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Author(s)	Higashikawa, Kei; Horiguchi, Sawako; Tarisawa, Makoto; Shibata, Yuki; Ohkura, Kazue; Yasui, Hironobu; Takeda, Hiroshi; Kuge, Yuji
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1 **Preclinical investigation of potential use of thymidine phosphorylase**  
2 **imaging probe for diagnosis of nonalcoholic steatohepatitis**

3

4 Kei Higashikawa<sup>1,2,\*</sup>, Sawako Horiguchi<sup>3</sup>, Makoto Tarisawa<sup>3</sup>, Yuki Shibata<sup>2</sup>, Kazue  
5 Ohkura<sup>4</sup>, Hironobu Yasui<sup>1,2</sup>, Hiroshi Takeda<sup>3</sup>, Yuji Kuge<sup>1,2</sup>

6 <sup>1</sup>Central Institute of Isotope Science, Hokkaido University, Hokkaido 060-0815, Japan

7 <sup>2</sup>Graduate School of Biomedical Science and Engineering, Hokkaido University,  
8 Hokkaido 060-0815, Japan

9 <sup>3</sup>Faculty of Pharmaceutical Sciences, Hokkaido University, Hokkaido 060-0812, Japan

10 <sup>4</sup>Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido,  
11 Hokkaido 061-0293, Japan

12

13 Running title: Decreased hepatic accumulation levels of TYMP imaging probe

14

15 **\*Corresponding Author:**

16 Kei Higashikawa, Ph.D.

17 Central Institute of Isotope Science, Hokkaido University

18 Kita 15, Nishi 7, Kita-ku, Sapporo, Hokkaido 060-0815, Japan

19 Tel.: +81-11-706-6092

20 Fax: +81-11-706-7862

21 E-mail: [higahikawa@ric.hokudai.ac.jp](mailto:higahikawa@ric.hokudai.ac.jp)

22

23

24 **Abstract**

25

26 **[Introduction]**

27 Although liver biopsy is the gold standard for the diagnosis of nonalcoholic  
28 steatohepatitis (NASH), it has several problems including high invasiveness and  
29 sampling errors. Therefore, the development of alternative methods to overcome these  
30 disadvantages is strongly required. In this study, we evaluated the potential use of our  
31 imaging probe targeting thymidine phosphorylase (TYMP),  
32 [<sup>123</sup>I]5-iodo-6-[(2-iminoimidazolidinyl)methyl]uracil ([<sup>123</sup>I]IIMU) for the diagnosis of  
33 NASH.

34

35 **[Methods]**

36 The mice used as the NASH model (hereafter, NASH mice) were prepared by feeding  
37 a methionine- and choline-deficient diet for 4 weeks. A control group was similarly  
38 given a control diet. The expression levels of the TYMP gene and protein in the liver  
39 were examined by real-time reverse-transcription polymerase chain reaction and  
40 western blot analyses. The localizations of [<sup>125</sup>I]IIMU and the TYMP protein in the liver  
41 were examined by autoradiography and immunohistochemical staining, respectively.

42 Finally, the mice were injected with [<sup>123</sup>I]IIMU and single-photon emission tomography  
43 (SPECT) imaging was conducted.

44

#### 45 **[Results]**

46 The hepatic expression levels of TYMP were significantly lower in the NASH mice  
47 than in the control mice at both mRNA and protein levels, suggesting that a decrease in  
48 TYMP level could be an indicator of NASH. [<sup>125</sup>I]IIMU was uniformly distributed in  
49 the liver of the control mice, whereas it showed a patchy distribution in that of the  
50 NASH mice. The localization of [<sup>125</sup>I]IIMU was visually consistent with that of the  
51 TYMP protein in the liver of the control and NASH mice. SPECT analysis indicated  
52 that the hepatic accumulation of [<sup>123</sup>I]IIMU in the NASH mice was significantly lower  
53 than that in the control mice [SUV (g/ml): 4.14 ± 0.87 (Control) vs 2.31 ± 0.29  
54 (NASH)].

55

#### 56 **[Conclusions]**

57 [<sup>123</sup>I]IIMU may provide a noninvasive means for imaging TYMP expression in the  
58 liver and may be applicable to the diagnosis of NASH.

59

60 KEYWORDS: NASH, TYMP, IIMU, Imaging

61

62 **1. Introduction**

63

64 Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver  
65 diseases affecting people who drink little to no alcohol. NAFLD covers a spectrum of  
66 conditions ranging from simple steatosis, which follows a benign clinical course, to  
67 nonalcoholic steatohepatitis (NASH), which progresses to liver fibrosis, cirrhosis, and  
68 hepatocellular carcinoma (HCC). Although progress has been made in the development  
69 of diagnostic methods for NASH, liver biopsy is the only reliable method to accurately  
70 diagnose and stage NAFLD/NASH severity [1]. However, liver biopsy has many  
71 drawbacks such as high invasiveness (risk of complications) and interobserver  
72 variability [2, 3]. In addition, biopsy sample is so small that diagnostic results tend to be  
73 different depending on the biopsy site (sampling errors) [4]. Therefore, it is not realistic  
74 to perform liver biopsies on all NAFLD patients, and the development of alternative  
75 methods to overcome these disadvantages is urgently desired.

76 Nuclear medicine imaging technologies with single-photon emission tomography  
77 (SPECT) and positron emission tomography (PET) enable the direct and noninvasive  
78 evaluation of whole organs. Therefore, diagnosis using these technologies has a

79 potential to become a reliable method to accurately diagnose and stage NAFLD/NASH  
80 severity if appropriate imaging biomarkers are found.

81 Tumor necrosis factor (TNF)- $\alpha$  plays a pivotal role in the development of fatty liver  
82 and subsequently NASH [5]. Treatment with TNF- $\alpha$  antibodies improved fatty liver  
83 formation and hepatic inflammation [6]. Therefore, factors induced by TNF- $\alpha$  may have  
84 the potential to be imaging biomarkers of NASH. Thymidine phosphorylase (TYMP) is  
85 induced by TNF- $\alpha$  and is also identical to the platelet-derived endothelial cell growth  
86 factor (PD-ECGF) [7, 8]. We previously synthesized radioiodine-labeled  
87 [<sup>123/125</sup>I]5-iodo-6-[(2-iminoimidazolidinyl)methyl]uracil (IIMU), a potent inhibitor of  
88 thymidine phosphorylase (TYMP) (Fig. 1) [9]. [<sup>123/125</sup>I]IIMU accumulated in rodent  
89 tumor cells and tissues, depending on TYMP expression level [10-13], and was safely  
90 administered in humans [14].

91 From these reports, we hypothesized that TYMP may function as an imaging  
92 biomarker of NASH, and [<sup>123</sup>I]IIMU provides a noninvasive means for imaging NASH.  
93 In this study, we aimed to investigate the feasibility of TYMP as an imaging biomarker  
94 of NASH, and we also evaluated the potential use of [<sup>123</sup>I]IIMU for the diagnosis of  
95 NASH.

