Table 1 Clinical characteristics of patients in the estimation and validation groups

Clinical parameter	Estimation group ($n = 177$)			Validation group ($n = 442$)		
	NASH ($n = 98$)	NonNASH ($n = 79$)	P value	NASH ($n = 244$)	NonNASH ($n = 198$)	P value
Age (years)	57.7 ± 15.2	47.8 ± 16.5	0.0001	54.2 ± 15.2	48.2 ± 14.3	<0.0001
Gender (female)	58 (59%)	28 (35%)	0.0024	127 (52%)	77 (39%)	0.0072
BMI (kg/m^2)	28.5 ± 5.0	26.6 ± 4.7	0.0075	28.4 ± 5.0	27.4 ± 4.7	0.0207
Obesity (BMI >25)	74 (76%)	46 (58%)	0.0311	187 (77%)	139 (70%)	0.1548
Dyslipidemia	57 (58%)	39 (49%)	0.2886	173 (71%)	149 (75%)	0.3339
Hypertension (yes)	33 (34%)	12 (15%)	0.0055	100 (41%)	47 (24%)	0.0002
Type 2 diabetes (yes)	50 (51%)	14 (18%)	<0.0001	121 (50%)	69 (35%)	0.0027
Hemoglobin (g/dl)	14.1 ± 1.6 ($n = 97$)	14.8 ± 1.5 ($n = 78$)	0.0029	14.7 ± 2.5 ($n = 239$)	14.7 ± 1.5 ($n = 195$)	0.9758
Platelet count ($\times 10^4/\mu l$)	21.3 ± 6.4 ($n = 97$)	24.8 ± 8.1 ($n = 78$)	0.0018	21.2 ± 6.7 ($n = 240$)	24.1 ± 5.7 ($n = 194$)	<0.0001
AST (IU/l)	70 ± 30	44 ± 25	<0.0001	88 ± 387	38 ± 22	0.0694
ALT (IU/l)	102 ± 53	79 ± 54	0.0002	111 ± 217	65 ± 43	0.0001
AST/ALT ratio	0.77 ± 0.32	0.63 ± 0.23	0.0022	0.75 ± 0.32	0.65 ± 0.21	0.0001
GGT (IU/l)	105 ± 128	86 ± 66 ($n = 78$)	0.1211	85 ± 73 ($n = 242$)	85 ± 102 ($n = 197$)	0.9852
Cholinesterase (IU/l)	365 ± 83 ($n = 93$)	390 ± 83 ($n = 78$)	0.0317	364 ± 89 ($n = 224$)	387 ± 85 ($n = 176$)	0.0091
Total cholesterol (mg/dl)	206 ± 43 ($n = 97$)	214 ± 42 ($n = 77$)	0.2431	207 ± 43 ($n = 195$)	210 ± 39 ($n = 125$)	0.5121
Triglyceride (mg/dl)	189 ± 106 ($n = 93$)	167 ± 81 ($n = 73$)	0.1365	172 ± 106 ($n = 241$)	173 ± 86	0.9038
Ferritin (ng/ml)	270.7 ± 231	160 ± 158	0.0011	346 ± 989	183 ± 159	0.0221
FPG (mg/dl)	108 ± 45	96 ± 17.0	0.0301	113 ± 63	105 ± 39	0.1081
IRI (μU/ml)	18.5 ± 14.7	9.6 ± 6.3	<0.0001	16.8 ± 12.9	11.9 ± 8.3	<0.0001
Hyaluronic acid (ng/ml)	95 ± 134	29 ± 30	<0.0001	67 ± 74 ($n = 211$)	34 ± 37 ($n = 181$)	<0.0001
Type IV collagen 7S (ng/ml)	5.4 ± 1.7	3.9 ± 0.7	<0.0001	5.2 ± 2.1	3.9 ± 0.8	<0.0001
Histological fibrosis						
0–1	47 (48%)			143 (59%)		
2	29 (30%)			56 (23%)		
3	18 (18%)			30 (12%)		
4	4 (4%)			15 (6%)		

Results are presented as numbers with percentages *in parenthesis* for qualitative data or as means ± SD for quantitative data

BMI Body mass index, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* gamma glutamyl transpeptidase, *FPG* fasting plasma glucose, *IRI* immunoreactive insulin

P values were calculated by *t* test or χ^2 analysis

(≥55 years), female sex, obesity, Hb ≤14.5 g/dl, presence of T2DM, platelet count ≤22 × 10^4/μl, AST ≥60 IU/l, ALT ≥90 IU/l, AAR ≥0.8, ferritin ≥200 ng/ml (female) or ≥300 ng/ml (male), FPG ≥100 mg/dl, IRI ≥10.0 μU/ml, HA ≥50 ng/ml, and type IV collagen 7S ≥5.0 ng/ml were significant variables. By multivariate analysis, three variables remained significant, including ferritin, IRI, and type IV collagen 7S. Thus, these three variables, ferritin ≥200 ng/ml (female) or ≥300 ng/ml (male), IRI ≥10.0 μU/ml, and type IV collagen 7S ≥5.0 ng/ml, were combined to form the NAFIC score. The score was weighted based on OR obtained from logistic regression analysis (Table 2). Ferritin was given 1 point, IRI 1 point, and type IV collagen 7S 2 points. A score ranging from 0 to 4, defined by the presence of laboratory parameters, was calculated. The score for NASH patients ($n = 98$, 2.36 ± 1.28) was significantly higher than that for nonNASH patients ($n = 79$, 0.66 ± 0.82, $P < 0.0001$). The percentage of NASH in NAFLD with an NAFIC score of 0, 1, 2, 3, and 4 was 14% (6/44), 44% (34/61), 74% (14/19), 100% (28/28), and 92% (23/25), respectively (Fig. 1). The score was significantly higher even in NASH patients without significant fibrosis (stage 0 or 1) ($n = 47$, 1.83 ± 1.15) than in nonNASH patients ($P < 0.0001$). Figure 2 shows the ROC curve for NAFIC score to differentiate NASH from NAFLD. This scoring system had an AUROC of 0.851. At a cutoff value of NAFIC score 2, the sensitivity, specificity, PPV, and NPV were 66, 91, 90, and 67%, respectively. At a cutoff value of NAFIC score 1, the sensitivity, specificity, PPV, and NPV were 94, 48, 31, and 86%, respectively.

Table 2 Results of univariate and multivariate analysis: independent predictors of NASH and assigned score values in the estimation group ($n = 177$)

Variables	Unadjusted (univariate)			Adjusted (multivariate)			Score value
	OR	95%CI	*P* value	OR	95%CI	*P* value	
Age ≥55 years	2.28	1.24–4.18	0.0077				
Gender (female)	2.64	1.43–4.87	0.0019				
Obesity (BMI ≥25)	2.10	1.09–4.04	0.0268				
Hemoglobin ≤14.5 g/dl	1.94	1.06–3.56	0.0312				
Hypertension	2.83	1.35–5.96	0.0060				
Type 2 diabetes	4.84	2.40–9.74	<0.0001				
Platelet count $\leq 22 \times 10^4/\mu l$	2.66	1.43–4.91	0.0019				
AST ≥60 IU/l	5.74	2.81–11.73	<0.0001				
ALT ≥90 IU/l	2.04	1.10–3.77	0.0230				
AST/ALT ratio ≥0.8	1.98	1.18–4.76	0.0153				
Cholinesterase ≤380 IU/l	1.55	0.83–2.90	0.1689				
Ferritin ≥200 ng/ml (female) or ≥300 ng/ml (male)	5.08	2.48–10.37	<0.0001	4.01	1.07–15.02	0.0396	1
FPG ≥100 mg/dl	2.25	1.19–4.26	0.0127				
IRI ≥10 μU/ml	5.33	2.78–10.22	<0.0001	5.59	1.71–18.31	0.0045	1
Hyaluronic acid ≥50 ng/ml	4.94	2.38–10.26	<0.0001				
Type IV collagen 7S ≥5.0 ng/ml	21.20	7.19–62.49	<0.0001	15.54	1.49–162.39	0.0219	2

OR Odds ratio, *CI* confidence interval, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* gamma glutamyl transpeptidase, *FPG* fasting plasma glucose, *IRI* immunoreactive insulin

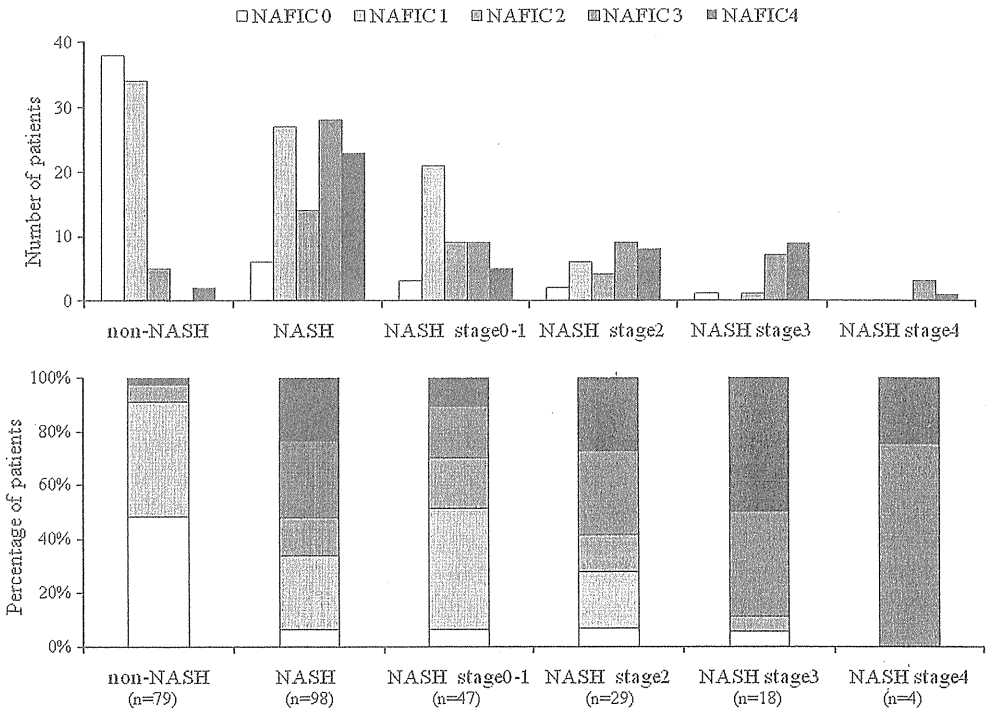

Fig. 1 Distribution of NAFIC scores in patients with NASH and nonNASH in the estimation group ($n = 177$)

