

Figure 1. Circulating AIM levels in healthy individuals. (A) AIM levels in different generations. Error bar: SEM. ***: $p < 0.001$ vs. the value of women in 20s. ###: $p < 0.001$ vs. the value of men in 20s. (B) Means \pm SD ($\mu\text{g/ml}$) of AIM levels in whole men and women. AIM levels were significantly higher in women than in men. (C) Correlation of AIM and IgM levels in men and women. IgM levels were analyzed by ELISA in 20 individuals exhibiting a variety of AIM levels in each generation in men and women. (D) Correlation in AIM levels and BMI, obesity index, % of fat mass or waist circumference, LDL cholesterol levels, HbA1C, FBS, systolic or diastolic blood pressure in women. (E) Correlation in AIM and AST or ALT levels in males and females. In C-E, r : correlation coefficients in single linear regression analysis, p : p value, n : number of samples. Blue dots: men, yellow dots: women.
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Fibrosis analysis

Fibrosis area determined by Sirius red staining was quantified using NIH Image J software. Five areas for each sample were assessed under a microscope (FSX 100, OLYMPUS, Tokyo).

Quantitative PCR assay

The quantitative evaluation of mRNA was performed by the $\Delta\Delta C_T$ method using a 7500Fast Real-Time PCR system (Life Technologies Japan, Tokyo) and Power SYBR Green PCR Master

Mix (Life Technologies). Sequences of the oligonucleotides used are below:

- f-GAPDH 5'-AACTTTGGCATTGTGGAAGG-3'
- r-GAPDH 5'-GGATGCAGGGATGATGTTCT-3'
- f-TNF α 5'-ACGGCATGGATCTCAAAGAC-3'
- r-TNF α 5'-AGATAGCAAATCGGCTGACG-3'
- f-IL1 β 5'-CTGGTGTGTGACGTTCCCATTA-3'
- r-IL1 β 5'-CCGACAGCAGGCTTT-3'
- f-IL 6 5'-CCAGTTGCCTTCTTGGGACT-3'

Table 1. The composition of examinees and the AIM level.

age	Male		Female	
	AIM (µg/ml)	n	AIM (µg/ml)	n
10s	5.62±1.66	28	6.39±1.65	15
20s	5.41±1.67	368	6.75±2.05	592
30s	5.04±1.69	806	6.53±2.03	791
40s	4.90±1.76	1153	6.14±2.09	1163
50s	4.84±1.78	988	5.64±1.95	977
60s	5.02±1.80	580	5.45±2.1	579
70s	5.11±1.79	120	4.91±1.57	87
80s	4.69±1.57	9	5.57±2.84	16
90s	7.55±3.60	3	4.22±2.03	5
whole	4.99±1.76	4055	6.06±2.09	4225

The AIM level is presented as mean±SD (µg/ml). n: sample number.
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r-IL 6	5'-GGTCTGTTGGGAGTGGTATCC-3'
f-MCP1	5'-ACTGAAGCCAGCTCTCTCTTCCTC-3'
r-MCP1	5'-TTCCTTCTGGGGTCAGCACAGAC-3'
f-CD11c	5'-GAGCCAGAACTTCCCAACTG-3'
r-CD11c	5'-TCAGGAACACGATGTCTTGG-3'
f-CD163	5'-CCTGGATCATCTGTGACAACA-3'
r-CD163	5'-TCCACACGTCCAGAACAGTC-3'
f-Arg-1	5'-CTCCAAGCCAAAGTCCTTAGAG-3'
r-Arg-1	5'-AGGAGCTGTCATTAGGGACATC-3'
f-MR	5'-CCACAGCATTGAGGAGTTTG-3'
r-MR	5'-ACAGCTCATCATTTGGCTCA-3'
f-TGF β	5'-TGGAGCAACATGTGGAACCTC-3'
r-TGF β	5'-CAGCAGCCGGTTACCAAG-3'
f-α SMA	5'-ACTCTCTTCCAGCCATCTTCA-3'
r-α SMA	5'-ATAGGTGGTTTCGTGGATGC-3'
f-Col4a1	5'-TTAAAGGACTCCAGGGACCAC-3'
r-Col4a1	5'-CCCCTGAGCCTGTCACAC-3'
f-CTGF	5'-TGACCTGGAGGAAAACATTAAGA-3'
r-CTGF	5'-AGCCCTGTATGTCTTCACACTG-3'
f-TIMP1	5'-GCAAAGAGCTTTCTCAAAGACC-3'
r-TIMP1	5'-AGGGATAGATAAACAGGGAAACACT-3'
f-mAIM	5'-GAGGACACATGGATGGAATGT-3'
r-mAIM	5'-ACCCTTGTGTAGCACCTCCA-3'

Results

Circulating AIM levels in healthy individuals

To investigate circulating AIM levels in healthy individuals, we performed a large-scale analysis of AIM using more than 8,000 blood samples of volunteers attending annual medical examinations in 2012 and 2013. For this study, we established an ELISA system by generating monoclonal antibodies that accurately estimated human AIM levels in blood. The composition of volunteers and the mean±SD AIM level (µg/ml) are shown in Table 1. AIM levels were highest in both men and women in their 20s and decreased with age (Fig. 1A). In individuals <50 years old, AIM levels were significantly higher in women (Fig. 1A), resulting in an overall higher mean AIM level in women (Fig. 1B). Consistent with our previous report [23], a strong correlation was observed between IgM and AIM levels (Fig. 1C).

The significance of the relationships between AIM levels and various clinical parameters is presented in Table S1. In particular, we focused on obesity-related parameters because AIM has lipolytic function and thus acts as an anti-obese factor in mice [29,30]. The relationship between AIM and various parameters was more significant in women, and AIM levels correlated negatively with body mass index, obesity index, % fat mass, and

Table 2. Number of patients in each type of liver injury.

	HCC		Non HCC	
	Men	Women	Men	Women
Whole	189 (100%)	86 (100%)	90 (100%)	56 (100%)
HBV	36 (19%)	6 (7%)	28 (31%)	14 (25%)
HCV	116 (61%)	61 (71%)	30 (33%)	26 (47%)
HBV and HCV	1 (1%)	1 (1%)	0 (0%)	0 (0%)
Alcoholic hepatitis	21 (11%)	2 (2%)	4 (5%)	3 (5%)
NAFLD	0 (0%)	0 (0%)	21 (23%)	8 (14%)
NASH	3 (2%)	5 (6%)	6 (7%)	4 (7%)
Cryptogenic or other	12 (6%)	11 (13%)	1 (1%)	1 (2%)

The percentage shows the proportion in total number of each gender with or without HCC.
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Table 3. Clinical features of patients analyzed for AIM.

	HCC		Non HCC	
	Men	Women	Men	Women
Age (year)	66±10 [32–87]	70±9 [33–87]	55±15 [23–84]	62±13 [31–80]
AIM (µg/ml)	5.7±2.8 [1.7–18.6]	5.8±2.6 [0.8–14.7]	5.1±2.7 [1.5–17.5]	5.8±2.9 [1.8–16.9]
IgM (mg/dl)	144.8±101 [24.2–708.0]	143.4±98.0 [1.6–769.2]	103.3±49.4 [18.7–216.4]	129.4±64.1 [42.8–346.0]
AST (U/L)	56.3±30.7 [14–170]	59.6±42.9 [17–312]	51.8±43.3 [13–287]	44.7±24.5 [14–139]
ALT (U/L)	53.6±37.6 [8–233]	48.0±39.5 [9–315]	60.3±66.8 [6–498]	40.4±26.6 [11–121]
TB (mg/dl)	0.98±0.54 [0.3–3.9]	1.02±0.61 [0.4–3.8]	1.13±0.85 [0.3–5.1]	1.07±1.17 [0.4–9.2]
DB (mg/dl)	0.35±0.32 [0.1–0.7]	0.38±0.32 [0.1–1.6]	0.29±0.28 [0.1–1.6]	0.25±0.13 [0.1–0.5]
ALB (g/dl)	3.69±0.46 [2.3–4.7]	3.64±0.49 [2.2–4.6]	4.06±0.49 [2.5–5.2]	3.89±0.58 [2.2–4.5]
PLT (×10 ⁶ /mm ³)	12.3±5.29 [3.0–28.3]	11.1±5.43 [4.2–31.9]	16.6±7.32 [2.1–35.6]	13.9±6.57 [2.3–26]
PT (%)	85.4±14.3 [53–100]	84.3±14.7 [42–100]	87.1±17.2 [28–100]	84.0±19.7 [32–100]
Cre (mg/dl)	0.81±0.19 [0.40–1.56]	0.61±0.14 [0.4–1.04]	0.83±0.20 [0.54–1.95]	0.66±0.26 [0.49–2.21]
ICG (%)	23.8±14.4 [2.7–72.4]	24.7±15.6 [0.9–71.2]	-	-

The mean ± SD as well as the range of diversity are presented for each parametric variable. The ICG score was only available in HCC patients.
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waist circumference (Fig. 1D). In line with these results, AIM levels also correlated negatively with low-density lipoprotein (LDL) cholesterol levels (Fig. 1D) and several diabetic markers including fasting blood sugar (FBS) and glycated hemoglobin (HbA1C) (Fig. 1D), as well as with blood pressure, in women (Fig. 1D). Thus, consistent with the findings of our previous study in mice [29,30], AIM levels correlated negatively with multiple parameters related to obesity, and these correlations were more prominent in women. Intriguingly however, a weak but significant positive correlation was found between AIM levels and biomarkers of hepatocyte injury, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), particularly in men (Fig. 1E). Taken together, it is likely that AIM levels increase along with the progression of liver injury. In both men and women, a significant negative correlation was unexpectedly found between AIM levels and red blood cell numbers, but the reason underlying this correlation is unclear (Table S1).