96

97

98 **2. Materials and Methods**

99

100 ***2-1. Preparation of mouse NASH model***

101

102 Male C57BL/6N mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The  
103 mice were housed under a 12-h light/12-h dark cycle with food and water supplied ad  
104 libitum. All experimental protocols were approved by the Laboratory Animal Care and  
105 Use Committee of Hokkaido University and performed in accordance with the  
106 Guidelines for Animal Experiments of the Graduate School of Medicine, Hokkaido  
107 University.

108 The mice (8 weeks old) were housed with control diet (A02082003B, Research Diets  
109 Inc., New Brunswick, NJ) for at least 1 week, and then they were divided into two  
110 groups, the NASH model and the control. The mice used as the NASH model (hereafter,  
111 NASH mice) were prepared by feeding a methionine/choline-deficient (MCD) diet  
112 (A02082002B, Research Diet, Inc.) for 4 weeks. The control group was maintained on  
113 the original diet for 4 weeks.

114

115 ***2.2. Physiological and biochemical assays***

116

117 The NASH and control mice were sacrificed under isoflurane anesthesia. Serum was  
118 collected and assayed for the levels of alanine aminotransferase (ALT) and aspartate  
119 aminotransferase (AST) using an Alanine Aminotransferase (ALT or SGPT) Activity  
120 Colorimetric/Fluorometric Assay Kit (BioVision, Inc., Milpitas, CA) and an Aspartate  
121 Aminotransferase (AST or SGOT) Activity Colorimetric Assay Kit (BioVision Inc.),  
122 respectively, in accordance with the manufacturer's instruction.

123

### 124 ***2.3. Real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis***

125

126 RNA was extracted by the same methods as in our previous studies [15, 16]. Hepatic  
127 total RNA was isolated from the NASH and control mice with TRIZOL® reagent  
128 (Thermo Fisher Scientific Inc., Waltham, MA) and purified with a PureLink™ RNA  
129 Mini Kit (Thermo Fisher Scientific Inc.). Total RNA (500 ng) was then reversely  
130 transcribed using a ReverTra Ace® qPCR RT Master Mix with a gDNA Remover  
131 (TOYOBO Co., Ltd., Osaka, Japan) and a PCR Thermal Cycler Dice (Takara Bio Inc.,  
132 Kusatsu, Japan). The messenger RNA (mRNA) expression level of TYMP was  
133 determined by real-time RT-PCR analysis using TaqMan™ Universal Master Mix II,

134 with UNG (Thermo Fisher Scientific Inc), primer-probe sets of TaqMan Gene  
135 Expression Assays (Mm00460357\_m1; Thermo Fisher Scientific Inc.), and Thermal  
136 Cyclor Dice Real Time System II (Takara Bio Inc.). The expression levels of TNF- $\alpha$ ,  
137 interleukin-1 $\beta$  (IL-1 $\beta$ ), cluster of differentiation 68 (CD68), collagen type I alpha 1  
138 (COA1A1), collagen type I alpha 2 (COA1A2),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and  
139 TATA-binding protein (TBP) were analyzed by real-time RT-PCR using FastStart  
140 Universal SYBR Green Master (Rox) (Roche Diagnostics, Indianapolis, IN), the primer  
141 set specific for each molecule, and Thermal Cyclor Dice Real Time System II. Primer  
142 sequences are listed in Supplementary Table S1. The expression level of each mRNA  
143 was normalized with that of TBP.

144

#### 145 ***2.4. Histopathological evaluation***

146

147 The excised livers were fixed in 10% formalin neutral buffer solution (FUJIFILM  
148 Wako Pure Chemical Co., Ltd., Osaka, Japan) and embedded in paraffin before  
149 sectioning and staining. Five-micrometer-thick liver sections were prepared,  
150 deparaffinized in xylene, rehydrated in an ethanol series, and washed in running water.  
151 The liver sections were stained with Mayer's hematoxylin solution (FUJIFILM Wako

152 Pure Chemical Co., Ltd.) and eosin Y solution (Muto Pure Chemicals Co., Ltd., Tokyo,  
153 Japan).

154

## 155 ***2.5. Western blot analysis***

156

157 The liver tissues were solubilized in RIPA Lysis and Extraction Buffer (Thermo Fisher  
158 Scientific, Inc.) containing a protease inhibitor cocktail (cOmplete™, Roche Co., Ltd.,  
159 Mannheim, Germany). One, ten, and twenty micrograms of the proteins were separated  
160 by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a  
161 5–20% gel (e-PAGEL, ATTO Co., Ltd., Tokyo, Japan) to evaluate the expression  
162 levels of TYMP, TBP, and uridine phosphorylase 1 (UPP1) and UPP2, respectively.  
163 Recombinant UPP1 (1 ng; Cloud-Clone Co., Katy, TX) and UPP2 (10 ng; Cloud-Clone  
164 Co.) were used as positive controls and also similarly separated by SDS-PAGE. Proteins  
165 were transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc.  
166 Hercules, CA). The membranes were washed with TBS buffer (25 mM Tris–HCl, 137  
167 mM NaCl, 2.68 mM KCl, pH 7.4), and then blocked with TBST buffer (50 mM  
168 Tris–HCl, 138 mM NaCl, 2.70 mM KCl, 0.5 ml/L Tween 20, pH 8.0) plus 5% skim  
169 milk. The membranes were incubated with a sheep polyclonal anti-TYMP primary

170 antibody (1:1000; R&D Systems, Inc., Minneapolis, MN) or a rabbit monoclonal  
171 anti-TBP primary antibody (1:1000; Cell Signaling Technology Inc., Beverly, MA), an  
172 anti-UPP1 primary antibody (1:400; Lifespan Biosciences Inc., Seattle, WA), and an  
173 anti-UPP2 primary antibody (1:3000; Abcam, Inc., Cambridge, UK) in TBST buffer  
174 plus 5% skim milk. The membranes were washed with TBST buffer and incubated  
175 with a horseradish peroxidase (HRP)-conjugated anti-sheep antibody (1:1000; R&D  
176 Systems, Inc.) or anti-rabbit antibody (1:4000; Promega Inc., Madison, WI) in TBST  
177 buffer plus 5% skim milk. Proteins were visualized and quantified using the  
178 Immobilon™ Western Chemiluminescent HRP Substrate (Millipore Inc. Billerica, MA)  
179 or ECL Prime Western Blotting Detection Reagent (GE Healthcare Inc., Chicago, IL),  
180 and the luminescent image analyzer LAS-4000 mini (Fuji Photo Film Co., Ltd., Tokyo,  
181 Japan). TBP was used as the protein-loading control for immunoblotting.

182

## 183 ***2.6 Biodistribution study***

184

185 Approximately 2.6 MBq/kg [<sup>125</sup>I]IIMU in saline was intravenously administered to the  
186 NASH and control mice via the tail vein under isoflurane anesthesia. Then, the mice  
187 were returned to their cages and allowed to move freely for 30 min. Subsequently, the

188 mice were sacrificed under isoflurane anesthesia, and their organs and blood were  
189 immediately removed. After washing the organs with water, the organs and the blood  
190 were weighed. The radioactivity in the organs and blood was counted with a gamma  
191 counter (2480 WIZARD 2 Automatic Gamma Counter, PerkinElmer Inc., Waltham,  
192 MA). Decay-corrected uptake was expressed as standardized uptake value (SUV) and  
193 organ-to-blood ratios. SUV was defined as {[tissue radioactivity concentration  
194 (Bq/g)/injected radioactivity (Bq)] × body weight (g)}.