Validation results

The diagnostic accuracy of the scoring system in separating patients with and without NASH was validated in 442 patients. Table 1 summarizes the clinical, laboratory, and liver biopsy data of the patient population in the validation group. Two hundred and two patients (46%) were female, and 326 (74%) patients were obese. Patients with NASH were significantly older, predominantly female, heavier, hypertensive, and more likely to have T2DM; had lower platelet count and ChE level; and had higher levels of AST, ALT, AAR, ferritin, IRI, HA, and type IV collagen 7S,

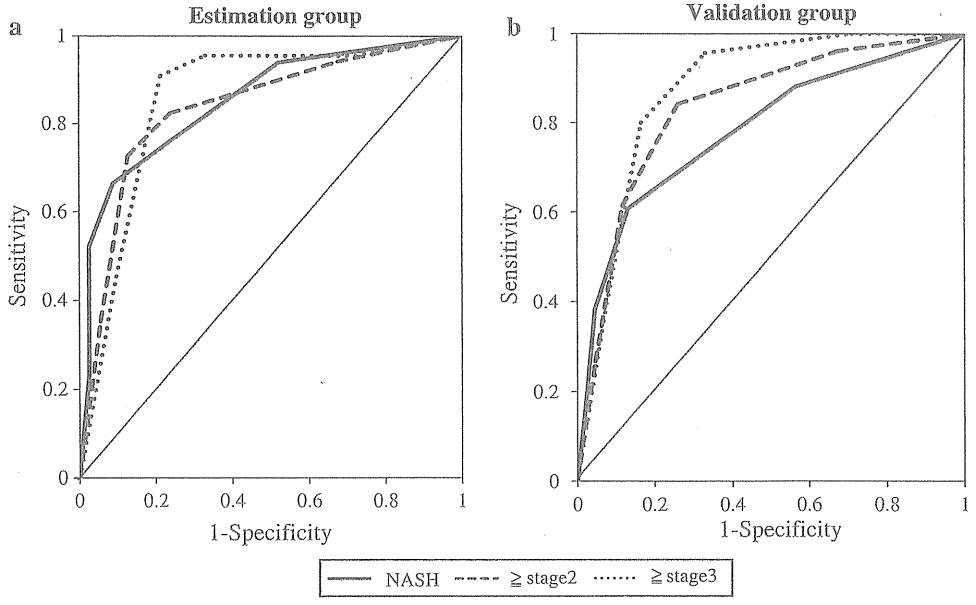

Fig. 2 ROC curves for the NAFIC score in the estimation (**a**) and validation (**b**) groups

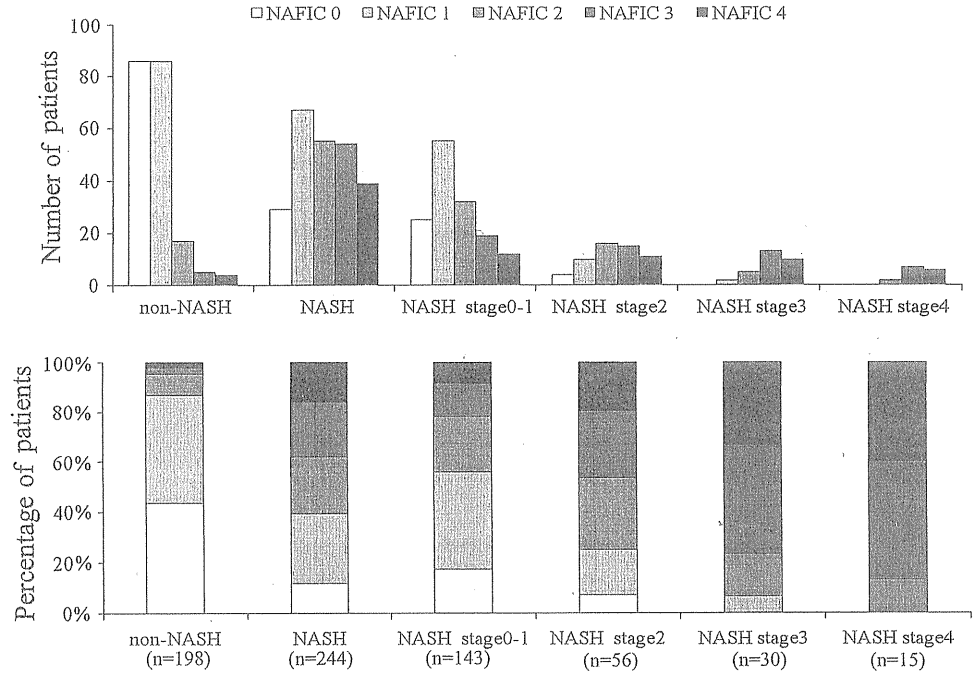

Fig. 3 Distribution of NAFIC score in patients with NASH and nonNASH in the validation group ($n = 442$)

than those with nonNASH NAFLD. The NAFIC score of NASH patients ($n = 244$, 2.03 ± 1.27) was significantly higher than that of nonNASH patients ($n = 198$, 0.76 ± 0.87, $p < 0.0001$). The percentage of NASH in NAFLD with an NAFIC score of 0, 1, 2, 3, and 4 was 25% (29/115), 44% (67/153), 76% (55/72), 92% (54/59), and 91% (39/43), respectively (Fig. 3). The score was significantly higher even in NASH patients without significant fibrosis (stage 0 or 1) ($n = 143$, 1.57 ± 1.17) than non-NASH patients ($p < 0.0001$). The AUROC remained relatively high in the validation set (0.782, Fig. 2; Table 3). At a cutoff value of NAFIC score 2, the sensitivity, specificity, PPV, and NPV were 60, 87, 85 and 64%, respectively. At a cutoff value of NAFIC score 1, the sensitivity, specificity, PPV, and NPV were 88, 43, 66, and 75%, respectively.

Comparing the NAFIC score to several previously established scoring systems

The AUROCs of various scoring systems that have been reported to differentiate NASH from NAFLD, or advanced from mild fibrosis, are shown in Table 3. To differentiate NASH from NAFLD, the AUROC in the validation group

Table 3 AUROC of NAFIC score and various scoring systems previously reported in the estimation and validation groups

Score	Group	AUROC for NASH	AUROC for significant fibrosis	AUROC for advanced fibrosis
NAFIC	Estimation ($n = 177$)	0.851	0.835	0.856
	Validation ($n = 442$)	0.782	0.833	0.874
	Total ($n = 619$)	0.803	0.834	0.869
HAIR [15]	Estimation ($n = 177$)	0.632	0.549	0.448
	Validation ($n = 432$)	0.636	0.620	0.631
	Total ($n = 609$)	0.631	0.593	0.566
Palekar et al. [17]	Estimation ($n = 173$)	0.784	0.794	0.847
	Validation ($n = 390$)	0.711	0.798	0.826
	Total ($n = 563$)	0.733	0.799	0.835
Modified Palekar et al.	Estimation ($n = 173$)	0.780	0.801	0.843
	Validation ($n = 390$)	0.709	0.810	0.830
	Total ($n = 563$)	0.730	0.808	0.837
Gholam et al. [18]	Estimation ($n = 177$)	0.829	0.784	0.713
	Validation ($n = 442$)	0.758	0.787	0.739
	Total ($n = 619$)	0.777	0.786	0.729
BAAT [19]	Estimation ($n = 164$)	0.672	0.533	0.473
	Validation ($n = 440$)	0.633	0.560	0.498
	Total ($n = 604$)	0.647	0.585	0.526
Modified BAAT	Estimation ($n = 164$)	0.741	0.615	0.566
	Validation ($n = 440$)	0.666	0.654	0.576
	Total ($n = 604$)	0.687	0.641	0.573
BARD [20]	Estimation ($n = 164$)	0.646	0.686	0.745
	Validation ($n = 440$)	0.621	0.689	0.731
	Total ($n = 604$)	0.627	0.688	0.734
Modified BARD	Estimation ($n = 164$)	0.647	0.709	0.734
	Validation ($n = 440$)	0.603	0.689	0.730
	Total ($n = 604$)	0.614	0.695	0.730
NAFLD fibrosis score [21]	Estimation ($n = 168$)	0.735	0.843	0.834
	Validation ($n = 420$)	0.663	0.805	0.862
	Total ($n = 588$)	0.685	0.817	0.853
N score (Nippon) [22]	Estimation ($n = 177$)	0.733	0.739	0.728
	Validation ($n = 408$)	0.642	0.715	0.698
	Total ($n = 585$)	0.668	0.720	0.704

was greatest for NAFIC (0.782, Fig. 2), then Gholam's score (0.758), followed by Palekar's score (0.711), modified Palekar's score (0.709), modified BAAT (0.666), NFS (0.663), *N* score (0.642), HAIR (0.636), BAAT (0.633), BARD score (0.621), and modified BARD score (0.603). Based on an evaluation of AUROC, NAFIC score outperformed other scoring systems in the estimation and validation groups to differentiate NASH from NAFLD. To differentiate NASH with significant fibrosis from NAFLD, the AUROC in the total cohort was greatest for NAFIC score (0.834), then NFS (0.817), followed by modified Palekar's score (0.808), Palekar's score (0.799), Gholam's score (0.786), *N* score (0.720), modified BARD (0.695), BARD (0.688), modified BAAT score (0.641), HAIR (0.593), and BAAT score (0.585). To differentiate NASH with advanced fibrosis from NAFLD, the AUROC in the total cohort was greatest for NAFIC score (0.869), then NFS (0.853), followed by modified Palekar's score (0.837), Palekar's score (0.835), BARD (0.734), modified BARD (0.730), Gholam's score (0.729), *N* score (0.704), modified BAAT (0.573), HAIR (0.566), and BAAT score (0.526). Among these prediction models, NAFIC score was superior to others in not only detecting NASH, but also predicting fibrosis stage.