AIM levels correlated strongly with liver function in liver injury

We next analyzed blood samples from patients with chronic hepatitis and liver cirrhosis. As depicted in Table 2, the cause of liver injury in the majority of the patients was hepatitis virus infection, whereas non-infected cases constituted a lower proportion of the patients with alcoholic liver failure and NAFLD/NASH. Patients with or without HCC were investigated. The clinical features of the patients tested are presented in Table 3 focusing on the liver function.

The positive correlation between AIM levels and AST/ALT for liver injury, which was already seen in individuals without severe liver damage (Fig. 1E) was notably more obvious in men and women with advanced liver damage. Highly significant correlations were observed between AIM and multiple biomarkers, thereby reflecting liver function, including total or direct bilirubin (TB or DB), albumin (ALB), platelet count (PLT), % prothrombin time (%PT), and the indocyanine green (ICG) test (Fig. 2). These correlations were obvious in individuals with or without hepatitis virus infection (Table S2) and in the presence or absence of HCC (Fig. 2). In HCC patients, there was no significant correlation in levels of AIM and several HCC markers including alpha

fetoprotein (AFP), des-gamma-carboxyprothrombin (DCP, also called prothrombin induced by vitamin K-absence II; PIVKA-II), and AFP fraction L3 (L3) (Fig. S1). As in healthy individuals, a significant correlation was also found between AIM and IgM levels in patients with liver injury (Fig. 3A); namely, IgM levels increased with the progression of liver damage (Fig. S2).

We then addressed which parameters correlated independently with AIM levels. To this end, we performed multiple regression analysis by the backward stepwise method including AIM. Table S3 shows the correlation coefficients between all parameters that reflect liver function and thus were candidates for determinant of AIM level. When confounding factors were eliminated, IgM, TB, ALB, and %PT in men ($R^2 = 54.0\%$) and IgM, TB, and ALB in women ($R^2 = 57.0\%$) were independent determinants of AIM in HCC patients (Table 4). In non-HCC patients, IgM, ALB, and PLT in men ($R^2 = 63.4\%$) and IgM and PLT in women ($R^2 = 53.6\%$) were the independent determinants of AIM (Table 4). In particular, in men, the t-value of TB in HCC patients was 5.39, but was not significant in non-HCC patients. In women, the t-value of ALB was -4.80 in HCC patients, but again was not significant in non-HCC patients (Table 4).

Similar results were obtained for the relationship between AIM and the grade of liver inflammation or fibrosis. Levels in inflammation (0–3) and fibrosis (F0–F4) were evaluated according to the Inuyama classification [32]. The number of patients in each category is presented in Table 5. Clinical information about inflammation and fibrosis were not available in a part of HCC patients and all of non-HCC patients. AIM levels were higher in those with liver inflammation (Fig. 3B). AIM also increased in line with the progression of liver fibrosis (Fig. 3C). Similarly, AIM levels were higher in patients with cirrhosis or ascites (Fig. 3D). Note that all cirrhotic patients tested in this study were under a compensated stage. Since some patients exhibited prominently high or low levels of AIM ($>10 \mu\text{g/ml}$ or $<3.0 \mu\text{g/ml}$), we assessed relationship in levels of AIM and various parameters in these populations. However, there was no remarkable correlation in AIM and any parameter (Table S4). No significant correlation was observed between AIM levels and alcohol intake (data not shown).

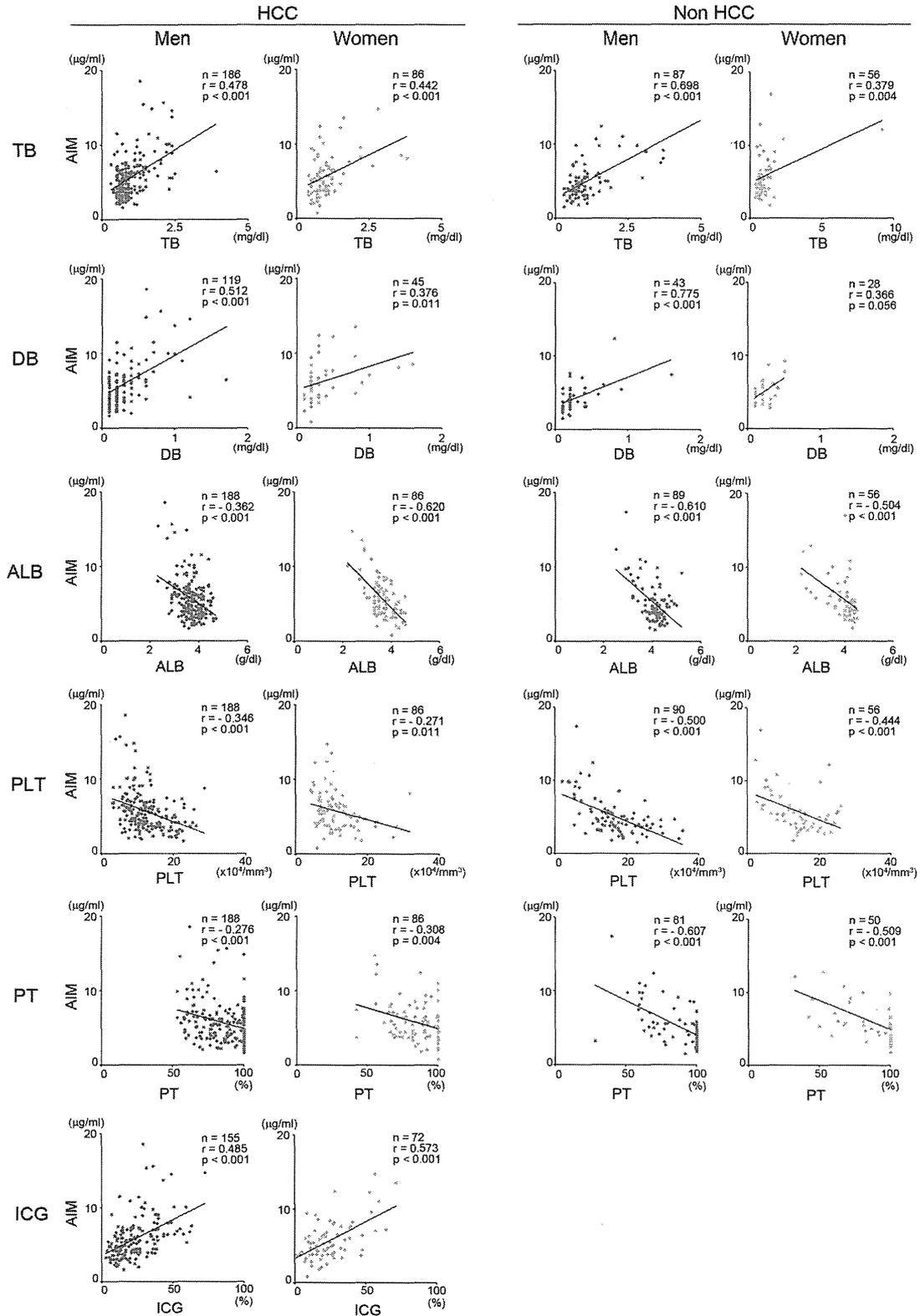


Figure 2. Correlation in the AIM level and the liver function under liver injury. Correlation in AIM levels and various biomarkers representing liver function in men (blue) and women (yellow), with or without HCC. ICG score was only available in HCC patients. doi:10.1371/journal.pone.0109123.g002

AIM^{-/-} mice had comparable liver damage to wild-type mice in response to CCl₄

Next, we assessed whether high AIM levels directly promote liver injury or whether AIM levels increase as a result of liver damage progression. To this end, we employed animal models of progressive liver injury induced by carbon tetrachloride. AIM^{-/-} and AIM^{+/+} mice were challenged with CCl₄ (1.6 g/kg body weight) injected twice a week for 12 weeks, and the state of liver injury was assessed. As demonstrated in Fig. 4A, AST and ALT levels were similar at multiple time points in AIM^{+/+} and AIM^{-/-} mice, indicating that the liver was comparably damaged in the presence or absence of AIM. Note that the average of circulating AIM levels without CCl₄ administration was 3.3 μg/ml in both males and females. Inflammatory states were investigated during the early phase (after 3 weeks of CCl₄ challenge) by measuring mRNA levels for various pro-inflammatory cytokines using quantitative RT-PCR (QPCR). No significant differences were observed in the increased expression levels of *TNFα*, *IL-1β*, *IL-6*, and *MCP-1* in both types of mice (Fig. 4B). In line with this result, similar expression profiles of the M1 and M2 macrophage marker genes (*CD11c* for M1; *CD163*, *Arg-1*, and *mannose receptor (MR)* for M2) were observed in AIM^{+/+} and AIM^{-/-} mice (Fig. 4C), suggesting that the absence of AIM did not influence the activation state or the M1/M2 polarity of liver macrophages in response to carbon tetrachloride, resulting in comparable liver inflammation progression in both types of mice. Consistent with this finding, liver fibrosis progressed comparably in AIM^{-/-} and AIM^{+/+} mice, and Sirius-red staining of liver specimens showed a similar increase in fibrotic areas in both types of mice (Fig. 4D). Accordingly,

mRNA levels of various markers of fibrosis progression such as *TGFβ*, *αSMA*, *Col4a1*, and *connective tissue growth factor (CTGF)* were also comparable in AIM^{-/-} and AIM^{+/+} mice (Fig. 4E). Taken together, these results clearly indicate that the presence or absence of AIM did not influence the state of liver injury in response to carbon tetrachloride. Thus, it is likely that the augmented AIM levels observed in humans (Fig. 2) were the result of liver damage. Further supporting this notion is the finding that AIM levels increased markedly with progression of liver damage in AIM^{+/+} mice (Fig. 4F, left). However, AIM mRNA levels in the AIM^{+/+} liver did not increase in response to CCl₄ (Fig. S3), suggesting that the increase in blood AIM was not brought about by enhancement of AIM production in liver Kupffer macrophages, one of the highest AIM-producing cell types [19].