195

## 196 ***2.7. Autoradiography (ARG) and immunohistochemical (IHC) staining analyses***

197

198 The livers were embedded in Optimal Cutting Temperature (O.C.T.) compound  
199 (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and frozen in isopentane/dry ice. For  
200 ARG and IHC staining experiments, 20- and 5- to 10- $\mu$ m-thick serial frozen tissue  
201 sections were prepared, respectively. The tissue sections were mounted on MAS-coated  
202 glass slides (Matsunami Glass Ind., Co., Ltd., Kishiwada, Japan) and fixed with  
203 M-Fix™ spray fixative (Merck, Darmstadt, Germany). For the autoradiography  
204 experiment, the liver tissue sections were exposed to a phosphor imaging plate (Fuji  
205 Photo Film Co., Ltd.). After the exposure, the imaging plate was scanned with a FLA

206 7000 Bio-Imaging Analyzer (Fuji Photo Film Co., Ltd.) and the images obtained were  
207 analyzed using Multi Gauge V3.0 (Fuji Photo Film Co., Ltd.). For the  
208 immunohistochemical staining, the liver tissue sections were washed in 100 mL of  
209 phosphate-buffered saline (-) [PBS (-)] containing a drop of 13-fold diluted Triton-X,  
210 and blocked with a blocking solution (protein block, serum-free; Agilent Technologies,  
211 Inc., Carpinteria, CA). The tissue sections were incubated with an anti-TYMP antibody  
212 (1:100; R&D Systems, Inc.) in 0.1% BSA/PBS (-), washed in the PBS (-) plus  
213 Triton-X solution, and subjected to endogenous peroxidase inactivation with 0.3%  
214 H<sub>2</sub>O<sub>2</sub>/methanol. Then, the tissue sections were washed in the PBS (-) plus Triton-X  
215 solution, incubated with an HRP-conjugated anti-sheep antibody (1:100; R&D Systems,  
216 Inc.) in 0.1% BSA/PBS (-), and washed with PBS (-). Immunocomplexes were  
217 visualized with a Peroxidase Stain DAB Kit (Nacalai Tesque Inc., Kyoto, Japan) and  
218 counterstained with hematoxylin.

219

## 220 ***2.8. SPECT and computed tomography (CT) analyses***

221

222 The NASH (n = 4) and control mice (n = 4) were injected with [<sup>123</sup>I]IIMU (3.3-4.7  
223 MBq) in saline containing 1% ascorbic acid via tail vein under isoflurane anesthesia. At

224 30 min after the injection, SPECT was performed for approximately 15 min using an  
225 Inveon SPECT/CT scanner (Siemens Healthineers Inc., Munich, Germany). A single  
226 pinhole collimator set with a 2.0 mm aperture (1-MHS-2.0) were used. Then, CT was  
227 performed for attenuation correction. Hepatic accumulation levels of [<sup>123</sup>I]IIMU were  
228 quantified using Inveon Research Workplace (Siemens Healthineers Inc.). To evaluate  
229 specific accumulation of [<sup>123</sup>I]IIMU in the liver, mice were injected with 25 µg tipiracil  
230 hydrochloride (TIP; a specific TYMP inhibitor) (SML1552; Sigma-Aldrich Co. Ltd.),  
231 into the NASH (n = 4) and control mice (n = 3) immediately before [<sup>123</sup>I]IIMU injection  
232 (3.9-4.8 MBq). The hepatic accumulation levels of [<sup>123</sup>I]IIMU was evaluated by  
233 SPECT/CT as described above.

234

## 235 **2.9. Statistical analysis**

236

237 All data are expressed as means ± standard deviation (SD). Statistical significance was  
238 determined using Welch's t-test or one-way analysis of variance (ANOVA) followed by  
239 Tukey-Kramer test. The level of significance was taken as  $p < 0.05$ . The tests were  
240 performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

241

242 **3. Results**

243

244 ***3.1. Physiological characteristics and hepatic pathology in NASH and control mice***

245

246 Anthropometric and biochemical parameters in the MCD-diet-induced NASH and  
247 control mice are shown in Table 1. No significant difference in liver weight was  
248 observed between the NASH and control mice. The NASH mice showed a lower body  
249 weight ( $p < 0.001$ ) and a higher liver/body weight ratio (%) ( $p < 0.01$ ) than the control  
250 mice. Serum ALT and AST levels were significantly higher in the NASH mice than in  
251 the control mice ( $p < 0.01$ ).

252 Figure 2 shows the hepatic expression levels of inflammation (TNF- $\alpha$ , IL-1 $\beta$ ), Kupffer  
253 cell (CD68), and fibrosis (COL1A1, COL1A2,  $\alpha$ -SMA) markers in the NASH and  
254 control mice. The expression levels of TNF- $\alpha$ , IL-1 $\beta$ , CD68, COL1A1, COL1A2, and  
255  $\alpha$ -SMA were significantly higher in the NASH mice than in the control mice.  
256 H&E-stained liver tissue sections from the NASH and control mice are shown in Fig. 3.  
257 Prominent steatosis, cellular infiltrations, and ballooning degeneration of hepatocytes  
258 were observed in the liver of the NASH mice but not in that of the control mice.

259

260 **3.2. Hepatic TYMP and UPP expressions in NASH and control mice**

261

262 The hepatic expression levels of TYMP were lower in the NASH mice than in the  
263 control mice at both mRNA [relative expression of mRNA:  $1.00 \pm 0.13$  (Control) vs  
264  $0.43 \pm 0.23$  (NASH),  $p < 0.01$ ] and protein levels [relative expression of protein:  $1.00 \pm$   
265  $0.51$  (Control) vs  $0.14 \pm 0.07$  (NASH),  $p < 0.05$ ] (Fig. 4). No UPP1 and UPP2  
266 expressions were detected in the liver of the NASH and control mice, whereas  
267 recombinant UPP1 (1 ng) and UPP2 (10 ng) (positive controls) were clearly detected  
268 (Fig. S1).

269

270 **3.3 Biodistribution analyses of [<sup>125</sup>I]IIMU in NASH and control mice**

271

272 The biodistributions of [<sup>125</sup>I]IIMU in the NASH, control mice, 30 min after  
273 administration are shown in Fig. 5. [<sup>125</sup>I]IIMU showed a markedly higher accumulation  
274 level in the liver than in other organs including the thymus, heart, lung, spleen, stomach,  
275 small intestine, large intestine, kidney, and thigh muscle (Fig. 5A). The hepatic  
276 accumulation level of [<sup>125</sup>I]IIMU was significantly lower in the NASH mice than in the  
277 control mice [SUV:  $6.65 \pm 1.44$  (Control) vs  $3.36 \pm 1.14$  (NASH),  $p < 0.05$ ; Fig. 5A].