We compared the diagnostic accuracy of NAFIC score to that of NFS in detecting advanced fibrosis (stage 3–4)

Table 4 Accuracy of the NAFIC score and NAFLD fibrosis score (NFS) in predicting advanced fibrosis (stage 3–4) and significant fibrosis (stage 2–4) in the total cohort

	NAFIC score			NAFLD fibrosis score		
	0–1	2	3–4	Low cutoff point (<−1.455)	Indeterminate (−1.455 to 0.676)	High cutoff point (>0.676)
Predicting advanced fibrosis (stage 3–4)						
N (%)	374 (60%)	90 (15%)	155 (25%)	330 (56%)	209 (36%)	49 (8%)
Stage 0–2	371	82	99	325	171	28
Stage 3–4	3	8	56	5	38	21
Se	96%		84%	92%		33%
Sp	67%		82%	62%		95%
PPV	26%		36%	23%		43%
NPV	99%		98%	98%		92%
LR (+)	2.913		4.660	2.427		6.141
LR (−)	0.067		0.200	0.126		0.710
Interpretation	Absence of advanced fibrosis (99% certainty)		Presence of advanced fibrosis (36% certainty)	Absence of advanced fibrosis (98% certainty)		Presence of advanced fibrosis (43% certainty)
	NAFIC score			NAFLD fibrosis score		
	0	1	2–4	Low cutoff point (<−1.455)	Indeterminate (−1.455 to 0.676)	High cutoff point (>0.676)
Predicting significant fibrosis (stage 2–4)						
N (%)	160 (26%)	214 (35%)	245 (40%)	330 (56%)	209 (36%)	49 (8%)
Stage 0–1	153	196	118	305	122	16
Stage 2–4	7	18	127	25	87	33
Se	95%		84%	86%		23%
Sp	33%		74%	69%		96%
PPV	32%		52%	47%		67%
NPV	96%		93%	92%		79%
LR (+)	1.416		3.266	2.657		6.301
LR (−)	0.141		0.070	0.250		0.801
Interpretation	Absence of significant fibrosis (96% certainty)		Presence of significant fibrosis (52% certainty)	Absence of significant fibrosis (92% certainty)		Presence of significant fibrosis (67% certainty)

Se Sensitivity, *Sp* specificity, *PPV* positive predictive value, *NPV* negative predictive value, *LR* likelihood ratio

and significant fibrosis (stage 2–4) (Table 4). To exclude advanced fibrosis using the low cutoff point (NFS < −1.455), 325 of 330 (98%) patients were correctly staged, whereas only 5 (2%) were understaged. The NPV of this cutoff for advanced fibrosis was 98%. Using the low cutoff point (NAFIC ≤1), 371 of 374 (99%) patients were correctly staged, whereas only 3 (1%) were understaged. The NPV of this cutoff for advanced fibrosis was 99%, which was equal to that of NFS. Using the high cutoff point (NFS >0.676), 21 of 49 (43%) patients were correctly staged, whereas 28 (57%) were overstaged. The PPV of this cutoff for advanced fibrosis was 43%. Using the high cutoff point (NAFIC ≥3), 56 of 155 (36%) patients were correctly staged, whereas 99 (64%) were overstaged. The PPV of this cutoff for advanced fibrosis was 36%, which was lower than that of NFS. The percentage of the undetermined range was much lower for the NAFIC score (15%) than for NFS (36%) (Table 4).

When the NFS low cutoff (NFS <−1.455) was applied to predict significant fibrosis, 305 of 330 (92%) patients were correctly staged, whereas 25 of 330 (8%) patients were understaged. The NPV for significant fibrosis was 92%. When their high cutoff (NFS >0.676) was applied to predict significant fibrosis, 33 of 49 (67%) patients were correctly staged, whereas 16 of 49 (33%) patients were overstaged. The PPV for significant fibrosis was 67%. When our low cutoff point (NAFIC = 0) was applied to exclude significant fibrosis, 153 of 160 (96%) patients were correctly staged, whereas only seven (4%) were understaged. The NPV of this cutoff for significant fibrosis was 96%, which

was slightly higher than NFS. Using our high cutoff point (NAFIC ≥2), 127 of 245 (52%) patients were correctly staged, whereas 118 (48%) were overstaged. The PPV of this cutoff for significant fibrosis was 52%, which was lower than that of NFS (67%) (Table 4).

Discussion

In this study, we developed and validated a simple scoring system to differentiate NASH from NAFLD. Our scoring system with the three variables ferritin, IRI, and type IV collagen 7S had an AUROC of 0.851 and 0.782 in the estimation and validation groups, respectively. Elevation of serum ferritin levels, a marker of iron storage, is associated with NASH [24, 25]. We previously reported high frequencies of hyperferritinemia and increased hepatic iron stores in Japanese NASH patients [11]. Yoneda and colleagues [26], our collaborative research group, also have reported that measurement of serum ferritin is useful to distinguish NASH from NAFLD. Their optimal cutoff value was 196 ng/ml, and their results for sensitivity, specificity, PPV, and NPV were 64, 77, 89, and 43%, respectively. Serum ferritin levels have been found to be a significant independent predictor of severe fibrosis in 167 Italian NAFLD subjects [27], but this has not been confirmed by other studies [28]. In Western countries, mildly increased serum ferritin does not necessarily indicate coexisting iron overload. However, it is well known that serum ferritin is closely associated with IR and can be considered a marker for metabolic syndrome [29].

Hyperinsulinemia (IRI ≥10.0 μU/ml) was also selected as an independent predictor of NASH. Hyperinsulinemia and increased IR could have important roles in the pathogenesis of NASH in both Western and Asian countries [30–33]. Hyperinsulinemia in NASH patients is attributable to increased insulin secretion, which compensates for reduced insulin sensitivity, and is not the consequence of decreased hepatic extraction of insulin, which occurs in all forms of CLD at the stage of advanced fibrosis or cirrhosis [30, 31]. The homeostasis model assessment (HOMA) has been validated and widely used for determining the degree of IR, and strongly predicts the development of T2DM [34]. Patients with NASH have a higher HOMA index compared with those with nonNASH NAFLD [30, 35]. Similarly, another study has reported the QUICKI model as being useful for predicting NASH [15]. However, appropriate cutoff values of these models have never been established. In contrast with these parameters that are mathematical transformations of FPG and IRI levels, fasting IRI, which has the advantage of being easily determined without calculations, was only applied to multivariate analysis in our study.

Type IV collagen is one of the extracellular matrices that are produced by hepatic fibroblasts. The 7S domain in the N-terminus of type IV collagen is inserted in tissues and released into the blood by turnover in connective tissues. Therefore, the serum 7S domain level increases in parallel with the amount of fibrosis and in synthesis from stellate cells and myofibroblasts following increased liver fibrosis [36]. In Japan, type IV collagen 7S is now widely used for assessing the extent of hepatic fibrosis in CLD because the test is covered by public health insurance. According to two reports [37, 38], a cutoff point of 5.0 or 4.25 ng/ml provided high NPV to exclude advanced fibrosis in Japanese NAFLD patients. Shimada et al. [39] have demonstrated that a cutoff point of 5.0 ng/ml provided sensitivity and specificity of 41 and 95%, respectively, to detect early-stage NASH. Serum HA levels are elevated during accelerated deposition of collagen in the extracellular space due to upregulation of HA production by activated stellate cells and myofibroblasts, and downregulation of its clearance by sinusoidal endothelial cells. Serum HA appears to be a relatively accurate predictor of advanced fibrosis stage in NAFLD, but less for distinguishing between minor degrees of fibrosis in NASH and nonNASH NAFLD [40, 41]. In our study, serum HA level was not an independent predictor of NASH by multivariate analysis. Moreover, HA increases in systematic inflammatory conditions, which might produce false-positive results. We believe that type IV collagen 7S is superior to HA in predicting the extent of fibrosis in NAFLD patients.

Currently, the NAFLD biomarkers have been evaluated for (1) distinguishing NASH from NAFLD and/or (2) diagnosing advanced fibrosis or cirrhosis. HAIR [15], Palekar's [17], and Gholam's [18] scores were derived for distinguishing NASH from NAFLD, and the others are for detecting significant or advanced fibrosis. The present study clearly demonstrated that NAFIC score was more useful than other scoring systems for detection of NASH and for prediction of fibrosis (Table 3). The HAIR score uses a combination of presence of hypertension, elevated ALT (>40 U/L), and IR, with at least two parameters that indicate NASH with high sensitivity and specificity [15]. None had an IR index >5 in our overall cohort (data not shown), and AUROCs were 0.632 and 0.634 in the estimation and validation groups, respectively. The score does not appear useful in a less obese population because it was described in a group of severely obese patients who were undergoing gastric bypass surgery. In Palekar's study [17], the presence of at least three out of six factors (age ≥50 years, female sex, AST ≥45 IU/l, BMI ≥30 kg/m^2, AAR ≥0.80, and HA ≥55 ng/ml) had a sensitivity and specificity for NASH diagnosis of 74 and 66%, respectively. In our estimation group, the presence of at least three of these factors had a sensitivity and specificity for

NASH diagnosis of 68 and 71%, respectively. In our validation group, the presence of at least three of these factors had a sensitivity and specificity for NASH diagnosis of 63 and 64%, respectively. AUROCs were 0.784 and 0.711 in the estimation and validation groups, respectively. Therefore, Palekar's score was not superior to NAFIC score for predicting NASH (Table 3).

Gholam's score [18], which consists of only two variables (AST and the presence of DM), is very simple and equally useful as NAFIC score for detection of NASH, but it was not superior to our score for predicting severe fibrosis in our cohort. Gholam et al. [18] have constructed other models that consist of ALT and HbA1c to detect the presence of fibrosis. We could not evaluate these models because HbA1c was only measured in a limited number of patients. Angulo et al. [21] have shown that the NFS, which consists of six variables (age, BMI, AAR, IFG/DM, platelet count, and albumin), can reliably predict advanced fibrosis. In ROC analysis, NFS is shown to be useful for prediction of advanced or significant fibrosis. The low cutoff point (NFS <-1.455) showed higher NPV (98%) than that in the estimation (93%) and validation (88%) cohort reported by Angulo et al. The low cutoff point in NAFIC score (≤ 1) had equally high NPV (99%). In contrast, the high cutoff point (NFS >0.676) showed lower PPV (43%) than that in the estimation (98%) and validation (80%) cohort reported by Angulo et al. The high cutoff point in NAFIC score (≥ 3) had lower PPV (36%). By applying the low cutoff score (NFS <-1.455, NAFIC ≤ 1), advanced fibrosis could be excluded with high accuracy. By applying the high cutoff score (NFS >0.676, NAFIC ≥ 3), the presence of advanced fibrosis could not be diagnosed with high accuracy. Consistent with our results, a separate validation study of NFS in 162 Chinese patients found that the NPV for excluding advanced fibrosis was 91%, but the PPV for predicting advanced fibrosis was 0% [42]. It is suggested that this low PPV might be due to lower prevalence of advanced fibrosis in the study of Wong et al. (11%) [42] than in that by Angulo et al. (27%) [21]. Similarly, the prevalence of advanced fibrosis was low (11%) in our study.