Interestingly, no significant difference in increase in IgM levels was observed in both AIM^{-/-} and AIM^{+/+} mice, suggesting that the increase in IgM was independent of AIM (Fig. 4F, right). This result is reminiscent of our previous finding of a similar increase in IgM levels in AIM^{-/-} and AIM^{+/+} mice in response to a HFD [23].

Diagnostic application of AIM for HCC

As demonstrated in Fig. 2, AIM levels increased in line with the progression of liver injury in patients with or without HCC. We then wondered whether HCC and non-HCC patients who show an equivalent score of certain liver biomarkers exhibited different AIM levels. Therefore, we normalized the level of AIM (AIM index) to that of each biomarker. The AIM level was divided by a biomarker score when both correlated positively (e.g. AST, ALT, TB, and DB), whereas the scores were multiplied when AIM and

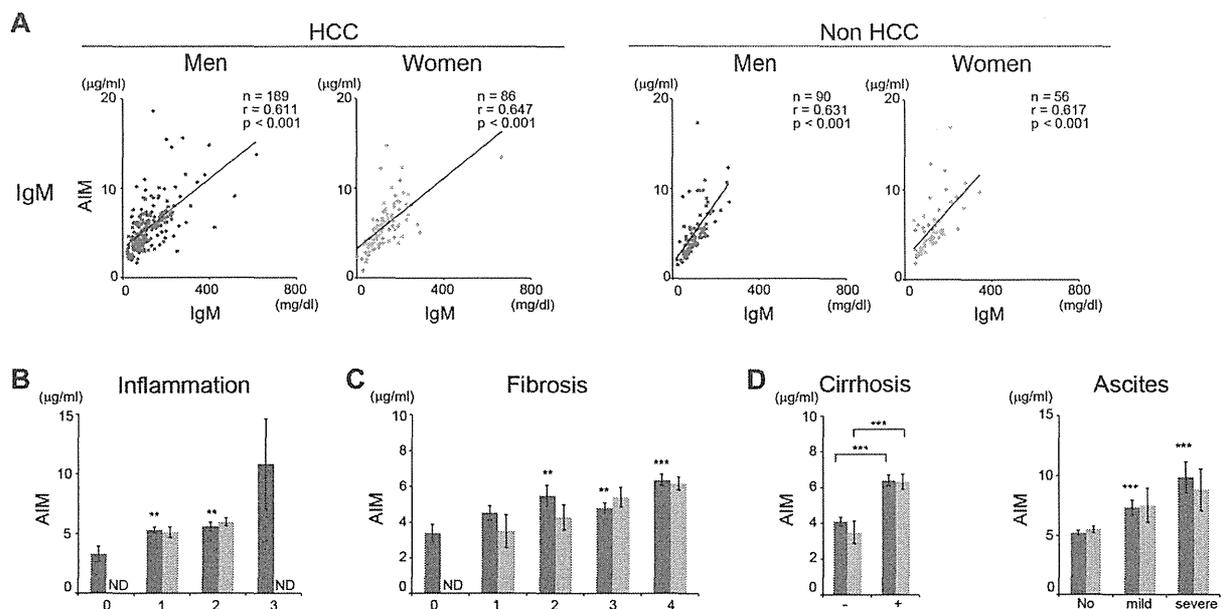


Figure 3. The AIM level increases with progression of liver inflammation and fibrosis. (A) Correlation in AIM and IgM levels in men (blue) and women (yellow), with or without HCC. (B) AIM levels in HCC patients with different inflammatory levels according to the Inuyama classification. (C) AIM levels in HCC patients with different fibrotic scores according to the Inuyama classification. (D) AIM levels in HCC patients with different grades of cirrhosis or ascites. In C-E, AIM levels are presented as means±SEM (μg/ml). doi:10.1371/journal.pone.0109123.g003

Table 4. Multiple linear regression analysis.

	HCC Men (n = 173)			Non-HCC Men (n = 89)		
	C	t	p	C	t	p
R ²	53.98%			63.44%		
Intercept	5.30	2.927	0.004	10.2	6.29	<0.001
IgM	0.0137	9.37	<0.001	0.0266	7.33	<0.001
TB	1.60	5.39	<0.001	–	–	–
ALB	–0.886	–2.53	0.012	–1.41	–3.59	<0.001
PLT	–	–	–	–0.127	–4.88	<0.001
PT	–0.0191	–1.77	0.079	–	–	–
	HCC Women (n = 86)			Non-HCC Women (n = 56)		
	C	t	p	C	t	p
R ²	57.02%			53.62%		
Intercept	9.58	4.82	<0.001	4.88	5.90	<0.001
IgM	0.0104	4.66	<0.001	0.0265	6.48	<0.001
TB	0.862	2.48	0.016	–	–	–
ALB	–2.17	–4.80	<0.001	–	–	–
PLT	–	–	–	–0.181	–4.53	<0.001

Independent determinants for AIM in men or women with or without HCC. R²: determination coefficient, C: regression coefficient, t: t-value, p: p-value, in multiple linear regression models. –: not independent.
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the biomarker correlated negatively (e.g. ALB, platelets, and %PT). As depicted in Fig. 5A, the ratio of the AIM-TB index to the AIM-ALB index in men was significantly higher in HCC patients than in non-HCC patients by analysis of covariance

(ANCOVA), but no significant differences were observed for the ratio of the TB score to the ALB score between HCC and non-HCC patients. Similar results were obtained for the ratio of the AIM-TB index to the AIM-PLT or AIM-AST index, although TB

Table 5. Number of patients showing different levels of inflammation, fibrosis, cirrhosis, or ascites.

		Men	Women
Whole		189	86
Inflammation (in %)	0	6 (3%)	0 (0%)
	1	88 (47%)	36 (42%)
	2	50 (26%)	28 (32%)
	3	2 (1%)	0 (0%)
	unknown	43 (23%)	22 (26%)
Fibrosis (in %)	0	7 (4%)	0 (0%)
	1	14 (7%)	3 (3%)
	2	19 (10%)	13 (15%)
	3	42 (22%)	11 (13%)
	4	81 (43%)	42 (49%)
	unknown	26 (14%)	17 (20%)
Cirrhosis (in %)	–	36 (19%)	6 (7%)
	+	82 (43%)	45 (52%)
	unknown	71 (38%)	35 (41%)
Ascites (in %)	No	159 (84%)	77 (90%)
	Mild (1–2L)	18 (10%)	5 (6%)
	Severe (3–5L)	10 (5%)	4 (4%)
	unknown	2 (1%)	0 (0%)

All patients here possessed HCC. The percentage shows the proportion in each level of identical phenotype. Unknown: Information was not available.
doi:10.1371/journal.pone.0109123.t005

