increased alongside increases in
hepatic integrin $\alpha_v$ and $\beta_3$ protein expression. These findings were in accordance with those of previous studies conducted in similar animal models [20,27,29]. Histopathological examination confirmed the development of NASH and fibrosis in CDHFD-fed rats. As shown by Kleiner et al. [30], CDHFD-feeding for 3–4- or 9–10 weeks caused borderline NASH and frank NASH, according to the NAFLD activity score. In addition, the area of fibrosis in the 9–10-week CDHFD-fed group was significantly larger than that in the normal diet-fed group, whereas the area of fibrosis in the 3–4-week CDHFD-fed group was not significantly higher than that in the 3–4-week normal diet-fed group. This indicates that 3–4 weeks of CDHFD feeding results in the development of NAFLD/NASH with minimal or no fibrosis and that 9–10 weeks of feeding results in NASH with moderate fibrosis.

HSC activation plays a pivotal role not only in the onset, but also in the progression of hepatic fibrosis [17]. The expression of receptors such as integrin $\alpha_v$$\beta_3$ has been shown to be a biomarker of HSC activation. [31]. In the present study, hepatic integrin $\alpha_v$ and $\beta_3$ protein expression in CDHFD-fed rats was higher from 3–4 weeks of feeding, prior to the development of significant fibrosis. Therefore, it seems that integrin $\alpha_v$$\beta_3$ protein expression increases, indicating HSC activation, before the development of fibrosis in rats with CDHFD-induced NASH.

The metabolite analysis confirmed that there is negligible metabolism of $^{18}$F-FPP-RGD$_2$ in the plasma and liver up to 90 min after intravenously administration. Hepatic TAC fitting revealed that the 2T3C model was more appropriate than the 1T2C model, and this was also quantitatively confirmed using the AIC values. Consistent with the results of a previous study [3], hepatic TACs for $^{18}$F-FPP-RGD$_2$ peaked rapidly after administration and then gradually decreased until the final time point, implying that $^{18}$F-FPP-RGD$_2$ kinetics are consistent with a reversible receptor-binding model. Therefore, in the present study, we did not evaluate an irreversible compartment model. These data indicate that $^{18}$F-FPP-RGD$_2$ binds to integrin $\alpha_v$$\beta_3$ proteins reversibly and that a 2T3C model is suitable for the analysis of $^{18}$F-FPP-RGD$_2$ kinetics. Moreover, we found that there is a strong correlation (Spearman's rank correlation $r_s = 0.943$, $P < 0.05$) between the $V_T$ (IDIF) and $V_T$ (AIF). Therefore, the $^{18}$F-FPP-RGD$_2$ activity in left ventricular blood as IDIF was used for kinetic analysis, instead of AIF, with the assumption that there was no inter-individual variation in haematocrit.

$^{18}$F-labelled RGD, which binds to integrin $\alpha_v$$\beta_3$, has previously been used to detect liver fibrosis in animal models [19,20]. A previous study showed the colocalization of integrin $\alpha_v$$\beta_3$ with $\alpha$-SMA in activated HSCs by immunofluorescence staining and that fluorescently labelled-RGD binds to activated HSCs [32]. In the present study, the hepatic SUV or $V_T$ (IDIF) for $^{18}$F-FPP-RGD$_2$ positively correlated with hepatic integrin $\alpha_v$ and $\beta_3$ protein expression, and the hepatic $V_T$ of $^{18}$F-FPP-RGD$_2$ was reduced by the co-administration of unlabelled RGD. These findings indicate that $^{18}$F-FPP-RGD$_2$ specifically binds to hepatic integrin $\alpha_v$$\beta_3$ on HSCs in a rat model of NASH.