In Asian patients, steatohepatitis and other metabolic complications tend to develop at a lower BMI, which is one of the factors in the equation of the NFS. Therefore, NFS and NAFIC score were applicable to exclusion rather than detection of significant or advanced fibrosis. NFS can be easily obtained in clinical practice, but this scoring system can be cumbersome and difficult to apply in every practice. The easily determined NAFIC score is at least equivalent to the more complex NFS. Our results suggest that liver biopsies can be avoided in NAFLD patients with a NAFIC score of 0 or 1 because they are likely to have NAFLD without advanced fibrosis. In contrast, liver biopsies should be recommended in NAFLD patients with an NAFIC score of ≥ 2 to assess the extent of hepatic fibrosis and predict prognosis. The BARD score developed by Harrison et al. is a weighted sum of three easily available variables [BMI ≥ 28 kg/m^2 (1 point), AAR ≥ 0.8 (2 points), and DM (1 point)], and the authors have shown that a score of 2–4 was associated with an OR of 17 for predicting advanced fibrosis [20]. Although the BARD score is simple to calculate, our validation study did not reveal an advantage of this score over others. In our cohort, when a BARD score of ≥ 2 was found, the sensitivity, specificity, PPV, and NPV for detecting advanced fibrosis were 73, 65, 19, and 95%, respectively. According to a study of 122 Japanese NAFLD patients by Fujii and colleagues [43], our collaborative research group, when a BARD score of ≥ 2 was used, the AUROC was 0.73 with an OR of 4.9 for detection of advanced fibrosis. It has been concluded that BARD score is less predictive of advanced fibrosis in Japanese NAFLD patients because they are not as obese as those in Western countries. Disappointingly, modified scores of Palekar's score, BAAT, and BARD could not improve the diagnostic accuracy for NASH or advanced fibrosis. The *N* score (the total number of the following risk factors: female sex, age >60 years, T2DM, and hypertension), which was established on the basis of data collection from 182 Japanese NAFLD patients in multiple centers in Nagasaki [22], is very simple, without the need for detailed laboratory tests. However, it was not superior to other scoring systems in our validation study.

Our study had several limitations. The fact that we excluded diabetic patients treated with exogenous insulin or insulin sensitizers (metformin or pioglitazone) from the analysis was a major limitation. In the future, we must find better scoring systems that are applicable to these patients. Other limitations include the largely retrospective study design and lack of complete data in many subjects. We included patients from different hepatology centers in Japan that have a particular interest in studying NAFLD, and thus, some referral bias could not be ruled out. Patient selection bias could also have existed because liver biopsy might have been considered for NAFLD patients who were likely to have NASH. We acknowledge that pathological diagnosis was mainly determined using liver tissues derived from percutaneous liver biopsy, which is prone to sampling error or interobserver variability [8, 9]. In fact, 11 patients of our total cohort were diagnosed as nonNASH in spite of an NAFIC score of 3 or 4. Although the exact reason was unknown, sampling error could have led to this misdiagnosis. These patients need follow-up care or repeat liver biopsies. It should be emphasized that we had a central pathology review by two hepatopathologists to prevent interobserver variability, although we were not able to quantify the effect on our results of some

intraobserver variability. Because all participants were Japanese, there is a possibility that our results might not be adaptable for NAFLD patients of other races. Due to these limitations, the present results need to be validated in independent populations by other investigators.

In conclusion, NAFIC score can predict NASH in Japanese NAFLD patients with sufficient accuracy and simplicity to be considered for clinical use, thus identifying a very high-risk group in whom liver biopsy would be very likely to detect NASH, as well as a low-risk group in whom liver biopsy can be safely delayed or avoided.

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Original Article

Accumulation of refractory factors for pegylated interferon plus ribavirin therapy in older female patients with chronic hepatitis C

Kazuaki Chayama,[1] C. Nelson Hayes,[1] Kentaro Yoshioka,[2] Hisataka Moriwaki,[3] Takeshi Okanoue,[4] Shotaro Sakisaka,[5] Tetsuo Takehara,[6] Makoto Oketani,[7] Joji Toyota,[8] Namiki Izumi,[9] Yoichi Hiasa,[10] Akihiro Matsumoto,[11] Hideyuki Nomura,[12] Masataka Seike,[13] Yoshiyuki Ueno,[14] Hiroshi Yotsuyanagi[15] and Hiromitsu Kumada[16]

[1]*Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima,* [2]*Division of Liver, Biliary Tract and Pancreas Diseases, Department of Internal Medicine, Fujita Health University, Aichi,* [3]*Department of Gastroenterology, Gifu University Graduate School of Medicine, Gifu,* [4]*Department of Gastroenterology and Hepatology, Saiseikai Suita Hospital,* [6]*Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka,* [5]*Department of Gastroenterology and Medicine, Fukuoka University School of Medicine, Fukuoka,* [7]*Department of Digestive and Lifestyle Related Disease, Health Research Course, Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima,* [8]*Department of Gastroenterology, Sapporo Kosei General Hospital, Sapporo, Hokkaido,* [9]*Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital,* [15]*Department of Internal Medicine, Graduate School of Medicine, University of Tokyo,* [16]*Department of Hepatology, Toranomon Hospital, Tokyo,* [10]*Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Matsuyama, Ehime,* [11]*Department of Medicine, Shinshu University School of Medicine, Matsumoto, Nagano,* [12]*The Center for Liver Diseases, Shin-Kokura Hospital, Kitakyushu City,* [13]*Department of Internal Medicine 1, Faculty of Medicine, Oita University, Oita, and* [14]*Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan*

Aim: Several host and viral factors have been reported to influence the effectiveness of pegylated interferon plus ribavirin combination therapy for chronic hepatitis C. In Japan, where the age of treated patients is comparatively high, recent studies have reported poor response to treatment in older female patients, but little is known about the relationship between advanced age in women and previously reported factors.

Methods: Using a database of 1167 patients chronically infected with hepatitis C virus (HCV) genotype 1b, we analyzed the amino acid sequences of the HCV core protein and interferon sensitivity determining region (ISDR) and examined the relationships among predictive factors.

Results: The proportion of patients with substitutions at core 70, which is associated with poor response to pegylated interferon plus ribavirin therapy, increased with age only in female patients. A similar trend was observed for ISDR wild type (wt). We also found that core 70 wt is associated with core 91 wt ($P = 5.4 \times 10^{-9}$) as well as ISDR wt ($P = 0.025$). HCV RNA levels were higher in patients with core and ISDR wt ($P < 0.001$). Furthermore, core amino acid mutations were associated with advanced fibrosis and higher inflammatory activity ($P = 0.028$ and 0.048, respectively) as well as higher gamma-glutamyltranspeptidase, alanine aminotransferase and low-density lipoprotein cholesterol levels ($P < 0.001$, 0.006 and 0.001, respectively).

Conclusion: A combination of factors account for poor response rate in older female patients in Japan. Elucidating the relationship between amino acid substitutions and metabolic alteration is an important step in understanding the mechanism of HCV interferon resistance.

Key words: combination therapy, core protein, genotype 1b, interferon sensitivity determining region, low-density lipoprotein cholesterol

Correspondence: Professor Kazuaki Chayama, Department of Medical and Molecular Science, Division of Frontier Medical Science Programs for Biomedical Research, Graduate School of Biomedical Science Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Email: chayama@hiroshima-u.ac.jp

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INTRODUCTION

HEPATITIS C VIRUS (HCV) is a causative agent of acute and chronic hepatitis as well as liver cirrhosis and hepatocellular carcinoma.[1–3] The single stranded RNA genome encodes one large open reading frame that is processed into at least 10 proteins by host and viral enzymes.[4,5] Some viral proteins are known to affect the outcome of pegylated interferon (PEG IFN) plus ribavirin combination therapy, the current standard of care for chronic hepatitis.[6–8] The number of amino acid substitutions in the IFN sensitivity determining region (ISDR) of the NS5A protein, which was initially reported to affect IFN monotherapy,[9,10] has recently been reported to affect PEG IFN plus ribavirin combination therapy as well.[11–14]

NS5A PKR binding domain (PKRBD),[15–19] variable region 3 (V3),[20–23] IFN/ribavirin resistance determining region (IRRDR),[24,25] and E2 PKR-eIF2α phosphorylation homology domain (PePHD)[26] have also been reported to affect therapy outcome, although these results need to be confirmed. More recently, amino acid (a.a.) substitutions in the core protein have been reported to negatively affect IFN plus ribavirin therapy.[27,28] Substitution at a.a. 70 of the core protein (core 70) has been reported to be associated with non-virological response (NVR), and this finding was confirmed by several groups.[29–31]

Several cytokines and adipokines have also been reported to be associated with the effectiveness of therapy. For instance, tumor necrosis factor (TNF)-α expression has been reported to be elevated in patients with HCV infection, and high expression levels are associated with poor response to IFN therapy.[32] IP-10 has also been reported to associate with response to therapy in patients with HCV and HIV co-infection.[33] Leptin and adiponectin levels are also reportedly associated with the effect of combination therapy.[34,35] In addition to these factors, there are many studies reporting relationships between common polymorphisms in the human genome and outcome of IFN therapy.[36–44] Among them, single nucleotide polymorphisms (SNP) in the interleukin (IL)-28B locus discovered through genome-wide association studies appear to have a large effect on outcome of PEG IFN plus ribavirin combination therapy[42–44] as well as spontaneous eradication of HCV.[45]

In addition to the above viral and host genetic factors, several metabolic factors such as obesity,[34] insulin resistance[46] and low-density lipoprotein (LDL) cholesterol levels[28,47] have been reported to be correlated with the effect of combination therapy. Furthermore, higher gamma-glutamyltranspeptidase (γ-GTP) levels, often associated with fatty liver, have also been reported to be associated with treatment outcome.[48,49] Although these factors may be mutually interdependent, their relationships with viral factors have not yet been analyzed.