278 The liver-to-blood ratios were also significantly lower in the NASH mice than in the  
279 control mice [ $58.3 \pm 15.1$  (Control) vs  $33.8 \pm 11.4$  (NASH),  $p < 0.05$ ; Fig. 5B]. The  
280 accumulation level and organ-to-blood ratios in the organs except for the liver were not  
281 significantly different between the NASH and control mice.

282

### 283 ***3.4. Hepatic localizations of [ $^{125}$ I]IIMU and TYMP protein in NASH and control mice***

284

285 Representative hepatic ARG images of [ $^{125}$ I]IIMU and IHC staining images of TYMP  
286 are shown in Fig. 6. [ $^{125}$ I]IIMU was uniformly distributed in the liver of the control  
287 mice (Fig. 6A, panel a), whereas it showed a patchy distribution in the liver of the  
288 NASH mice (Fig. 6B, panel a). The hepatic localization of [ $^{125}$ I]IIMU was visually  
289 consistent with that of the TYMP protein in the control (Fig. 6A, panels a and b) and  
290 NASH mice (Fig. 6B, panels a and b).

291

### 292 ***3.5. SPECT/CT imaging of [ $^{123}$ I]IIMU in NASH and control mice***

293

294 SPECT/CT images and mean hepatic [ $^{123}$ I]IIMU uptake values are shown in Fig. 7.  
295 The hepatic accumulation of [ $^{123}$ I]IIMU in the NASH mice was significantly lower than

296 that in the control mice [SUV (g/ml):  $4.14 \pm 0.87$  (Control) vs  $2.31 \pm 0.29$  (NASH),  $p <$   
297  $0.01$ ]. Blocking with TYMP inhibitor TIP significantly reduced the hepatic  
298 accumulation of [ $^{123}\text{I}$ ]IIMU in the both NASH and control mice [SUV (g/ml):  $0.52 \pm$   
299  $0.42$  (Control-TIP),  $0.13 \pm 0.05$  (NASH-TIP),  $p < 0.001$ ]. However, [ $^{123}\text{I}$ ]IIMU SPECT  
300 could not visualize a patchy distribution of TYMP in the liver of NASH.

301

302 **4. Discussion**

303

304 In this study, we investigated whether TYMP has a potential as an imaging biomarker  
305 and also evaluated the potential use of [<sup>123</sup>I]IIMU for the diagnosis of NASH. This is  
306 the first study to evaluate the hepatic expression levels of TYMP in NASH mice and to  
307 examine the usability of a TYMP imaging probe for the evaluation of the state of NASH.  
308 We hypothesized that the expression levels of TYMP could be increased in the liver of  
309 NASH mice, because TYMP is induced by TNF- $\alpha$ , which plays a pivotal role in the  
310 development of fatty liver and subsequently NASH [5]. However, both the mRNA and  
311 protein expression levels of TYMP in the liver of NASH mice were decreased (Fig. 4).  
312 Moreover, as in the results of TYMP expression analyses, the hepatic accumulation  
313 levels of [<sup>125</sup>I]IIMU were significantly lower in the NASH mice than in the control mice  
314 (Fig. 5). Furthermore, we found that [<sup>125</sup>I]IIMU also showed a patchy distribution in the  
315 liver of NASH mice, and the distribution of [<sup>125</sup>I]IIMU was visually consistent with that  
316 of the TYMP protein in the liver of NASH mice (Fig. 6). SPECT analysis also indicated  
317 that the hepatic accumulation levels of [<sup>123</sup>I]IIMU were significantly lower in the NASH  
318 mice, and [<sup>123</sup>I]IIMU SPECT can visually distinguish between the NASH and control  
319 mice (Fig. 7). Although these results were different from our initial hypothesis, they

320 suggested that SPECT imaging using [<sup>123</sup>I]IIMU may be applicable to the noninvasive  
321 evaluation of the entire liver in NASH patients.

322 In this study, we utilized the MCD-diet-induced NASH as the disease model because it  
323 is one of the most widely used NASH models, in which the expression levels of TNF- $\alpha$   
324 are also increased in the liver [17], and its hepatic histopathological changes are  
325 morphologically similar to those observed in NASH patients [18]. Also in our study, the  
326 main histopathological features of NASH, such as prominent steatosis, cellular  
327 infiltrations, and ballooning degeneration of hepatocytes, were observed in the liver of  
328 NASH mice, but not in that of control mice (Fig. 3). Moreover, the serum ALT and AST  
329 levels (Table 1) and the hepatic expression levels of markers of inflammation (including  
330 TNF- $\alpha$ ), Kupffer cells, and fibrosis (Fig. 2) were significantly higher in the NASH mice  
331 than in the control mice, indicating that the NASH model was successfully prepared in  
332 our study.

333 In our previous studies, we clearly demonstrated that TYMP mRNA and protein were  
334 more highly expressed in the normal liver and small intestine than in the other organs  
335 including the heart, lung, stomach, spleen, kidney, large intestine, muscle, and brain  
336 [12]. Radiolabeled IIMU also accumulated mostly in the liver and small intestine of  
337 mice [9, 10, 12]. Moreover, the hepatic accumulation level of this probe was reduced to

338 15% in blocking experiments with non-radiolabeled IIMU [10]. These results in these  
339 studies indicate that the high accumulation levels of radiolabeled IIMU in the liver and  
340 small intestine are attributable to its binding to the TYMP physiologically expressed in  
341 the organs. Also in this study, radiolabeled IIMU accumulated mostly in the liver and  
342 small intestine compared with the other organs, which is consistent with the results of  
343 our previous studies. The ARG and IHC studies also indicated that the hepatic  
344 distribution of [<sup>125</sup>I]IIMU in the NASH and control mice was visually consistent with  
345 that of the TYMP protein (Fig. 6).

346 Since liver is a main organ involved in drug metabolism, [<sup>123/125</sup>I]IIMU has the  
347 potential to be metabolized in the liver and accumulate in it nonspecifically. However,  
348 the metabolite analysis of it in the liver has not been conducted. This is a limitation of  
349 our study. On the other hand, we performed the blocking study in NASH and control  
350 mice using TIP (TYMP inhibitor), to confirm whether [<sup>123</sup>I]IIMU accumulates in the  
351 liver specifically (Fig. 7). TIP is also a uracil derivative and has a similar structure of  
352 IIMU. Therefore, it is expected that the hepatic accumulation of IIMU is inhibited by  
353 TIP when the accumulation is specific to TYMP. This blocking study also indicated that  
354 the hepatic accumulation of [<sup>123</sup>I]IIMU in the both NASH and control mice is  
355 significantly reduced (Fig. 7B). This result suggested that most hepatic accumulation of

356 [<sup>123</sup>I]IIMU in the both NASH and control mice is specific and not due to hepatic  
357 metabolism (non-specific).