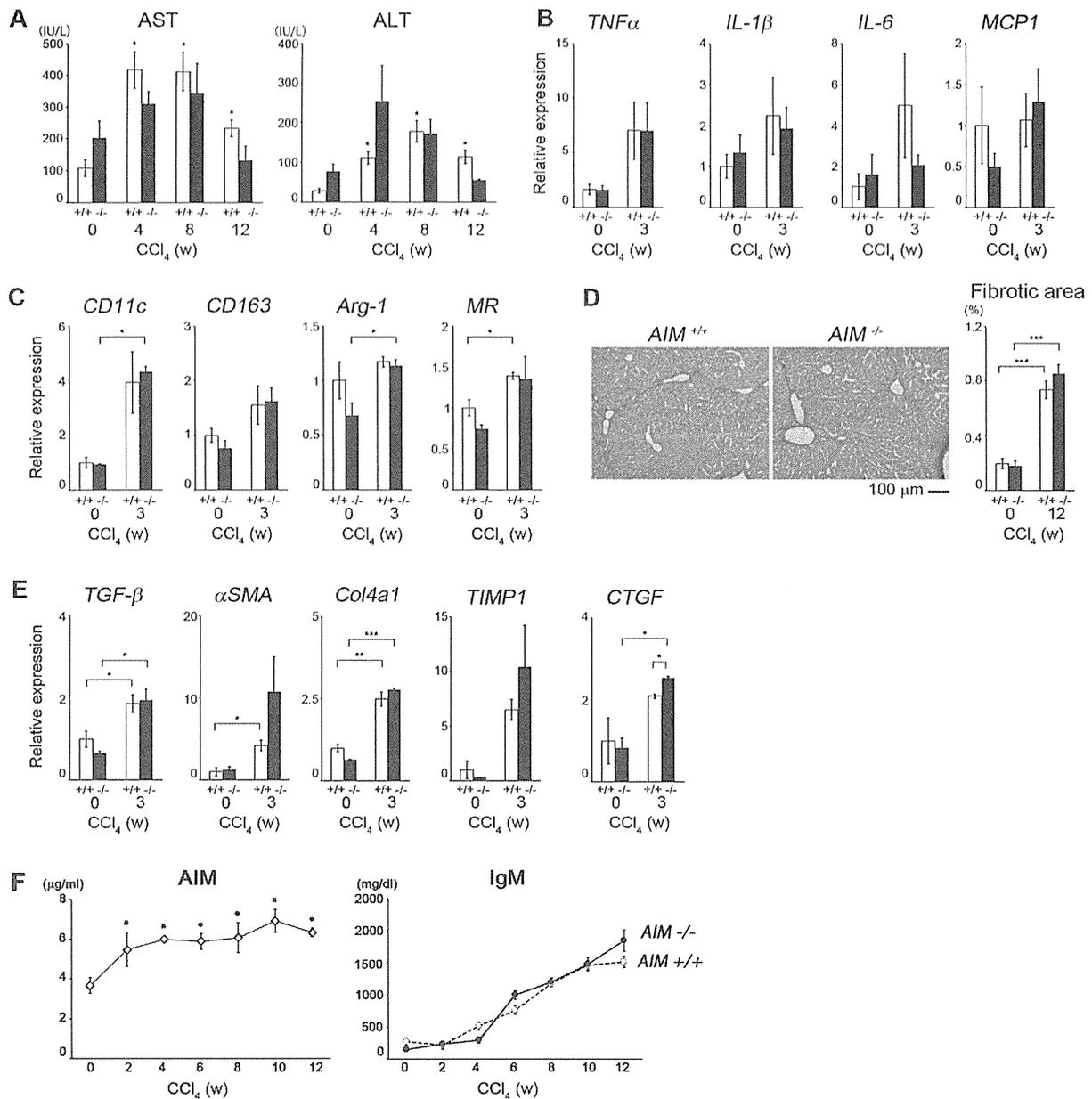


Figure 4. *AIM*^{+/+} and *AIM*^{-/-} mice exhibit comparable liver damage in response to CCl₄. (A) AST and ALT at 0, 4, 8, 12 wk after administration of CCl₄ (1.6 g/kg body weight, twice injection per week for 12 weeks) in *AIM*^{+/+} mice (+/+) and *AIM*^{-/-} mice (-/-). n = 3 for each. Error bar: SEM, *; p < 0.05 vs. before CCl₄ administration (0 w). (B, C) mRNA levels of *TNFα*, *IL-1β*, *IL-6* and *MCP-1* (B); or *CD11c*, *CD163*, *Arg-1* and *MR* (C) were assessed by QPCR using RNA isolated from liver after administration of CCl₄ for 3 weeks. n = 3 for each. Error bar: SEM, *; p < 0.05. (D) Sirius-red staining of the liver specimens after administration of CCl₄ for 12 weeks to *AIM*^{+/+} mice (+/+) and *AIM*^{-/-} mice (-/-). Bar: 100 μm. Right graph shows the quantification of fibrotic area. (E) mRNA levels of *TGFβ*, *αSMA*, *Col4a1* and *CTGF* were assessed by QPCR using RNA isolated from liver from mice after administration of CCl₄ for 3 weeks. n = 3 for each. Error bar: SEM; *; p < 0.05, ***; p < 0.001. (F) Left: Serum AIM levels were measured by ELISA from wild-type mice after administration of CCl₄. n = 6 for each. Error bar: SEM, *; p < 0.05 vs. before CCl₄ administration (0 w). Right: Serum IgM levels were measured by semi-quantitative immunoblotting using sera from *AIM*^{+/+} (+/+) mice and *AIM*^{-/-} (-/-) after administration of CCl₄. Purified mouse IgM clone (3F3) was used as standard. Quantification of signals from immunoblotting was performed by using ImageQuant TL software (GE Healthcare, Little Chalfont, UK). n = 6 for each. Error bar: SEM; *; p < 0.05, ***; p < 0.01, vs. before CCl₄ administration in *AIM*^{+/+} (+/+) mice; ###; p < 0.001 vs. before CCl₄ administration in *AIM*^{-/-} mice. doi:10.1371/journal.pone.0109123.g004

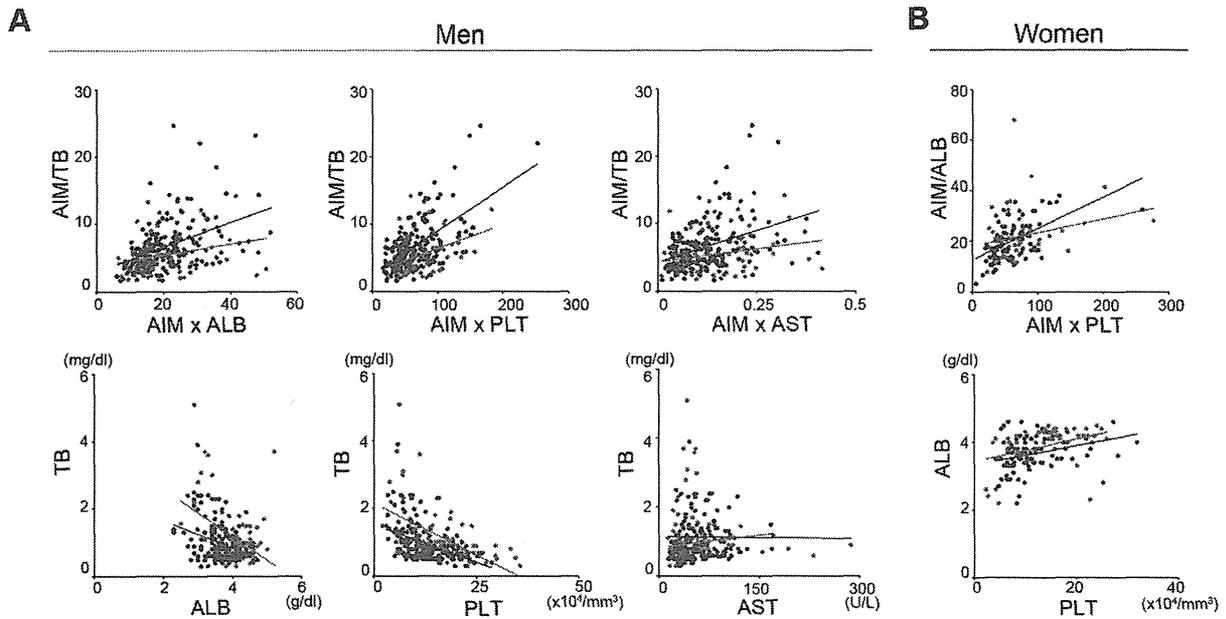


Figure 5. AIM-index distinguishes HCC and non-HCC patients. (A) AIM-TB index vs. AIM-ALB index, AIM-PLT index or AIM-AST index in men with or without HCC. TB vs. ALB, PLT or AST are also presented. (B) AIM-ALB index vs. AIM-%PT index in women with or without HCC. ALB vs. %PT is also presented. r : correlation coefficients; p : p value determined by ANCOVA. Blue dots and bars: HCC patients, red dots and bars: non-HCC patients. doi:10.1371/journal.pone.0109123.g005

correlated with neither PLT nor AST (Fig. 5A). In women, a comparison of the AIM-ALB and AIM-%PT indexes revealed similar differences between the HCC and non-HCC patients (Fig. 5B). Note that TB in male HCC patients and ALB in female HCC patients produced high t -values in multiple regression analysis when assessing the determinants of AIM (Table 3).

Discussion

This study provides the first large-scale description of the circulating AIM levels in the general population and in the context of liver function parameters in humans. A variety of new findings were obtained as follows. Firstly, relatively higher AIM levels were observed in the younger generation, especially in women, suggesting potential involvement of estrogen in the increase in circulating AIM levels. Accumulating evidence of estrogen-associated physiology including suppression of triacylglycerol (TG) storage in fat and liver tissues [33–36], reduction of expression and enzymatic activity of FASN [37], and preventive effect for foam cell formation and the development of atherosclerosis [38–41], which are all reminiscent of AIM function [20,29,30,42,43]. Although *AIM* mRNA is expressed under transcriptional regulation by LXR [20–22], the impact of estrogen on LXR activation is controversial. For instance, Wang et al. recently reported that E2 activates LXR α [41], whereas suppression of LXR α by E2 was also reported in hepatocytes [34], adipocyte [36], and pancreatic β cells [37]. Alternatively, evidence has shown that estrogen stimulates natural IgM production through B lymphocyte activation [44,45]. This effect certainly increases AIM levels based on the strong correlation between AIM and IgM. However, further studies are required to clarify the precise involvement of estrogen in the regulation of age-dependent AIM levels in humans. It is noteworthy that in individuals without advanced liver damage, AST and ALT showed a weak positive

correlation with AIM levels only in men (Fig. 1E and Table S1). It might be possible that the sex-dependent difference of AIM levels obscured the correlation in women.

Secondly, patients with advanced liver damage exhibited high AIM levels. We postulate that the mean \pm SD AIM level ($\mu\text{g/ml}$) in each generation in men and women presented in Table 1 can be defined as the “normal range” of AIM levels. Certainly, however, whether AIM levels that are higher or lower than this range mean pathological may depend on the type of disease. At least, patients with progressive liver damage exhibited significantly higher levels than the normal range. Based on the results of mouse experiments demonstrating that the presence or absence of AIM does not influence the state of liver injury in response to CCl $_4$, and that AIM levels increase in response to CCl $_4$ in wild-type mice, it is likely that AIM levels increase as a result of liver damage. Thus, AIM can be used as a novel biomarker for liver injury. The mRNA level of liver *AIM* did not increase in response to CCl $_4$, indicating no enhanced AIM production by liver Kupffer macrophages. It remains possible, however, that inflammatory stimuli caused by carbon tetrachloride will increase *AIM* expression in macrophages in other tissues such as in the peritoneal cavity and splenic marginal zone, but additional experiments are required to assess this possibility. Alternatively, liver damage might increase AIM stability in the blood. Natural IgM is catabolized mainly in the liver [46–48]. Therefore, it is possible that the progression of liver damage prolongs the half-life of IgM, resulting in advanced accumulation of circulating AIM. This scenario can also be applied in humans, and may explain the more profound increase in AIM levels in cirrhotic patients compared with non-cirrhotic patients. A precise assessment of the half-life of IgM and AIM in the presence or absence of liver injury can evaluate this possibility.