Recent papers have reported poor response to therapy in older female patients,[50–52] but little is known about the relationship between age, sex and other predictive factors. To analyze these associations, we constructed a database consisting of 1425 patients with chronic hepatitis C. Using this database, we analyzed the relationship between viral and metabolic data and found that a.a. substitutions in the core and ISDR are associated with metabolic change, which may be related to disease progression and response to therapy.

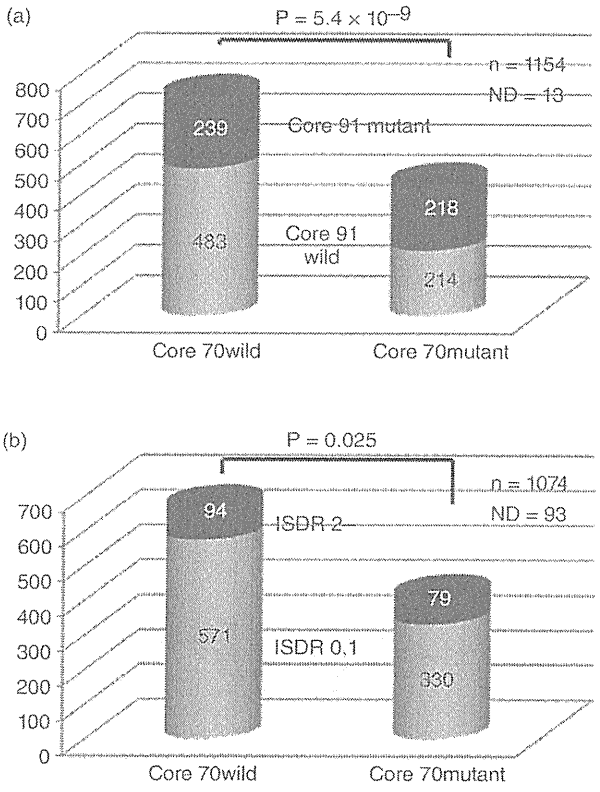


Figure 1 Association of core amino acid 70, amino acid 91 and interferon sensitivity determining region (ISDR). The relationship between hepatitis C virus core 70 and core 91 wild type and mutant amino acids (a) and the ISDR (b) were examined. Statistical significance was assessed using the χ^2-test. ND, not determined due to polymerase chain reaction or sequence calling failure.

Table 1 Clinical profile of 1167 patients

	All patients $n = 1167$	Tx naive $n = 570$ (48.84%)	Prev. tx $n = 597$ (51.16%)	*P*-value
Sex (male/female)	606/561	259/311	347/250	1.45E-05
Age	55.1 ± 10.7	55.2 ± 11.0	55.0 ± 10.5	0.604
Body weight	60.6 ± 10.8	59.5 ± 10.5	61.7 ± 11.0	0.001
BMI	27.0 ± 7.38	24.3 ± 5.46	29.6 ± 8.02	0
Fibrosis stage (0–2/3–4/ND)	815/192/160	422/78/70	393/114/90	0.005
Activity stage (0–1/2–3/ND)	531/465/171	263/234/73	268/231/98	0.803
Steatosis (present/absent/ND)	207/428/532	103/175/292	104/253/240	0.034
White blood cells (/mm^3)	4808 ± 1428	4871 ± 1395	4748 ± 1457	0.127
Hemoglobin (g/dL)	14.1 ± 1.88	14.0 ± 1.39	14.3 ± 2.23	0.001
Platelets ($\times 10^4$/mm^3)	16.6 ± 5.06	16.5 ± 5.31	16.7 ± 4.82	0.288
ALT (IU/L)	66 ± 52	67 ± 48	65 ± 55	0.265
AST (IU/L)	65 ± 54	58 ± 37	71 ± 66	0.001
γ-GTP (IU/L)	56 ± 58	57 ± 62	55 ± 54	0.942
Albumin (g/dL)	4.00 ± 0.375	4.04 ± 0.402	3.97 ± 0.347	0.001
Total cholesterol (mg/dL)	173 ± 32.1	175 ± 32.7	172 ± 31.6	0.206
Fasting blood sugar (mg/dL)	101 ± 24.9	102 ± 27.2	99.8 ± 22.2	0.715
HCV RNA (KIU/mL: amp)	2999 ± 4523	2822 ± 4365	3169 ± 4668	0.048
ISDR (0–1/≥2/ND)	908/178/81	440/85/45	468/93/36	0.863
Core 70 (wild/mutant/ND)	722/433/12	349/218/3	373/215/9	0.509
Core 91 (wild/mutant/ND)	697/457/13	349/217/4	348/240/9	0.39

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, gamma-glutamyltranspeptidase; HCV, hepatitis C virus; ND, not determined; tx., treatment.

METHODS

Study subjects

WE COLLECTED DATA from 1425 participating patients with chronic hepatitis C from 16 centers in Japan. Inclusion criteria included testing positive for HCV RNA over a period of more than 6 months and testing negative for both hepatitis B virus surface antigen and anti-HIV antibody. Patients with confounding liver conditions were excluded, as well as patients who were lost to follow up or who did not have high viral load (≥5 log IU/mL) for HCV genotype 1b (Fig. 1). Patient data was not used when we failed to determine core 70, core 90 and ISDR sequences. In total, data from 1167 patients were included in the analysis. All subjects gave written informed consent to participate in the study according to the process approved by the ethical committee of each hospital and conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

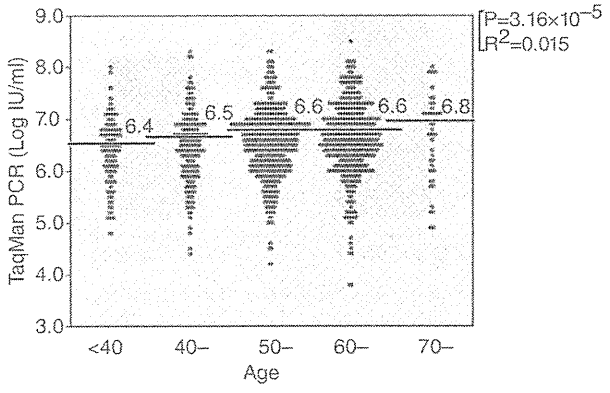


Figure 2 Relationship between age and virus titer. Virus titers were plotted according to age. The median titer within each 10-year age group is shown as horizontal bars.

Patients received weekly injections of PEG IFN-α-2b for either 48 or 72 weeks using the following doses: 60 μg for 35–45 kg bodyweight; 80 μg for 46–60 kg; 100 μg for 61–75 kg; 120 μg for 76–90 kg; and 150 μg for 91–120 kg. Ribavirin was administrated p.o., and the dose was determined based on the patient's bodyweight (600 mg for <60 kg, 800 mg for 60–80 kg, 1000 mg for >80 kg). Ribavirin dosage was reduced when hemoglobin levels reduced to 10.0 g/dL and stopped if hemoglobin levels reached 8.5 g/dL. Bio-

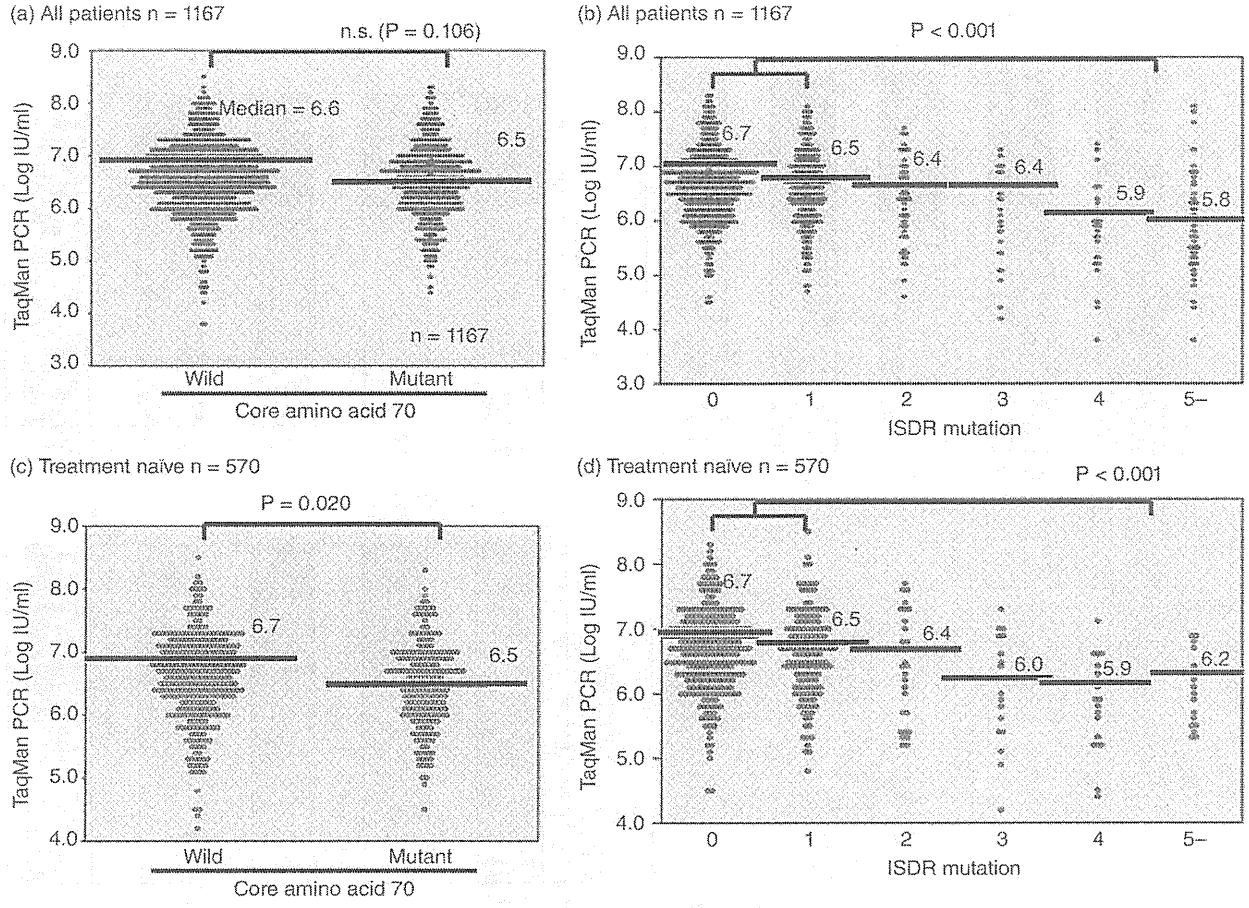


Figure 3 Analysis of virus load by core amino acid 70 substitution and number of amino acid substitutions in the interferon sensitivity determining region (ISDR). Virus titers of all 1167 patients were classified according to core 70 wild type and mutant amino acids (a) or by the number of substitutions in the ISDR (b). The 570 interferon therapy naive patients were also examined separately (c,d).

chemical tests were performed by center, and pathological diagnosis was made according to the criteria of Desmet *et al.*[53] Successful treatment was ascertained based on sustained virological response (SVR), defined as HCV RNA negative 6 months after cessation of therapy.