Next, we conducted 90-min dynamic PET scans using $^{18}$F-FPP-RGD$_2$ and determined the most suitable kinetic model for the evaluation of the liver in rats with CDHFD-induced NASH. In the 3–4-week CDHFD-fed groups, the image-derived input function was similar to that of the normal diet-fed groups. However, in the 9–10-week CDHFD-fed group, although there was no difference in the image-derived input function during the late phase, it was slightly lower during the early phase (0–1 min after administration). Moreover, the $K_1$, extravasation rate of the PET probe in the CDHFD-fed groups was lower than in the respective normal diet-fed groups. The low input function implies that the liver uptake of $^{18}$F-FPP-RGD$_2$ might have been slightly underestimated in the 9–10-week CDHFD-fed group. The hepatic SUVs of the CDHFD groups were significantly higher than those of the respective
normal diet-fed groups and significantly increased with the duration of the feeding period. These data indicate that hepatic integrin \(\alpha_v\beta_3\) protein expression in CDHFD-fed rats is higher than that in normal diet-fed rats. The \(V_T^{(IDIF)}\) for \(^{18}\text{F-FPP-RGD}_2\), evaluated using the 2T3C model, was significantly higher in the CDHFD-fed groups than in the normal diet-fed groups and strongly positively correlated with hepatic integrin \(\alpha_v\) (\(r_s = 0.717\)) and \(\beta_3\) (\(r_s = 0.644\)) protein expression. The strengths of these correlations were similar to those between \(\text{SUV}_{60-90\,\text{min}}\) and hepatic integrin \(\alpha_v\) (\(r_s = 0.680\)) or \(\beta_3\) (\(r_s = 0.776\)) protein expression. Indeed, the clinical usefulness of \(V_T\) has recently been reported for pulmonary fibrosis related to other causes than cancer [33]. Our data suggest that hepatic \(V_T\), obtained non-invasively using kinetic modelling, might be useful as a predictor of fibrosis and for determining the efficacy of new drugs in pre-clinical and clinical studies. Moreover, the correlation analyses (Fig. 6) suggest that hepatic SUV, which can be easily obtained using a static scan in the clinic, could be used as a predictor of fibrosis with similar efficacy to hepatic \(V_T\) obtained using a dynamic scan.

The hepatic blood supply is derived from two vessels: the hepatic artery and the portal vein. However, in the present study, the left ventricular time-activity curves were used as the IDIF without considering the input function from the portal vein. Although the hepatic \(V_T^{(IDIF)}\) of \(^{18}\text{F-FPP-RGD}_2\) strongly positively correlated with hepatic integrin \(\alpha_v\) and \(\beta_3\) protein expression in the present study, further studies are required to evaluate the \(V_T^{(IDIF)}\) using input functions from both the hepatic artery and portal vein.

**Conclusions**

Kinetic modelling studies using dynamic \(^{18}\text{F-FPP-RGD}_2\) PET scans are feasible, on the basis of an image-derived input function, in a diet-induced animal model of NASH. Moreover, the kinetic modelling analysis performed in this study will be useful for the quantitative evaluation of \(^{18}\text{F-FPP-RGD}_2\) binding to hepatic integrin \(\alpha_v\beta_3\) proteins. The present findings indicate that higher hepatic \(V_T\) values for \(^{18}\text{F-FPP-RGD}_2\) may offer novel strategies for the prediction of fibrosis associated with NASH.

**Declarations**

*Ethics approval*

The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Shionogi & Co., Ltd. and Osaka University Graduate School of Medicine.

*Consent for publication*

Not applicable

*Availability of data and material*

All data generated or analysed during this study are included in this published article and its supplementary information files.

*Competing interests*

The authors declare that they have no competing interests.
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Author’s contributions

SH performed the PET experiments and data analysis, and drafted the manuscript. TR and MI performed the PET experiments. HI synthesized the PET probe. IM and HM performed the biochemical and protein analyses. KF performed the histological analysis. KM and ES were the supervisors of this study. KA coordinated and designed the study and drafted the manuscript. All authors read and approved the final version of the manuscript.