358 Furthermore, since mouse UPPs also have TYMP activity unlike human UPPs [19],  
359 [<sup>123/125</sup>I]IIMU has potential to accumulate organs expressing the UPPs. Although the  
360 activity is not necessarily proportional to the expression level, an organ with the highest  
361 TYMP activity derived from UPPs is a small intestine [19] and hence the observed  
362 accumulation of IIMU in the small intestine may be partly due to UPPs. On the other  
363 hand, our study clearly indicated that no UPP1 and UPP2 expressions were detected in  
364 the liver of the NASH and control mice (Fig. S1), indicating that UPPs are not involved  
365 in the accumulation of the TYMP imaging probe in the liver. These results supported  
366 the finding that [<sup>123/125</sup>I]IIMU binds specifically to TYMP in the liver of the NASH and  
367 control mice.

368 A key question not addressed in this study is the reason why the hepatic expression  
369 levels of TYMP were lower in the NASH mice than in the control mice, even when the  
370 TNF- $\alpha$  expression levels were higher in the NASH mice. Although the reason for this  
371 unexpected finding is currently unknown, it strongly suggests that certain TYMP  
372 downregulators, which are more potent than TNF- $\alpha$ , may exist in the liver of NASH  
373 mice. In the NASH liver, the repetitive cycles of hepatic injury and enhanced

374 compensatory regeneration/proliferation of hepatocytes occur, which may lead to the  
375 increased demand for thymidine. Since TYMP is a key enzyme involved in the  
376 thymidine salvage pathway [20] and a recent study suggested that TYMP lowers the  
377 thymidine levels within the hepatocytes [21], it is possible to consider that the increase  
378 in thymidine requirement suppressed the hepatic expression levels of TYMP in NASH  
379 mice. Further mechanistic studies are warranted to evaluate this possibility.

380 Although the MCD-diet-induced NASH is commonly used as the disease model in  
381 basic research, the problems with these mice are their body weight loss and lack of  
382 insulin resistance [22], which are well-known symptoms in patients with NASH. It is  
383 necessary to examine in future experiments whether obesity and insulin resistance affect  
384 the thymidine metabolism and biodistribution of [<sup>123</sup>I]IIMU.

385 Other imaging techniques such as MRI and CT can assess the hepatic steatosis with  
386 higher spatial resolution image in comparison with SPECT. Therefore, further studies  
387 are needed to find advantages of [<sup>123</sup>I]IIMU for imaging NASH. As a potential  
388 advantage, unlike the liver with NASH, several cancers including HCC highly express  
389 TYMP, in comparison with normal organs [20, 23]. Therefore, [<sup>123</sup>I]IIMU may be used  
390 for the clear discrimination between NASH and NASH-related HCC. Further studies  
391 are currently under way to examine how the expression levels of TYMP change

392 according to the progression of NASH.

393

## 394 **5. Conclusions**

395

396 In this study, we demonstrated that the hepatic accumulation levels of [<sup>125</sup>I]IIMU were  
397 decreased with a patchy distribution in NASH mice. These results were consistent with  
398 the expression level and localization of TYMP in the liver. These results also suggest  
399 the feasibility of TYMP as an imaging biomarker of NASH. Moreover, SPECT imaging  
400 using [<sup>123</sup>I]IIMU may provide a noninvasive means for the diagnosis of NASH.

401

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403

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407 for the Promotion of Science and the Ministry of Education, Culture, Sports, Science,  
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409

410 **Conflict of Interest Statement**

411

412 Kazue Ohkura, Yuji Kuge, Hokkaido University, Health Sciences University of

413 Hokkaido, and Nihon Medi-Physics Co.,Ltd. have patent rights for [<sup>123</sup>I]IIMU. Yuji

414 Kuge has received grants from Nihon Medi-Physics Co.,Ltd., outside the submitted

415 work. There are no other potential conflicts of interest relevant to this article.

416

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489

490 **Figure legends**

491

492 **Figure 1: Chemical structure of radioiodine-labeled**  
493 **5-iodo-6-[(2-iminoimidazolidinyl)methyl] uracil (IIMU).** The \*I in the structure  
494 indicates the position of radioiodine.

495 **Figure 2: Gene expression analysis in liver tissues from NASH and control mice by**  
496 **real-time RT-PCR.** Relative hepatic expression levels of inflammatory (TNF- $\alpha$ , IL-1 $\beta$ ),  
497 Kupffer cell (CD68), and fibrosis (COL1A1, COL1A2,  $\alpha$ -SMA) markers in NASH (n =  
498 5) and control (n = 5) mice. Each expression level was normalized to that of TBP, and  
499 each bar represents means  $\pm$  SD. For statistical evaluation, Welch's t-test was applied.  
500 The symbols \*\* and \*\*\* denote  $p < 0.01$  and  $p < 0.001$  vs control mice, respectively.

501 **Figure 3: Assessment of histopathological findings of livers from NASH and**  
502 **control mice.** A. Microscopic observations of the liver from control mice (n = 3). B.  
503 Microscopic observations of the liver from NASH mice (n = 3). Representative  
504 H&E-stained liver sections are shown. Scale bar = 100  $\mu$ m. The yellow and blue arrows  
505 indicate cellular infiltration and ballooning degeneration of hepatocytes, respectively.

506 **Figure 4: TYMP expression in liver tissues from NASH and control mice evaluated**  
507 **by real-time RT-PCR and western blot analyses.** A. mRNA expression levels of  
508 TYMP in the livers from NASH (n = 5) and control (n = 5) mice. Each TYMP  
509 expression level was normalized to that of TBP, and each bar represents mean  $\pm$  SD.  
510 For statistical evaluation, Welch's t-test was applied. The symbol \*\* denotes  $p < 0.01$  vs  
511 control mice. B. Representative western blot images of TYMP in the livers from NASH  
512 (n = 5) and control (n = 5) mice (left), and the densitometric quantification values of the  
513 immunoblot bands (right). The expression level of TBP is shown as an internal control.

514 **Figure 5: Biodistribution analyses of [<sup>125</sup>I]IIMU in NASH and control mice.** A.  
515 SUVs of [<sup>125</sup>I]IIMU in NASH (n = 4) and control (n = 4) mice. B. Organ-to-blood ratios  
516 of [<sup>125</sup>I]IIMU in NASH (n = 4) and control (n = 4) mice. For statistical evaluation,  
517 Welch's t-test was applied. Data are expressed as means  $\pm$  SD. The symbol \* denotes  $p$   
518  $< 0.05$  vs control mice.

519 **Figure 6: Hepatic localizations of TYMP and [<sup>125</sup>I]IIMU in NASH and control mice.**  
520 A. Representative ARG images of [<sup>125</sup>I]IIMU in the liver of a control mouse (panel a; n  
521 = 4). Representative IHC staining images of TYMP in the liver from a control mouse  
522 (panel b; n = 4). B. Representative ARG images of [<sup>125</sup>I]IIMU in the liver from a NASH

523 mouse (panel a; n = 4). Representative IHC staining images of TYMP in the liver of a  
524 NASH mouse (panel b; n = 4). The yellow arrows indicate similar hepatic distribution  
525 patterns of [<sup>125</sup>I]IIMU and TYMP protein in a NASH mouse. Scale bar (blue) = 2.0 mm.