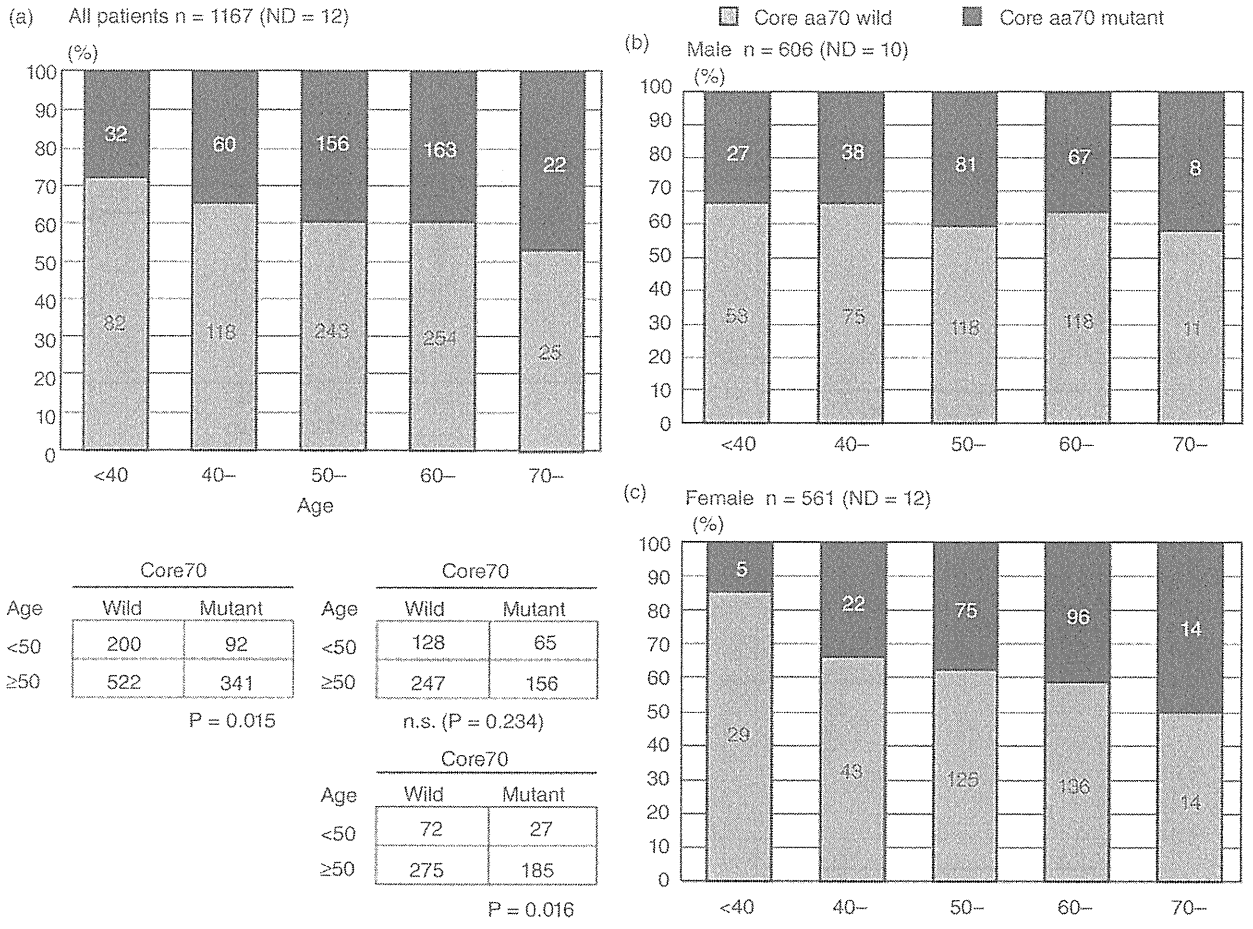
Analysis of viral titer and a.a. sequences in the core and ISDR region

The HCV RNA level was analyzed using reverse transcription polymerase chain reaction (RT–PCR)-based methods (Amplicor Hepatitis C Virus test: Roche Diagnostics, Basel, Switzerland; high range test: Cobas Amplicor, Roche Diagnostics, Basel, Switzerland; or TaqMan RT–PCR test: Applied Biosystems, Foster city, CA, USA). The measurement ranges of these assays were 5–5000 KIU/mL and 1.2–7.8 log IU, respectively. For values exceeding the measurable range, the titer was determined after dilution of the serum samples.

Sequences were determined by direct sequencing of PCR fragments following extraction and RT of serum HCV RNA. For core 70 and 91, arginine and leucine were considered wild type (wt) according to Akuta *et al.*[27,28] The number of a.a. substitutions in the ISDR was determined as described previously.[9,10,53]

Statistical analysis

The χ^2-test and Mann–Whitney *U*-test were applied to detect significant associations using PASW ver. 18



Core70

Age	Wild	Mutant
<50	200	92
≥50	522	341

P = 0.015

Core70

Age	Wild	Mutant
<50	128	65
≥50	247	156

n.s. (P = 0.234)

Core70

Age	Wild	Mutant
<50	72	27
≥50	275	185

P = 0.016

Figure 4 Age-dependent increase in core amino acid 70 mutants in female patients. Percentages of core wild type (arginine) and mutant amino acids for all patients (a), as well as for male (b) and female (c) patients are shown. Note that the age-dependent increase in mutant frequency was observed only in female patients. Statistical analysis was performed by χ^2-test. ND, not determined.

(SPSS, Chicago, IL, USA). All statistical analyses were two sided, and $P < 0.05$ was considered significant. Simple and multiple regression analyses were used to examine the association between viral substitutions and clinical factors using $P < 0.05$ as the criterion for inclusion in the multivariate model. Continuous variables were split into indicator variables based on the median, except for age which was divided into 10-year intervals. Multivariate logistic regression analysis was performed using the Design package in R (www.r-project.org) with fast backward elimination and validation based on AIC score.

RESULTS

Patient characteristics

PATIENT PROFILES ARE shown in Table 1. Results are presented separately for patients who were naive to IFN therapy and those who had had previous IFN therapy but failed to eradicate the virus.

Virus titer and a.a. substitutions in the core and the ISDR

We found a significant positive correlation between patient age and virus titer ($P = 3.16 \times 10^{-5}$, $R^2 = 0.015$,

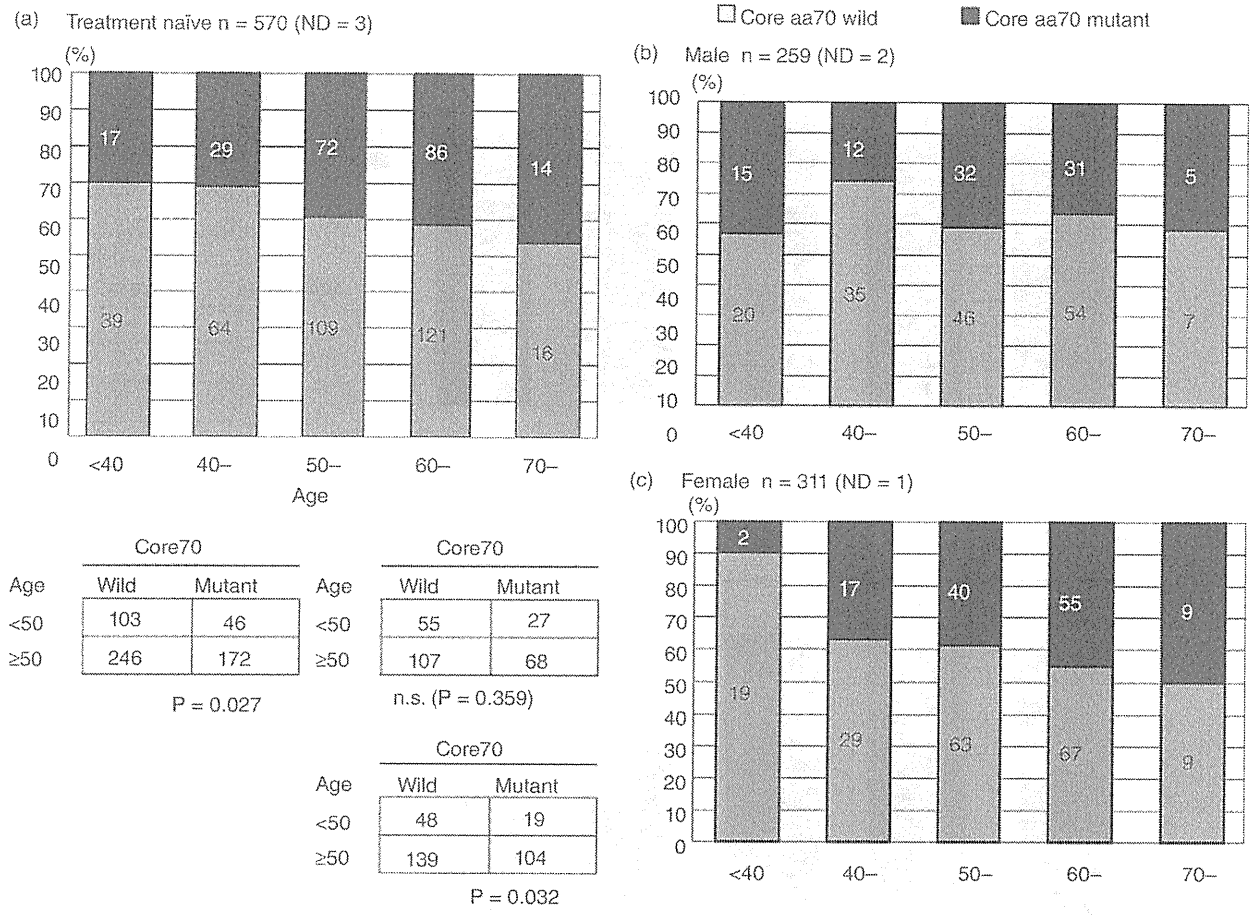


Figure 5 Age-dependent increase in core amino acid 70 mutants in treatment-naive female patients. Percentage of core wild type (arginine) and mutant amino acid were analyzed as in Figure 5 using only interferon treatment-naive patients. Results for all 561 patients (a), as well as for male (b) and female (c) patients are shown. ND, not determined.