More notably, we found that use of the AIM index, which is the blood AIM level normalized to the liver biomarker score,

appeared to be useful for distinguishing HCC and non-HCC patients. The AIM-TB index of men with an equivalent AIM-ALB or AIM-PLT index was significantly higher in HCC patients than in non-HCC patients. This is not secondary effect of the correlation in TB and ALB or PLT, as there was no significance in the difference in these correlations in HCC and non-HCC groups. The same conclusion was obtained for the AIM-ALB and AIM-platelets indexes in women. Thus, the presence of HCC may increase the AIM-TB index in men and the AIM-ALB index in women. If this is the case, the AIM index can serve as a novel tumor marker and will be useful for the diagnosis of HCC. Further analysis using HCC-bearing mouse models in the presence or absence of AIM may be appropriate for evaluating this possibility. Alternatively, one could also speculate that individuals who show an enhanced increase in certain AIM indexes (*i.e.* AIM-TB index in men and AIM-ALB index in women) in response to liver injury might be more susceptible to HCC. However, further study such as prospective cohort study of HCC development in patients with similar levels of liver damage and different levels of circulating AIM is certainly needed to assess this possibility. In either case, further investigation will corroborate the applicability of the AIM index for the early detection of HCC.

Since HCC is one of the most common malignant tumors with an increasing incidence, identifying serological biomarkers are extremely needed, especially because most of HCC cases are diagnosed at a late stage. Thus, our study could be the bases of application of circulating AIM level as a diagnostic and/or prognostic marker of HCC, either solo or in combination with other biomarkers.

Supporting Information

Figure S1 Correlation between AIM levels and various HCC markers. Men: blue dots, women: yellow dots. In HCC patients, no significant correlation was observed in levels of AIM and either HCC marker. (TIF)

Figure S2 Correlation between IgM levels and various biomarkers representing liver function. Men: blue dots, women: yellow dots. ICG score was only available in HCC patients. (TIF)

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Figure S3 AIM expression did not increase in the liver in response to CCl₄. mRNA levels of AIM in the liver from wild-type mice after administration of CCl₄ for 3 wk. n = 3 for each. Error bar: SEM. (TIF)

Table S1 Correlation in AIM level and different clinical parameters. Number of samples, and the correlation coefficients and p values in the correlation with AIM levels in separate tested item. n: sample numbers. (DOCX)

Table S2 Correlation between AIM and liver function in patients with or without hepatitis viral infection. Number of samples, and the correlation coefficients and p values in the correlation with AIM levels in the indicated tested item, in patients with or without hepatitis viral infection. n: sample number. (DOCX)

Table S3 Correlation coefficients between all variables that are candidates for determinant of AIM. C: single regression coefficient, p: p-value. Cre: creatinine. (DOCX)

Table S4 AIM and various clinical markers in populations who exhibit very high or very low AIM levels. Number of samples (n), and the correlation coefficients and p values in the correlation with AIM levels in identical parameter. Low: patients who exhibited less than 3.0 μ g/ml of AIM, High: patients who exhibited more than 10.0 μ g/ml of AIM. (DOCX)

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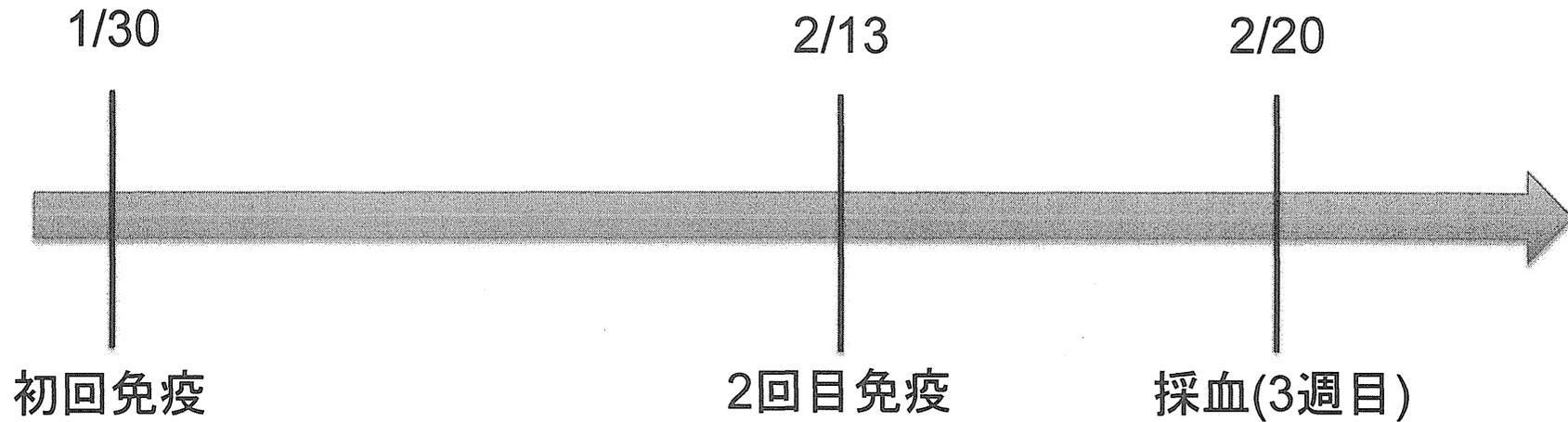
Author Contributions

Conceived and designed the experiments: SA TM. Performed the experiments: TY M. Mori SA AN KA. Analyzed the data: TY TK M. Mori. Contributed reagents/materials/analysis tools: RT MA MB M. Maeda TA K. Izumino JT SH TT TG SO SS YY YH KK KIY K. Inoue. Wrote the paper: TM.

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ジーンガンによる免疫スケジュール



Animal	150130 免疫	150212 免疫
BALB/c #1	tIFN γ (plasmid + gold)	tIFN γ (plasmid + gold)
BALB/c #2	tIFN γ (plasmid + gold)	tIFN γ (plasmid + gold)
BALB/c #3	tIFN γ (plasmid)	tIFN γ (plasmid)
GANP #1	tIFN γ (plasmid + gold)	tIFN γ (plasmid + gold)
GANP #2	tIFN γ (plasmid + gold)	tIFN γ (plasmid + gold)
GANP #3	tCD4 (plasmid + gold)	tCD4 (plasmid + gold)
GANP #4	tCD8 (plasmid + gold)	tCD8 (plasmid + gold)
GANP #5	tCD4 (plasmid + gold)	tCD4 (plasmid + gold)
GANP #6	tCD8 (plasmid + gold)	tCD8 (Electroporation)
GANP #7	tIFN γ (plasmid)	tIFN γ (plasmid)
GANP #8	tCD4 (plasmid)	tCD4 (plasmid)
GANP #9	tCD8 (plasmid)	tCD8 (plasmid)

ジーンガンによる免疫

<初回 (150130)>

Group	Plasmid + gold	Plasmid
tIFNg (BALB/c)	#1, #2	#3
tIFNg (GANP)	#1, #2	#7
tCD4 (GANP)	#3, #5	#8
tCD8 (GANP)	#4, #6	#9

Plasmid + gold → Plasmid 1ug, gold 1mg/shot
Plasmid → Plasmid 20ug/shot

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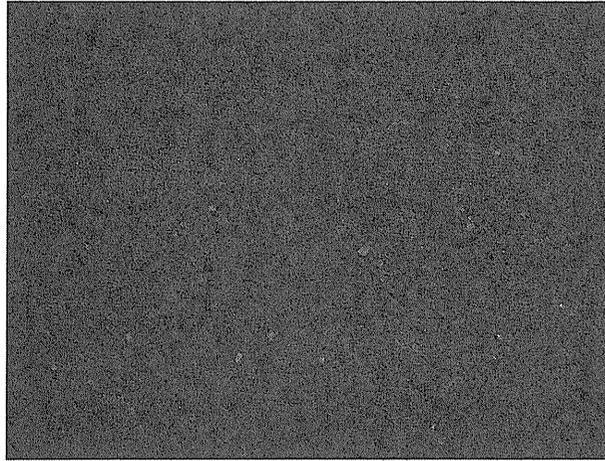
Group	Plasmid + gold	Plasmid	Electroporation
tIFNg (BALB/c)	#1, #2	#3	
tIFNg (GANP)	#1, #2	#7	
tCD4 (GANP)	#3, #5	#8	
tCD8 (GANP)	#4,	#9	#6

Plasmid + gold → Plasmid 1ug, gold 1mg/shot
Plasmid → Plasmid 20ug/shot
Electroporation → Plasmid 100ug/shot