526 **Figure 7: SPECT/CT analyses of [<sup>123</sup>I]IIMU in NASH and control mice. A.**

527 Representative SPECT/CT images of [<sup>123</sup>I]IIMU in the NASH and control mice with or  
528 without blockade of TIP (TYMP inhibitor) at 30 min post injection under isoflurane  
529 anaesthesia (3.3-4.8 MBq injected dose). The imaging time was 15 min. B. Hepatic  
530 accumulation levels of [<sup>123</sup>I]IIMU in NASH and control mice with or without blockade  
531 of TIP. Data are expressed as means ± SD (n = 3-4, for each). For statistical evaluation,  
532 one-way ANOVA followed by Tukey-Kramer test was applied. The symbols \*\* and \*\*\*  
533 denote  $p < 0.01$  and  $p < 0.001$  vs control mice, respectively.

534

535 **Table 1: Biochemical parameters of MCD-diet-induced NASH and control mice.**

536

	Control	NASH
Serum ALT (U/L)	30.6 ± 8.3	254.9 ± 76.4**
Serum AST (U/L)	58.9 ± 13.2	89.4 ± 12.7**

537 ALT, alanine aminotransferase; AST, aspartate transaminase.

538 Data are expressed as means ± standard deviation (SD). For statistical evaluation,

539 Welch's t-test was applied. The symbols \*\* denotes  $p < 0.01$  vs control mice,

540 respectively. n = 5.

Figure 1

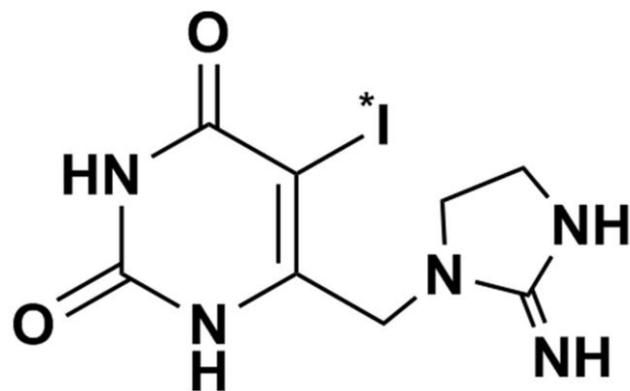


Figure 2

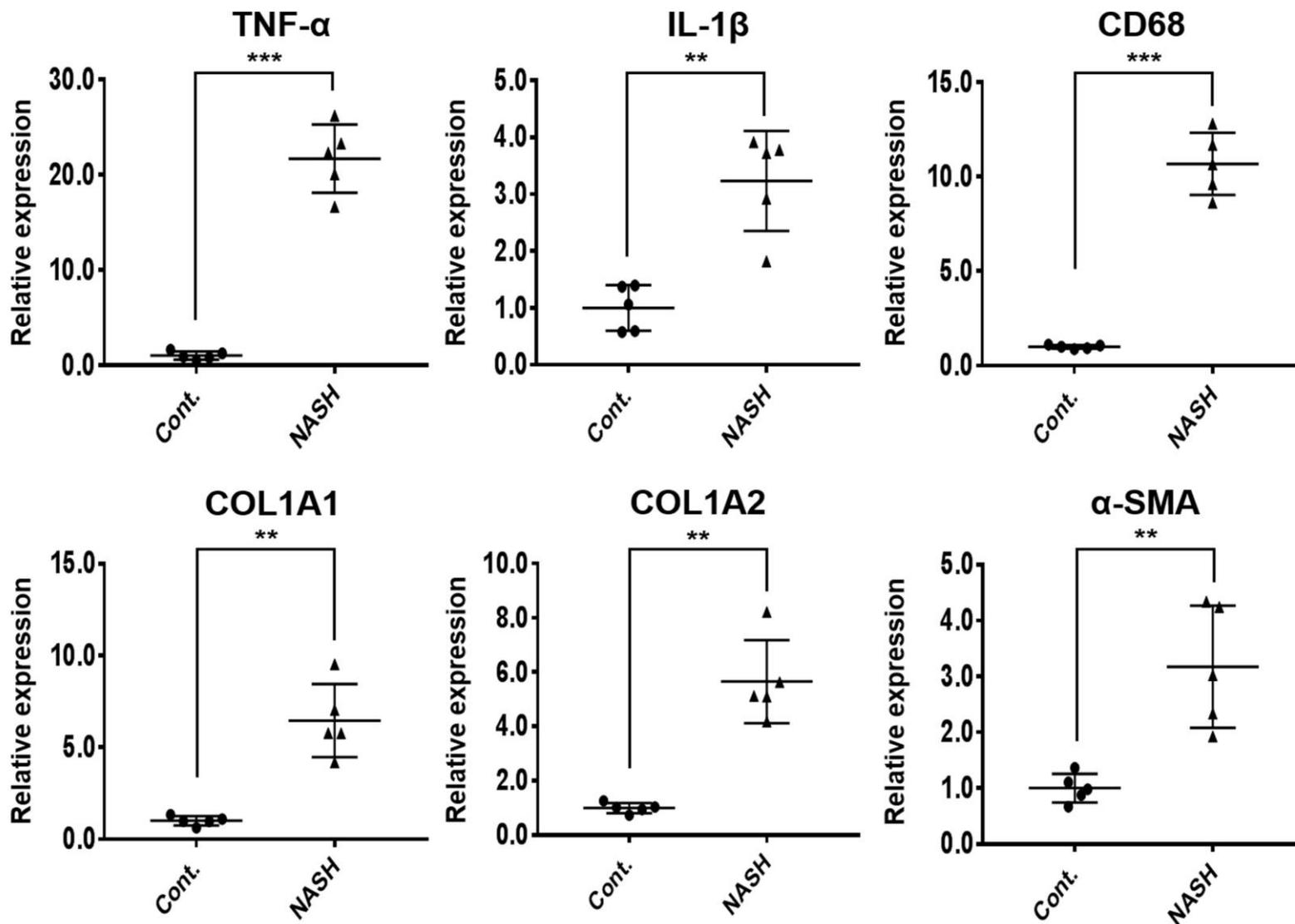
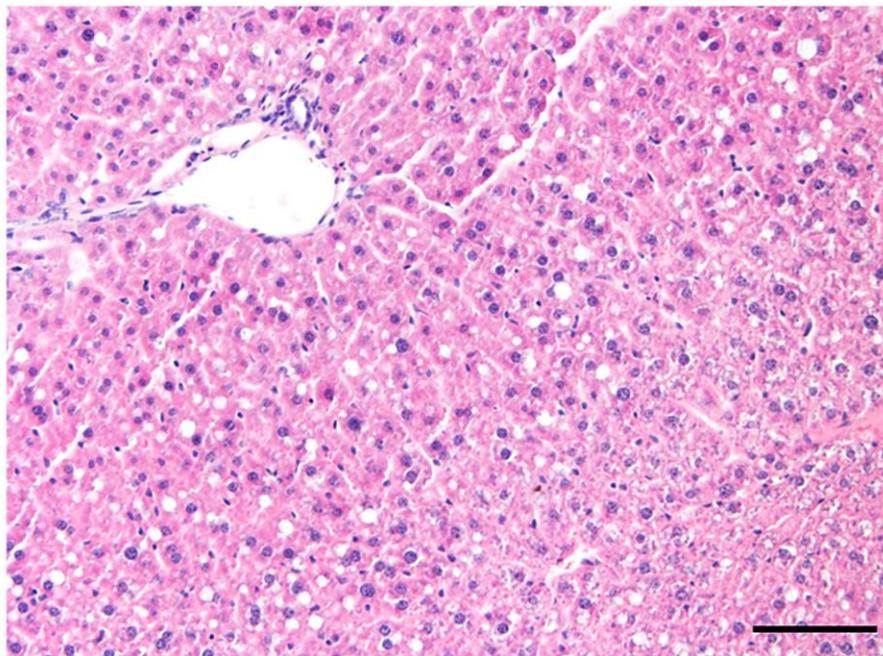