Fig. 2). Wt core 70 was associated with wt core 91, with 40% of patients wt for both core 70 and core 91 and 20% of patients non-wt for both (Fig. 1, $P = 5.4 \times 10^{-9}$). Virus titer did not differ in patients with wt core 70 compared to non-wt when all patients were included (Fig. 3a), but when treatment-naive patients were analyzed separately, virus titer was significantly higher in patients with core 70 wt ($P = 0.02$, Fig. 3c). We found a significant negative linear relationship between virus titer and the number of substitutions in the ISDR ($P < 0.001$, Fig. 3b), regardless of treatment history ($P < 0.001$, Fig. 3d).

Amino acid substitution and age

The proportion of patients with core 70 substitutions increased with age among female patients (Figs 4,5), and the proportion of patients without substitutions in the ISDR tended to increase with age among treatment-naive females ($P = 0.0581$, Fig. 6).

Core 70 a.a. substitution and histological findings

Fibrosis stage and activity were higher in patients with core 70 mutants ($P = 0.028$ and $P = 0.048$, respectively; Fig. 7). There was no apparent correlation between his-

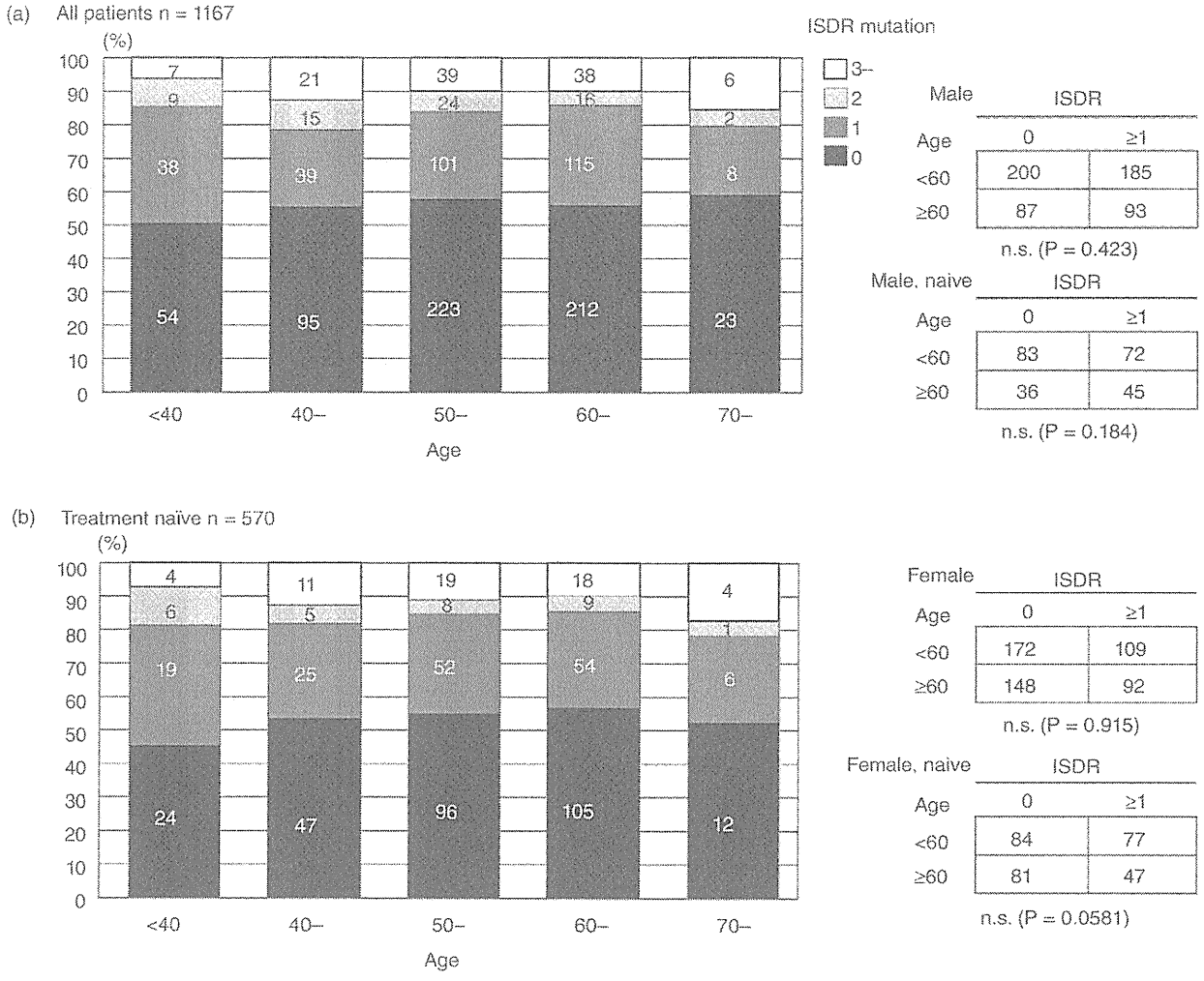


Figure 6 Age-dependent increase in number of amino acid substitutions in the interferon sensitivity determining region (ISDR). The relationship between age and the number of amino acid substitutions in the ISDR was examined. All patients (a) and only naive patients (b) were analyzed. Statistical analysis was performed using the χ^2-test.

tological findings and the number of a.a. substitutions in the ISDR (data not shown).

Correlation between viral a.a. substitutions and clinical conditions

We compared γ-GTP, ALT, LDL cholesterol levels and other clinical conditions between patients with core 70 wild and mutant types (Fig. 8). ALT and γ-GTP levels were significantly higher in patients with core 70 substitutions (Fig. 8a,b). In contrast, LDL cholesterol levels and platelet counts were significantly higher in patients with core 70 wt (Fig. 8c,d). However, only sex, fibrosis, γ-GTP and core 91 substitution were independently associated with core 70 substitution (Table 2). Only viral load and core 70 substitutions are independent predictive factors for the presence of two or more ISDR substitutions (Table 3).

DISCUSSION

WE FOUND THAT factors previously reported to be associated with poor response to IFN-based treatment for chronic hepatitis C tended to be most strongly associated with older female patients. Studies on difficult-to-treat older female patients have so far only been reported in Japan, probably due to the rela-

Table 2 Factors associated with HCV core protein amino acid 70 substitutions

Variable	Simple			Multiple			
	n	OR	*P*	*n*	OR	(95% CI)	*P*
Age (in 10-year increments)	331	1.1	0.3536				
Sex (male vs female)	365	1.58	0.04178	214	2.09	(1.11–3.95)	0.0234
BMI (kg/m^2)	363	0.763	0.2229				
Diabetes	312	1.77	0.08053				
Fibrosis (F0–1 vs F2–4)	252	2.12	0.007444	214	2.18	(1.15–4.13)	0.017
Activity (A0–1 vs A2–4)	246	1.73	0.04849				
ALT (IU/L)	329	0.866	0.5461				
Platelets (×104/mm^3)	329	0.937	0.7836				
γ-GTP (IU/L)	305	1.69	0.03427	214	1.59	(0.841–3.02)	0.153
Albumin (g/dL)	190	0.765	0.3981				
Fasting blood sugar (mg/dL)	250	0.898	0.6878				
TaqMan PCR (log IU/mL)	327	0.748	0.2232				
HDL cholesterol (mg/dL)	202	1.64	0.1025				
LDL cholesterol (mg/dL)	165	1.25	0.5085				
Total cholesterol (mg/dL)	321	0.907	0.6847				
Core 91 (wild vs others)	365	2.22	0.000393	214	2.68	(1.43–5.02)	0.002
ISDR (0,1 vs >1)	343	1.82	0.03102	214	1.85	(0.853–4)	0.1197

Simple and multiple logistic regression were used to examine the association between substitution at core amino acid 70 and patient and viral factors.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; γ-GTP, gamma-glutamyltranspeptidase; HCV, hepatitis C virus; HDL, high-density lipoprotein; ISDR, interferon sensitivity determining region; LDL, low-density lipoprotein; ND, not determined; OR, odds ratio.

Table 3 Factors associated with viral ISDR substitutions (0–1 vs >1 mutations)

Variable	Simple			Multiple			
	n	OR	*P*	*n*	OR	(95% CI)	*P*
Age (in 10-year increments)	311	1	0.9735				
Sex (male vs female)	345	0.644	0.1247				
BMI (kg/m^2)	343	1.14	0.6254				
Diabetes	293	0.818	0.6509				
Fibrosis (F0–1 vs F2–4)	235	1.28	0.4545				
Activity (A0–1 vs A2–4)	229	1.3	0.4281				
ALT (IU/L)	309	1.15	0.646				
Platelets (×104/mm^3)	309	0.668	0.1707				
γ-GTP (IU/L)	287	1.47	0.2115				
Albumin (g/dL)	172	0.979	0.9622				
Fasting blood sugar (mg/dL)	233	1.36	0.3641				
TaqMan PCR (log IU/mL)	307	0.517	0.02527	305	0.529	(0.30–0.95)	0.03223
HDL cholesterol (mg/dL)	189	1.23	0.617				
LDL cholesterol (mg/dL)	152	0.463	0.1199				
Total cholesterol (mg/dL)	303	0.656	0.1537				
Core 70 (wild vs others)	343	1.82	0.03102	305	1.82	(1.01–3.3)	0.04763
Core 91 (wild vs others)	344	0.699	0.2038				

Simple and multiple logistic regression was used to examine the association between the number of substitutions in the ISDR region and patient and viral factors.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; γ-GTP, gamma-glutamyltranspeptidase; HCV, hepatitis C virus; HDL, high-density lipoprotein; ISDR, interferon sensitivity determining region; LDL, low-density lipoprotein; ND, not determined; OR, odds ratio.