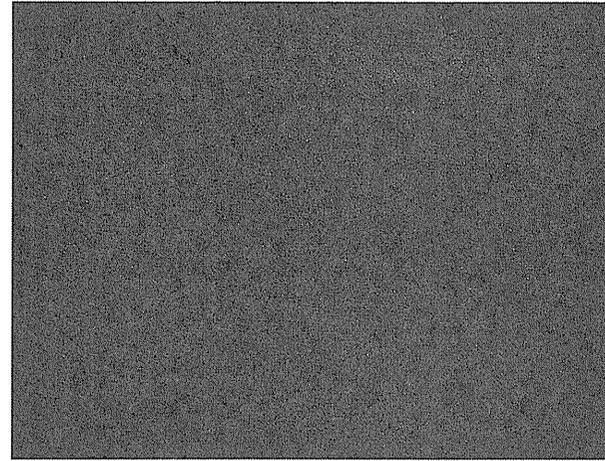
免疫マウス血清(免疫3週目)IFA結果

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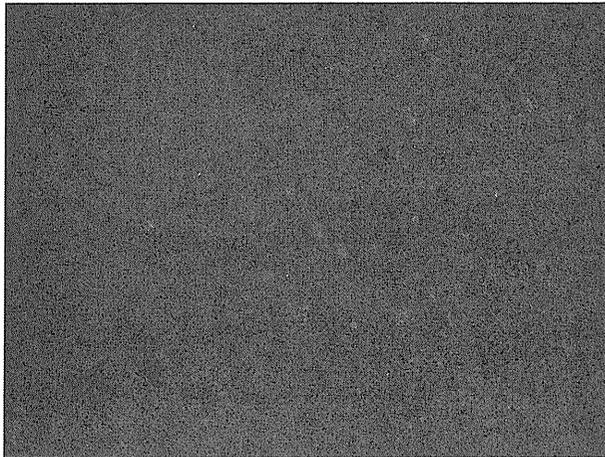
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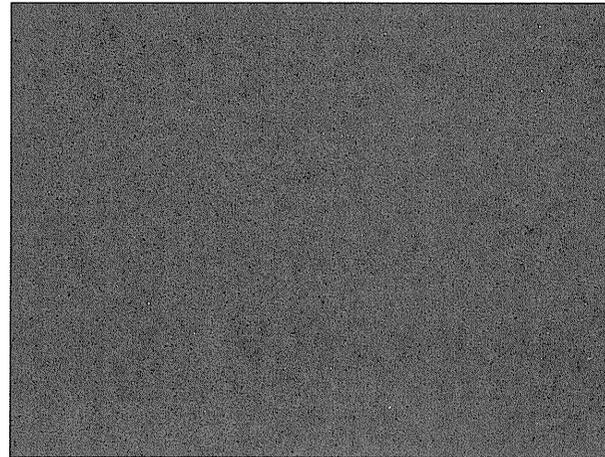
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Serum, 1:10



#2 (plasmid + gold)
Serum, 1:10



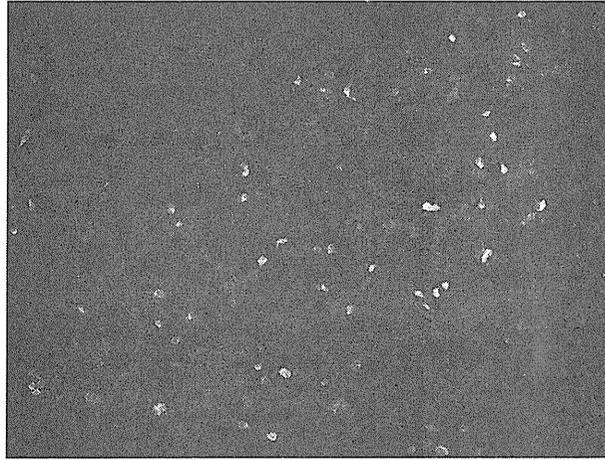
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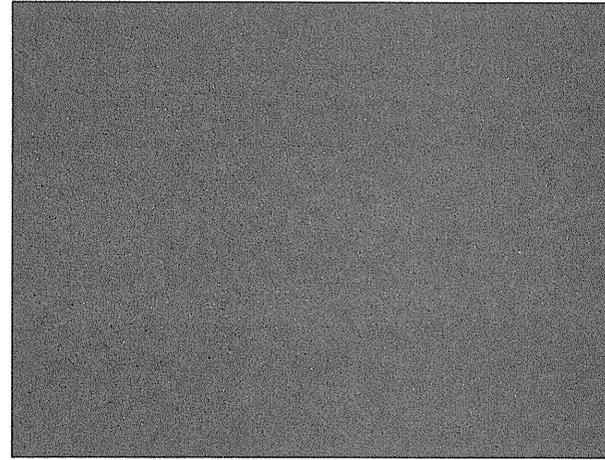
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GANP (IFN gamma), X10

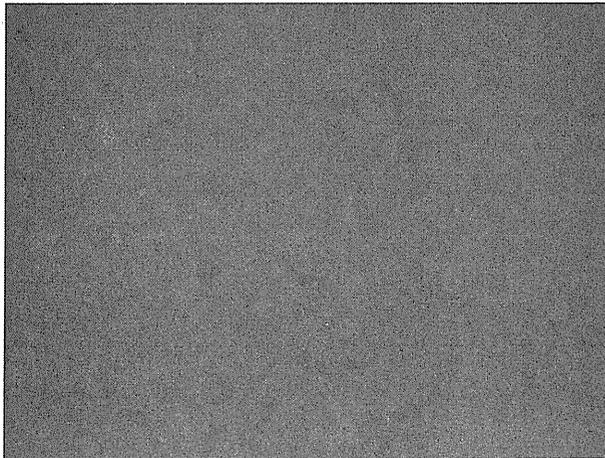
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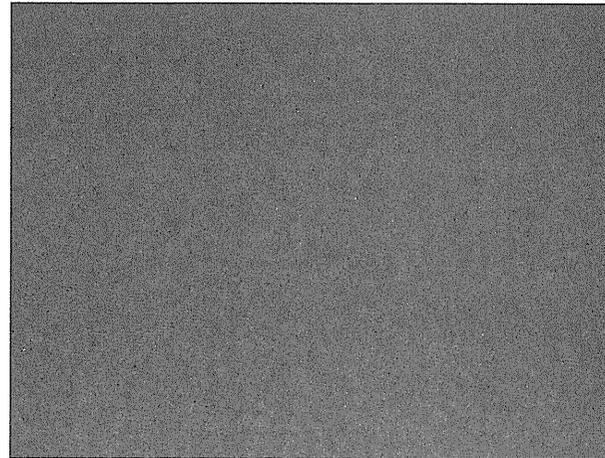
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Serum, 1:10



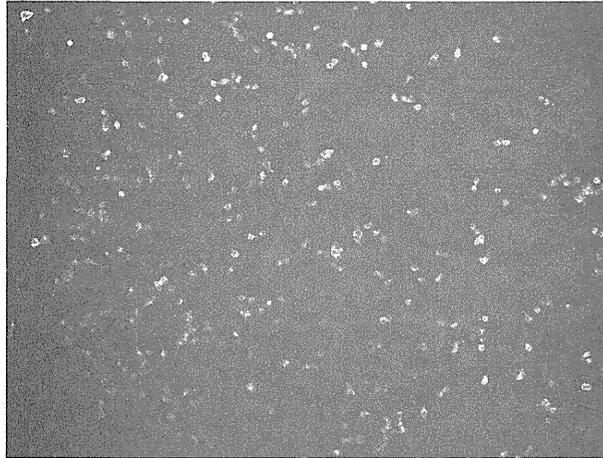
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Serum, 1:10



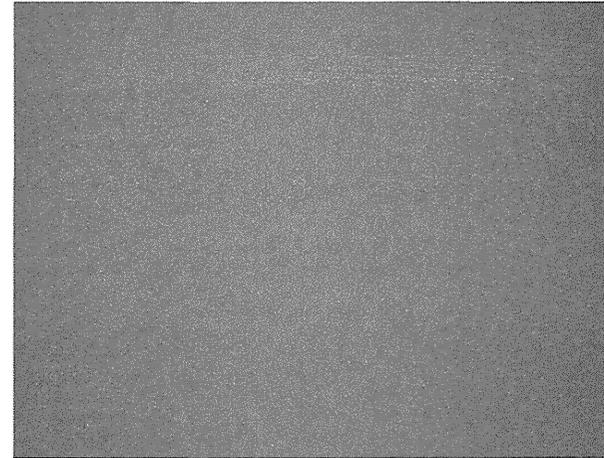
免疫マウス血清(免疫3週目)IFA結果

GANP (CD4), X10

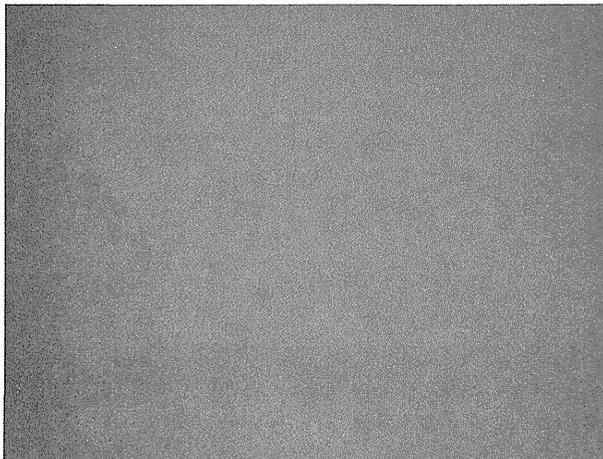
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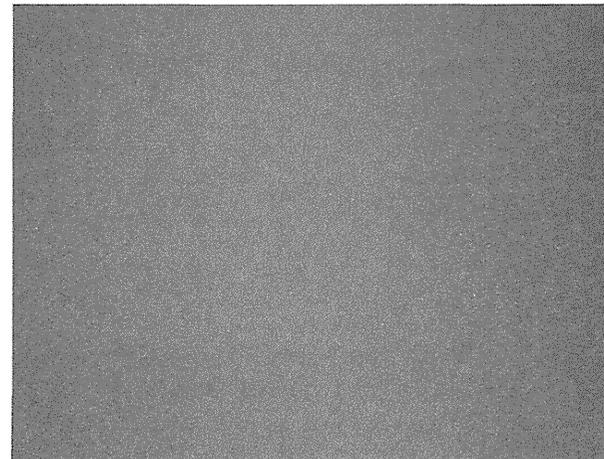
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Serum, 1:10