Figure 3

**A**



**B**

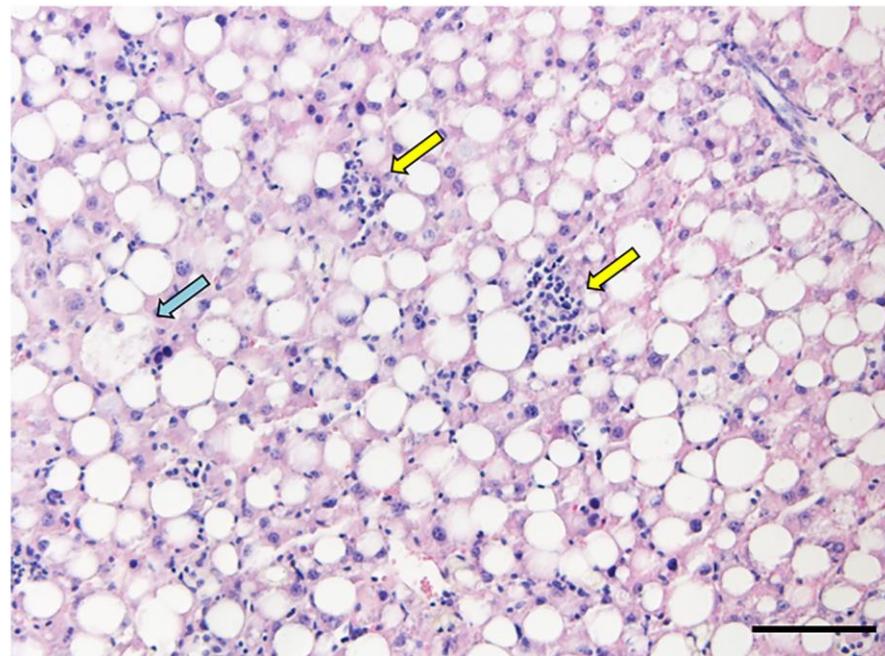


Figure 4

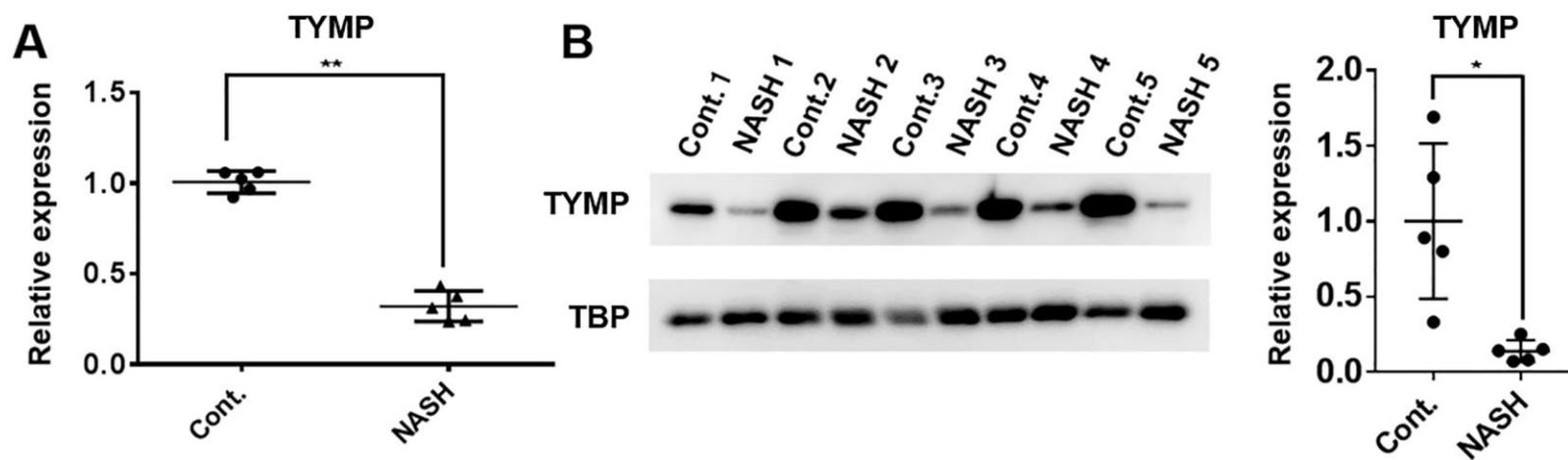
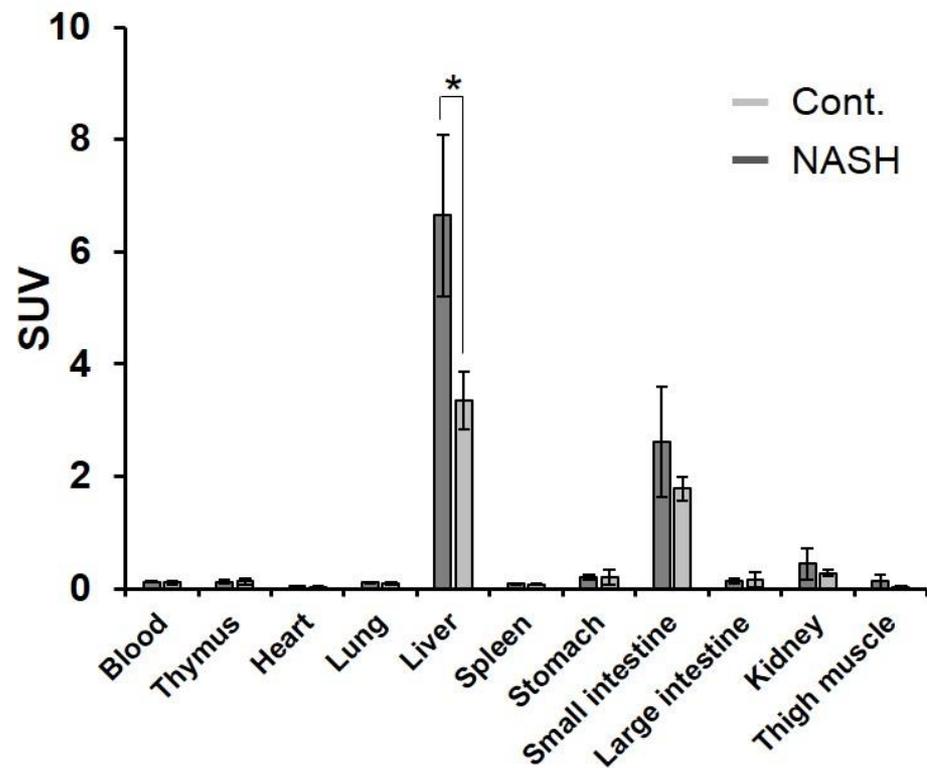