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Tables
Table 1: Body and liver masses, liver/body mass ratio and biochemical parameters

| Parameters                  | Group         | 3-4 weeks | 9-10 weeks | 3-4 weeks | 9-10 weeks |
|-----------------------------|---------------|-----------|------------|-----------|------------|
|                            | Normal diet   | CDHFD     | Normal diet | CDHFD     | CDHFD     |
| Body weight (g)             | 299.1 ± 20.5  | 206.2 ± 15.7 | 433.3 ± 16.2 | 246.0 ± 11.9 |
| Liver weight (g)            | 9.9 ± 0.8     | 13.9 ± 2.1  | 12.1 ± 12   | 18.8 ± 2.9  |
| Liver/Body weight ratio     | 0.033 ± 0.001 | 0.067 ± 0.007 | 0.028 ± 0.002 | 0.076 ± 0.008 |
| AST (IU/L)                  | 74.9 ± 12.6   | 564 ± 438  | 62.7 ± 16.6 | 566 ± 229  |
| ALT (IU/L)                  | 45.1 ± 13.1   | 760 ± 528  | 38.6 ± 15.9 | 229 ± 94.6 |
| TC (mg/dL)                  | 56.4 ± 4.4    | 31.4 ± 3.1  | 51.7 ± 10.4 | 66.6 ± 23.7 |
| TG (mg/dL)                  | 96.2 ± 33.4   | 4.6 ± 3.2   | 184 ± 131   | 8.1 ± 4.6   |
| GLU (mg/dL)                 | 192 ± 20.1    | 143 ± 13.8  | 201 ± 18.1  | 127 ± 16.6  |
| TBA (mg/dL)                 | 7.6 ± 4.6     | 117 ± 91.3  | 13.2 ± 12.2 | 138 ± 75.1  |
| ALB (g/dL)                  | 2.2 ± 0.1     | 2.3 ± 0.1   | 2.3 ± 0.1   | 2.1 ± 0.2   |
| ALP (IU/L)                  | 518 ± 75.7    | 1109 ± 427 | 316 ± 69.7  | 1757 ± 818  |
| T-BIL (mg/dL)               | 0.11 ± 0.02   | 0.44 ± 0.24 | 0.09 ± 0.02 | 0.66 ± 0.41 |

Values are expressed as means ± SDs (n = 5). ▫ P < 0.05, ▫ P < 0.01, compared with the respective normal diet-fed group.
CDHFD, choline-deficient, low-methionine high-fat diet; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TC, total cholesterol; TG, triglyceride; GLU, glucose; TBA, total bile acids; ALB, albumin; ALP, alkaline phosphatase; T-BIL, total bilirubin.
Table 2: Histological parameters, evaluated using H&E and SR staining

| Parameters                      | Group          | 3-4 weeks |          | 9-10 weeks |          |
|--------------------------------|----------------|-----------|----------|------------|----------|
|                                |                | Normal diet | CDHFD    | Normal diet | CDHFD    |
| Steatosis score                |                | 0.0 ± 0.0  | 3.0 ± 0.0 | 0.0 ± 0.0  | 3.0 ± 0.0 |
| Inflammation score             |                | 0.0 ± 0.0  | 1.4 ± 0.55 | 0.20 ± 0.45 | 2.6 ± 0.55 |
| Ballooning score               |                | 0.0 ± 0.0  | 0.20 ± 0.45 | 0.0 ± 0.0  | 1.0 ± 0.0  |
| NAFLD activity score           |                | 0.0 ± 0.0  | 4.6 ± 0.89 | 0.20 ± 0.45 | 6.6 ± 0.55 |
| Fibrosis area (%)              |                | 1.0 ± 0.0  | 1.4 ± 0.5  | 1.0 ± 0.0  | 19.8 ± 10 |

Values are expressed as means ± SDs (n = 5). \( \ddagger \) \( P < 0.05 \), \( \ddagger \ddagger \) \( P < 0.01 \), compared with the respective normal diet-fed groups; \( \dagger \) \( P < 0.05 \); \( \dagger \dagger \) \( P < 0.01 \), compared with the 3–4-week CDHFD-fed group.

H&E, haematoxylin and eosin; SR, Sirius red; CDHFD, choline-deficient, low-methionine high-fat diet; NAFLD, non-alcoholic fatty liver disease.