#5 (plasmid + gold)
Serum, 1:10



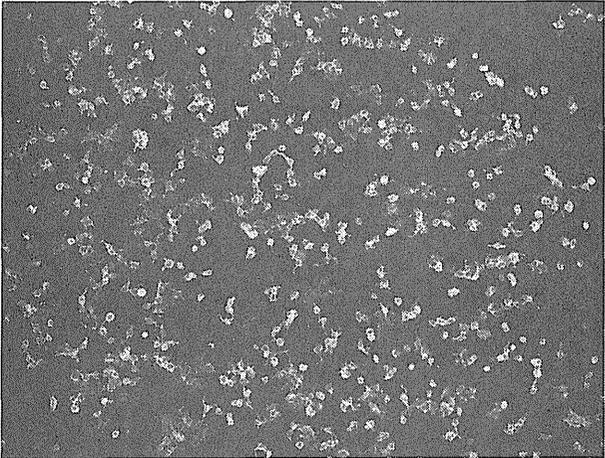
#8 (plasmid)
Serum, 1:10



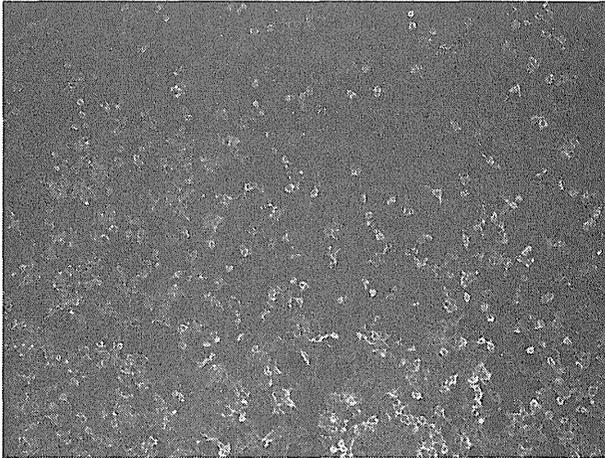
免疫マウス血清(免疫3週目)IFA結果

GANP (CD8), X10

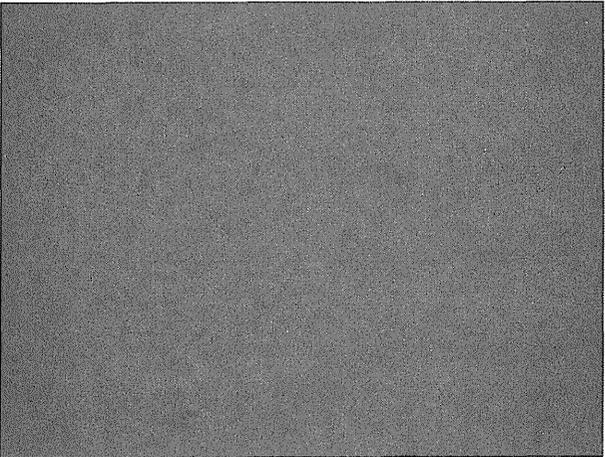
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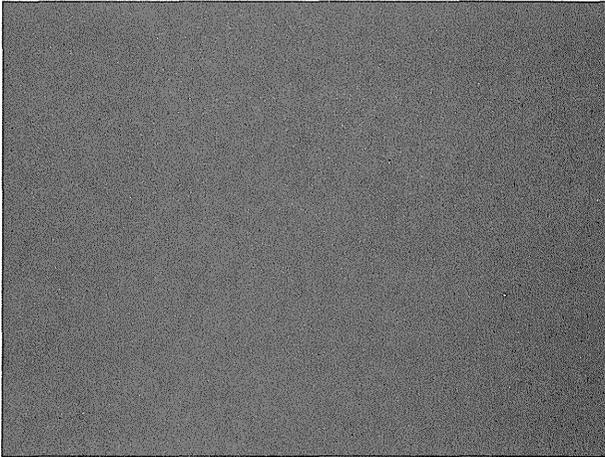
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#6 (plasmid + gold)
Serum, 1:10



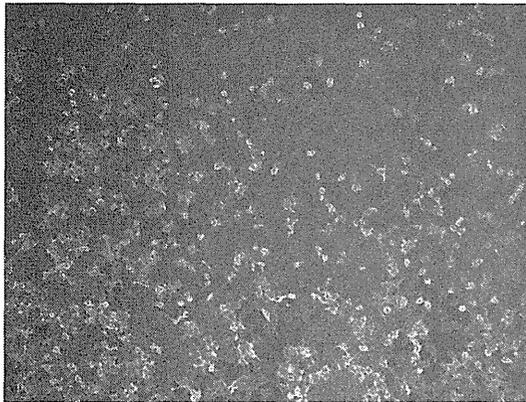
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Serum, 1:10



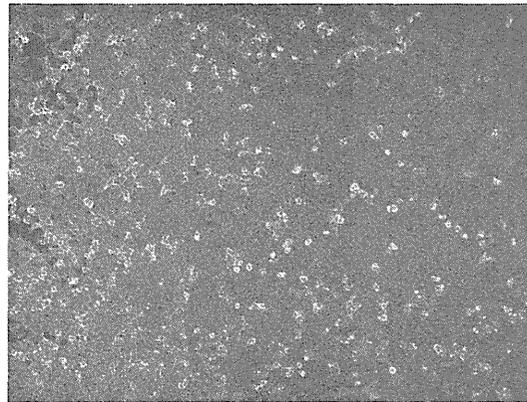
免疫マウス血清(免疫3週目)IFA結果

GANP (CD8), X10

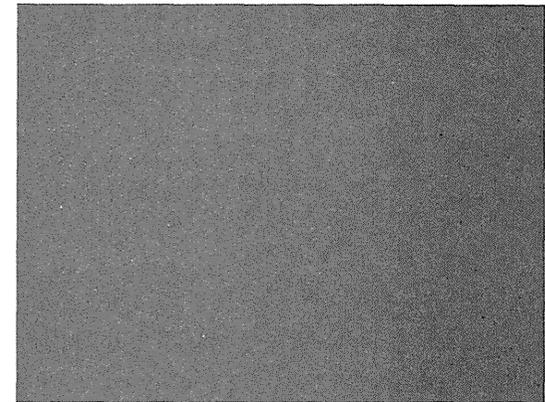
#4 (plasmid + gold)
Serum, 1:10



#4 (plasmid + gold)
Serum, 1:100



#4 (plasmid + gold)
Serum, 1:1,000



Nonalcoholic Fatty Liver Disease: Portal Hypertension Due to Outflow Block in Patients without Cirrhosis¹

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Purpose:

To prospectively evaluate whether the characteristics of hepatic blood flow change during the early stages of fibrosis in patients with nonalcoholic fatty liver disease (NAFLD).

Materials and Methods:

All participants provided written informed consent, and the study protocols were approved by the institutional ethics committee. A total of 121 patients with NAFLD that was diagnosed at histologic examination were enrolled. Hepatic blood flow was measured by means of Doppler ultrasonography (US), and the ratio of arterial to portal blood flow (arterioportal ratio) was calculated. Elasticity of the spleen was measured by means of real-time tissue elastography. The elastic ratio was measured as the value in the small splenic vessel divided by the value of the splenic parenchyma. The arterioportal ratio and splenic elasticity were compared in patients with all stages of fibrosis (F0–F4) and with all levels of platelet counts. Correlations among the resistive index of the hepatic artery and that of the splenic artery, the fibrosis 4 index, hepatic elasticity, arterioportal ratio, splenic elasticity, and platelet counts were analyzed. Univariate and multivariate logistic regression analyses were performed. Correlations in patients with platelet counts greater than 200 000/ μ L also were analyzed.

Results:

The mean \pm standard deviation arterioportal ratio was significantly higher at fibrosis stage 2 (2.4 ± 0.6) than at fibrosis stage 0 (1.8 ± 0.4) in patients with NAFLD ($P < .01$). Increased splenic stiffness at earlier stages of fibrosis also was observed in patients with NAFLD (fibrosis stage 2, 4.4 ± 2.3 ; fibrosis stage 0, 3.2 ± 1.9 ; $P < .05$). In patients with NAFLD with platelet counts higher than 200 000/ μ L, pericellular fibrosis was the only significant predictor of hepatic hemodynamic change at multivariate analysis (odds ratio, 7.17; 95% confidence interval: 1.33, 57.13; $P = .021$).

Conclusion:

Change in hepatic blood flow occurred during the earliest stage of hepatic fibrosis in patients with NAFLD because of outflow block in the sinusoidal area.

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Online supplemental material is available for this article.

Untreated nonalcoholic fatty liver disease (NAFLD) encompasses a wide spectrum of pathologic conditions, from simple steatosis to nonalcoholic steatohepatitis, which may progress to liver cirrhosis, and is associated with hepatocellular carcinoma (1–5). To prevent this progression, diagnosis of NAFLD and early initiation of therapy is important.

Liver specimens from patients with NAFLD show pericellular fibrosis around the central vein in the early stages of the disease, with gradual progression to fibrosis connecting the central veins in neighboring lobules. In patients with chronic hepatitis, portal tract damage with pathologic changes to the portal vein generally precedes central vein involvement. The lumina of portal veins in the portal tracts

tend to be preserved in the noncirrhotic stage of nonalcoholic steatohepatitis (6). This raises the concern that factors that contribute to the development of portal hypertension in patients with NAFLD may differ from those in patients with chronic viral hepatitis. Mendes et al (7) reported that portal hypertension could occur in some patients with NAFLD who show only mild or no fibrosis. Evaluation of splenic elasticity is superior to other methods for the estimation of portal hypertension (8). Portal hypertension, as measured according to splenic elasticity, has been reported (9) to occur in patients with NAFLD who have milder fibrosis.