Figure 5

**A**



**B**

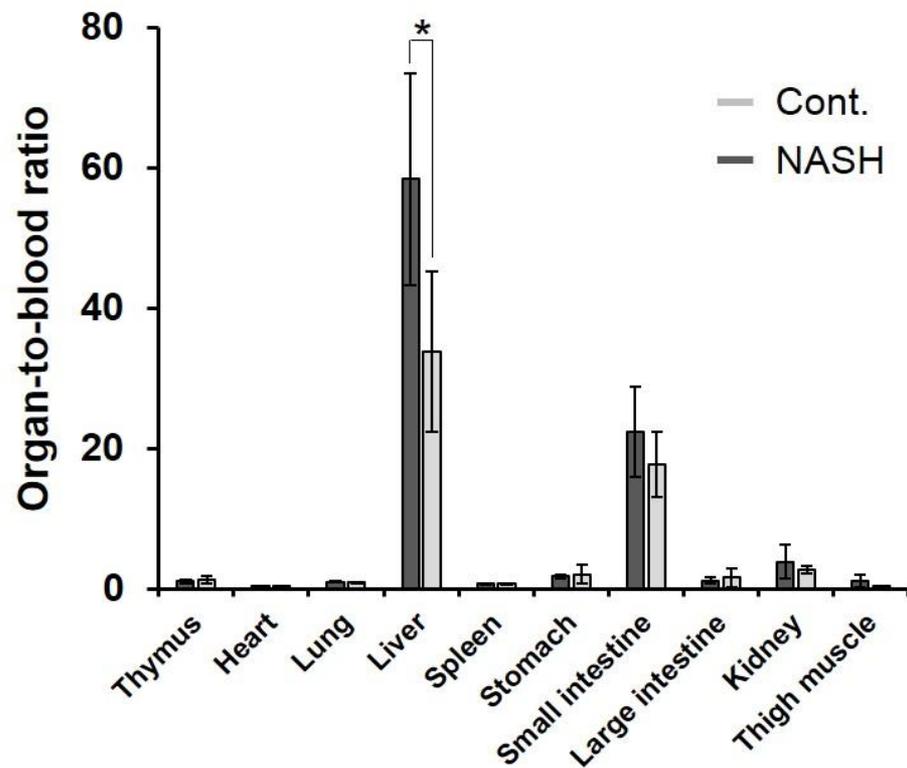


Figure 6

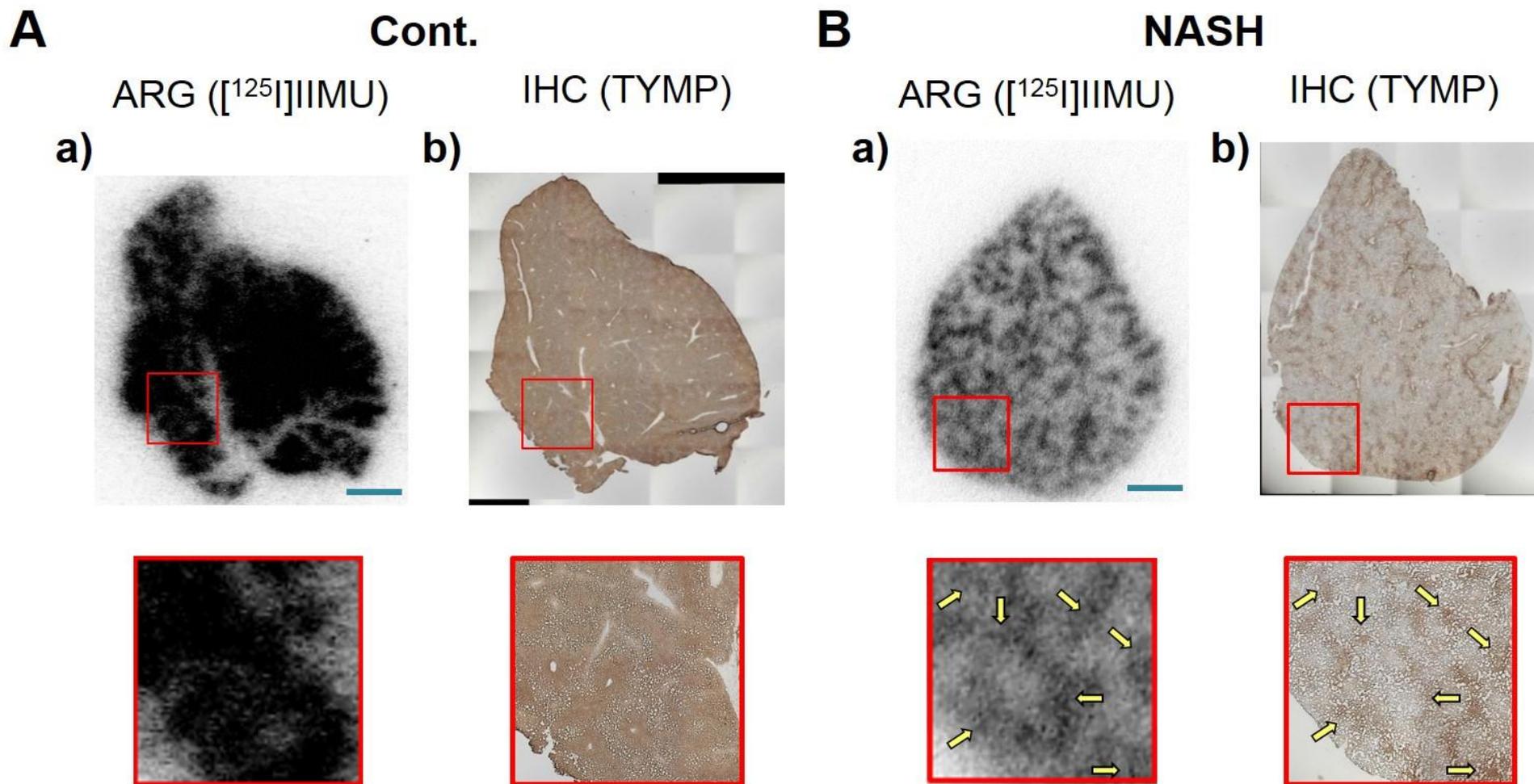


Figure 7