Table 3: Kinetic parameters, determined using compartment analysis

| Group    | Model   | \( K_1 \) | \( k_2 \) | \( k_3 \) | \( k_4 \) | AIC  |
|----------|---------|-----------|----------|----------|----------|------|
| 3-4 weeks | Normal diet | 1T2CM     | 1.06     | 2.08     | -        | -    | 89.33 |
|          |          | 2T3CM     | 1.43     | 3.63     | 0.05     | 0.03 | 35.96 |
|          | CDHFD    | 1T2CM     | 0.74     | 1.15     | -        | -    | 93.31 |
|          |          | 2T3CM     | 0.90     | 1.94     | 0.05     | 0.02 | 41.14 |
| 9-10 weeks | Normal diet | 1T2CM     | 0.87     | 1.56     | -        | -    | 97.59 |
|          |          | 2T3CM     | 1.26     | 3.00     | 0.05     | 0.02 | 43.93 |
|          | CDHFD    | 1T2CM     | 0.42     | 0.51     | -        | -    | 100.3 |
|          |          | 2T3CM     | 0.69     | 1.19     | 0.07     | 0.02 | 32.12 |

Values are expressed as means (n = 5).

CDHFD, choline-deficient, low-methionine high-fat diet; 1T2CM, one-tissue two-compartment model; 2T3CM, two-tissue three-compartment model; AIC, Akaike information criterion.
## Figures

|               | 3-4 weeks | 9-10 weeks |
|---------------|-----------|------------|
| Normal diet   | ![Image](image1) | ![Image](image2) |
| CDHFD         | ![Image](image3) | ![Image](image4) |
| Normal diet   | ![Image](image5) | ![Image](image6) |
| CDHFD         | ![Image](image7) | ![Image](image8) |

### Figure 1

Representative photomicrographs of hepatic histopathology in H&E- and Sirius red-stained sections CDHFD, choline-deficient, low-methionine high-fat diet.
Figure 2

Hepatic integrin αv and β3 protein expression. Values are protein expression relative to that of the mean value for the 3–4-week normal-diet-fed group; mean ± SD (n = 5). The vertical axis values are the integrin αv or β3 protein expression relative to that of the mean of the 3–4-week normal diet-fed group. *, P < 0.05; ***, P < 0.001 compared with the equivalent normal diet-fed groups. CDHFD, choline-deficient, low-methionine high-fat diet.
Figure 3

SUV profile and representative colour-coded PET images (a) Left ventricular time-activity curve and (b) hepatic time-activity curve after 18F-FPP-RGD2 administration. (c) Mean SUV and (d) representative PET/CT fusion images 60–90 min after 18F-FPP-RGD2 administration. Values are expressed as means ± SDs (n = 5). \( \mathbb{I} \), \( P < 0.05 \); \( \mathbb{II} \), \( P < 0.01 \); \( \mathbb{III} \), \( P < 0.001 \), compared with the respective normal diet-fed groups; \( \dagger \), \( P < 0.05 \) compared with 3–4-week CDHFD-fed groups. CDHFD, choline-deficient, low-methionine high-fat diet.
Figure 4

Curve fitting for the compartment analysis and correlation analysis with VT (a) Quality of curve fitting for a representative hepatic time-activity curve and (b) correlation between VT obtained using IDIF and VT obtained using AIF. Saline or unlabelled RGD (0.1, 0.3, 1, or 3 mg/kg) were administered intravenously 5 min before 18F-FPP-RGD2. 1T2C model, one-tissue two-compartment model; 2T3C model, two-tissue three-compartment model; IDIF, image-derived input function; AIF, arterial input function.
Hepatic VT analysed by 2T3C model analysis. Values are expressed as means ± SDs (n = 5). *, P < 0.05; † †, P < 0.01 compared with the equivalent normal diet-fed groups; † † †, P < 0.01 compared with the 3–4-week CDHFD-fed group. CDHFD, choline-deficient, low-methionine high-fat diet.
Figure 6

Correlation analysis of SUV60–90 min or VT and integrin Correlation between SUV60–90 min and (a) integrin αv and (c) β3 protein expression. Correlation between VT obtained using the 2T3C model and (b) integrin αv and (d) β3 protein expression. Vertical axis values are the integrin αv or β3 protein expression relative to the mean value for the 3–4-week normal diet-fed group. CDHFD, choline-deficient, low-methionine high-fat diet.

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