Hepatic fibrosis is accompanied by several regional hepatic hemodynamic changes, including increased hepatic arterial blood flow, formation of intrahepatic shunts, and the existence of a hyperdynamic circulatory state, which affects parameters of hepatic hemodynamics such as the resistance index, hepatic blood flow, and the velocity of blood flow in the portal vein and hepatic arteries (9–13). These parameters can be evaluated by means of duplex Doppler ultrasonography (US). Hirata et al (14) reported that the ratio of peak relative blood velocity in the hepatic artery and portal vein, or the arteriportal ratio, as evaluated with duplex Doppler US, is useful for diagnosing the degree of hepatic fibrosis in patients with chronic hepatitis due to the hepatitis C virus. In addition, duplex Doppler US has shown clinical utility in the diagnosis of portal hypertension (12,13,15).

Implications for Patient Care

- Portal hypertension can occur in patients with NAFLD who do not have cirrhosis.
- This hemodynamic change occurs even in patients with lower platelet counts ($\geq 140\,000/\mu\text{L}$ but $< 200\,000/\mu\text{L}$).
- Surveillance for portal hypertension is needed in patients with NAFLD even if their fibrosis is not advanced.

Several groups have reported hepatic hemodynamic changes in patients with NAFLD (16,17); however, whether such hemodynamic changes occur at earlier stages of fibrosis remains unclear. In addition, histologic parameters that correlate with hemodynamic changes in patients with NAFLD have not been determined, particularly in patients with NAFLD who have only mild fibrosis. Thus, our study was a prospective evaluation to determine if the alteration of hepatic blood flow occurs at earlier stages of fibrosis.

Materials and Methods

Patients

All participants provided written informed consent before enrollment, and the study protocols were approved by the institutional ethics committee. All patients ($n = 123$) who were admitted for liver biopsy underwent abdominal US at Ehime University Hospital (Toon, Japan) between April 2009 and December 2012. The diagnosis of fatty liver at B-mode US was made on the basis of the appearance of contrast on images of the liver and kidney and the brightness of the liver (18). Liver biopsy was performed within 3 days after B-mode US for all patients. After biopsy, Doppler US and elastography were performed. The grade of steatosis was assessed

Advances in Knowledge

- Hemodynamic changes in patients with nonalcoholic fatty liver disease (NAFLD) start in the earliest stage of fibrosis; the median hepatic arteriportal ratios in patients with fibrosis stage F0, F1, F2, F3, and F4 were 1.8, 2.0, 2.5, 3.2, and 3.7, respectively.
- This hemodynamic change occurs even in patients with lower platelet counts ($\geq 140\,000/\mu\text{L}$ but $< 200\,000/\mu\text{L}$).
- Splenic stiffness in patients with NAFLD also starts in the earliest fibrosis stage; median splenic elasticity for portal hypertension (splenic elasticity times, 1.63–2.88) scores in patients with stage F0, F1, F2, F3, and F4 were 2.9, 3.8, 4.3, 5.4, and 10.7, respectively; the mean \pm standard deviation splenic elasticity was 3.1 ± 1.9 , 3.6 ± 1.7 , 4.4 ± 2.3 , 5.8 ± 2.6 , and 9.2 ± 4.0 , respectively.
- Patients with hemodynamic changes in the earliest fibrosis stage of NAFLD showed pericellular fibrosis (odds ratio, 7.17 [95% confidence interval: 1.33, 57.13]; $P = .021$).

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Abbreviations:

CI = confidence interval
 HVPG = hepatic venous pressure gradient
 NAFLD = nonalcoholic fatty liver disease
 SEP = splenic elasticity for portal hypertension

Author contributions:

Guarantor of integrity of entire study, Y.H.; study concepts/study design or data acquisition or data analysis/interpretation, all authors; manuscript drafting or manuscript revision for important intellectual content, all authors; approval of final version of submitted manuscript, all authors; literature research, Y.H.; clinical studies, all authors; experimental studies, Y.H.; statistical analysis, Y.H.; and manuscript editing, Y.H.

Conflicts of interest are listed at the end of this article.

histologically, and to meet inclusion criteria, the area of hepatic steatosis was confirmed to be greater than 5%. The exclusion criteria are detailed in Appendix E1 (online). We were unable to obtain US images in two of the 123 patients, and these patients were excluded after enrollment. No other patients met any of the remaining exclusion criteria; therefore, 121 patients were enrolled in this study. We reported previously on elastography in patients with NAFLD (9); 75 of the 121 patients enrolled in the present study also were included in our previous study. The parameters we analyzed in the present study, in which we focused on hemodynamic changes in patients in the early stages of fibrosis, were different from those in the previous study (9). Moreover, we performed the present study to clarify the changes in histologic factors.

Imaging Studies: Real-time Tissue Elastography and Doppler US

All participants fasted overnight and refrained from smoking cigarettes. Hepatic and splenic elasticity and Doppler flow were measured by using a real-time tissue elastographic probe (EUB-7500; Hitachi Medical Systems, Tokyo, Japan) with a linear probe (EUP-L52, central frequency, 5.5 MHz; Hitachi Medical Systems) for elastography and a 3.5-MHz probe for Doppler US. These measurements were obtained by one gastroenterologist (M.H., 15 years of experience with US). This operator had performed at least 300 evaluations of liver stiffness before the start of this study.

Regions of interest were simultaneously placed on small intrahepatic veins and hepatic parenchyma. Elastography was performed without the addition of pressure from the probe. The hepatic or splenic elastic ratio (elasticity of small veins and elasticity of hepatic or splenic parenchyma) was then calculated. The means of five measurements were calculated as described elsewhere (8,19). Splenic elasticity has been reported to correlate well with the hepatic venous pressure gradient (HVPG), and this value was termed the splenic elasticity for portal hypertension

(SEP) score (splenic elasticity times, 1.63–2.88; $r = 0.854$; $P < .0001$) (8,9). The HVPG of patients with portal hypertension was greater than 6 mm Hg. Thus, an elevated SEP score (≥ 6) suggested portal hypertension.

Doppler US measurements were obtained five times, as previously described (8,19). Maximal blood velocities in the right portal vein and right hepatic artery were calculated. The sample volume size for the Doppler mode was set at 4–5 mm for the right portal vein and 2–3 mm for the right hepatic artery. The angle was always less than 60°. Color Doppler US allowed identification of the main branches of the splenic artery at the splenic hilum by enabling confirmation of a pulsation wave. The ratio of the maximal blood velocity in the right hepatic artery to maximal blood velocity in the right portal vein (arterioportal ratio) was calculated (14).

Clinical Data Collection

Collected clinical data are described in Appendix E1 (online). If the fibrosis stage of NAFLD is advanced, the platelet count is known to decrease (20). In patients with NAFLD and fibrosis, the platelet count is typically lower than 200 000/ μL (20). When we examined the association of platelet count with an SEP score greater than or equal to 6, we found the optimal cutoff value for the platelet count to be 146 000/ μL . Thus, participants were classified into the following three categories according to platelet count: category 1, platelet count lower than 140 000/ μL ; category 2, platelet count higher than or equal to 140 000/ μL but lower than 200 000/ μL ; and category 3, platelet count higher than or equal to 200 000/ μL .

Histologic Assessment

Histologic assessment was performed as previously described (9). Fibrosis was staged on a five-point scale from F0 to F4 as follows: F0, absence of fibrosis; F1, perisinusoidal or periportal fibrosis; F2, combined perisinusoidal and portal/periportal fibrosis; F3, bridging fibrosis; and F4, cirrhosis. Fibrosis stage was defined by two pathologists in

consensus. Early fibrosis was defined as F2 or less. Platelet counts higher than 200 000/ μL have been reported (20) to correspond to this early stage of fibrosis (20). The details of the histologic assessments are in Appendix E1 (online).

Statistical Analysis

To clarify the level of the arterioportal ratio in patients with portal hypertension, the receiver operating characteristic curve was prepared by using software (JMP version 8; SAS Institute Japan, Tokyo, Japan). Sensitivity, specificity, positive predictive value, and negative predictive value were calculated by using the cutoff values obtained from the receiver operating characteristic curves. To examine the associations of arterioportal ratio and platelet count with an SEP score greater than or equal to 6, optimal cutoff values for the arterioportal ratio platelet count were selected to maximize the calculated value of the equation $SE - (1 - SP)$, where SE is sensitivity and SP is specificity. The normality assumption was analyzed by means of the Shapiro-Wilk test. Because the arterioportal ratio and SEP score were not normally distributed, these factors were compared with the categories of platelet counts by using the Kruskal-Wallis nonparametric analysis. Because the fibrosis stage is an ordinal scale, pair-wise comparisons were performed in order. Four pair-wise comparisons were performed (stage F0 vs stages F1–F4, stages F0–F1 vs stages F2–F4, stages F0–F2 vs stages F3–F4, and stages F0–F3 vs stage F4). This analysis was an assessment of how hepatic blood flow and splenic elasticity changed with changes in the platelet counts. Steatosis and platelet counts also were analyzed by means of pair-wise comparisons. Correlations between the resistive index of the hepatic artery and the splenic artery, fibrosis 4 index, hepatic elasticity, arterioportal ratio, splenic elasticity, and platelet counts were analyzed by using Spearman correlation coefficients. To clarify the changes in imaging factors for hepatic fibrosis, univariate and multivariate logistic regression analyses were performed with