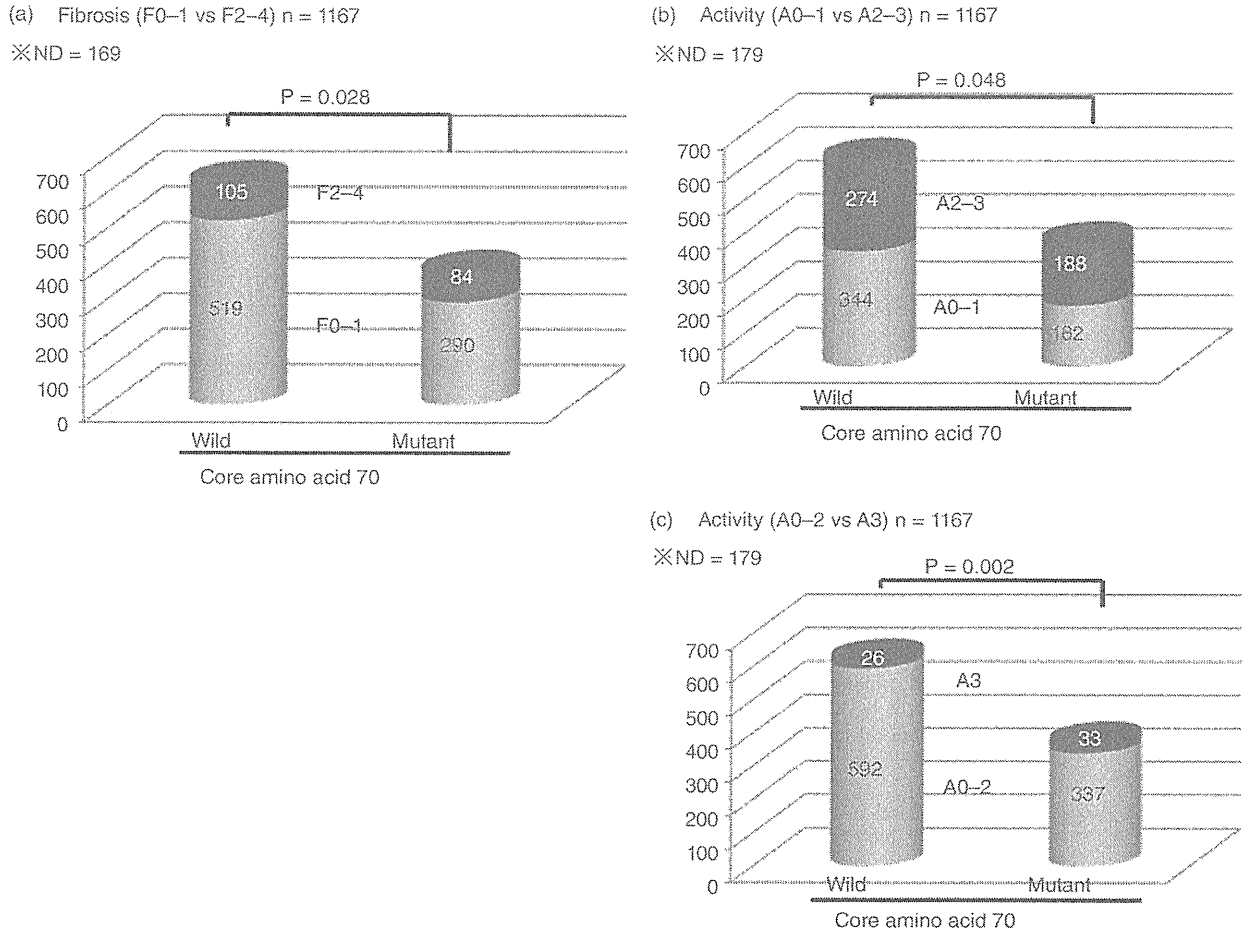


Figure 7 Histological findings and core amino acid 70 substitutions. Relationships between core amino acid 70 (wild type or mutant) and degree of fibrosis (F0–1 and F2–4) (a) and activity (b,c) were examined. Activity was divided into A0–1 and A2–3 (b) or A0–2 and A3 (c) and compared with amino acid 70. ND, not determined.

tively higher age at treatment. The mechanism underlying this association is unknown. Recently, SNP in the IL-28B locus were found to be associated with response to combination therapy as well as to spontaneous eradication of the virus,[42–44] although differences in the eradication rate between men and women have not been reported so far. We have previously reported that incidence of wt core 70 is significantly higher in patients with the IL-28 protective allele.[54] Therefore, it seems reasonable that the wt core 70 confers a selective advantage for the virus in patients with the IL-28 protective allele. During the time when IFN monotherapy was still the standard treatment, female sex, or perhaps the lower iron concentration associated with female sex, had been reported as one of the predictive factors for a favorable response to monotherapy.[55–57] It is possible that spontaneous eradication of the virus occurs during the natural course of chronic hepatitis through IFN produced naturally as a result of liver inflammation in young female patients with wt core 70, resulting in accumulation of core mutant viruses as the patient ages. Further prospective observations are necessary to address this issue.

In this study, we found that each of the previously reported predictive factors that we examined also correlated with HCV a.a. substitutions. Interestingly, a.a. substitutions in the virus are associated with metabolic factors such as LDL and high-density lipoprotein cholesterol and fatty liver-related γ-GTP, and in particular, we found that substitution in the core protein (and possibly ISDR) is correlated with LDL cholesterol. The virus appears to influence expression of genes involved

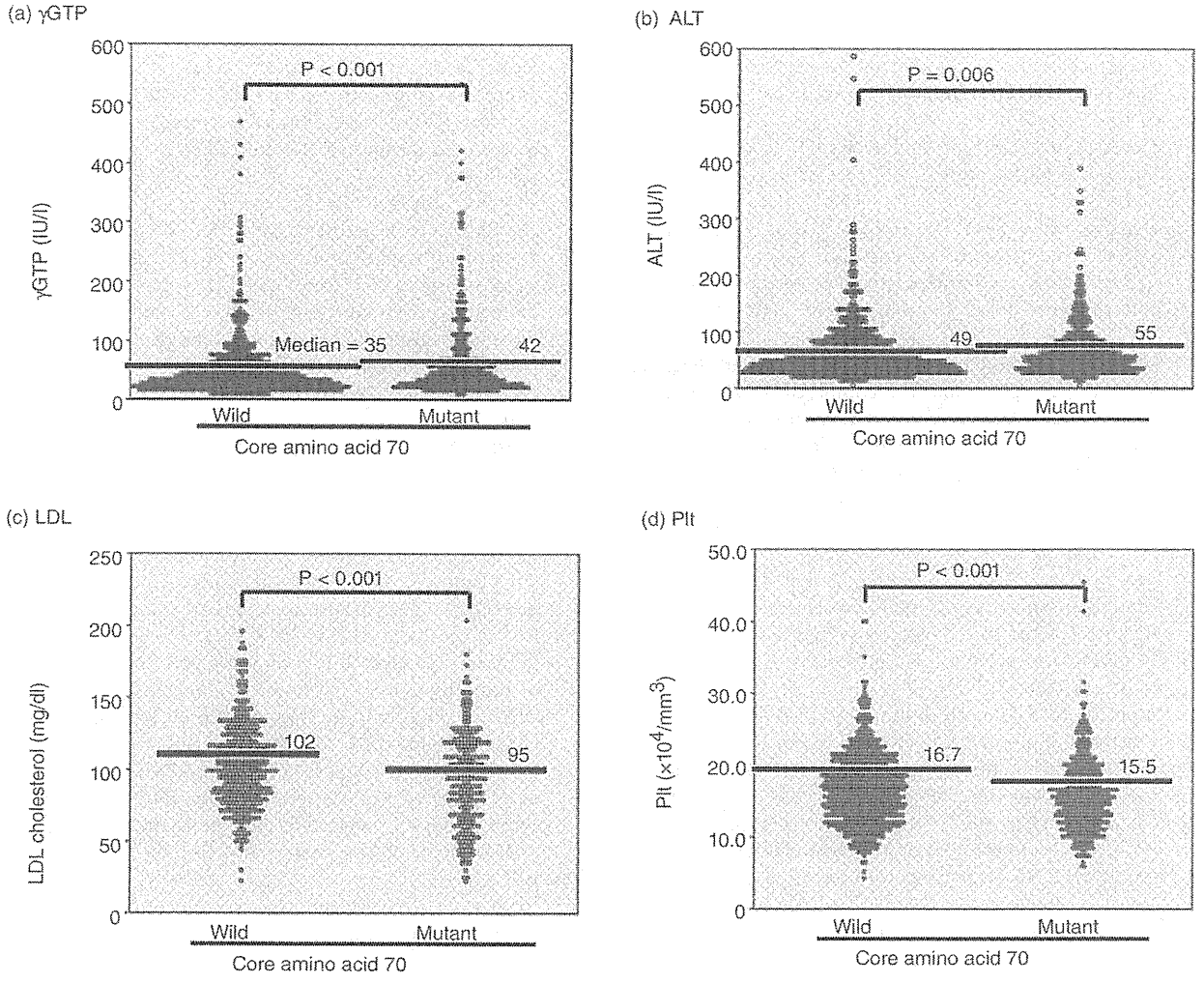


Figure 8 Relationship between blood test findings and core amino acid 70 substitutions. Relationships between core amino acid 70 (wild type or mutant) and gamma-glutamyltranspeptidase (γ-GTP) (a), alanine aminotransferase (ALT) (b), low-density lipoprotein (LDL) cholesterol (c) and platelet count (Plt) (d) were examined. Bars represent the median.

in host cell lipid metabolism to enhance its own replication and secretion.[58] Consequently, metabolic changes induced by infection by different strains of HCV should be investigated further to understand viral mechanisms of IFN resistance and to develop effective personalized therapies.

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