Table 2 Factors correlating with liver stiffness of the patients with extrahepatic cholestasis

	Linear regression analysis		Multiple regression analysis	
	r	*P*	β	*P*
Cause (benign diseases/carcinomas)		$P = 0.0401$*		NS
Sex (female/male)		NS		
Age (year)		NS		
Total bilirubin (mg/dL)	$r = 0.726$	$P < 0.0001$	$\beta = 0.774$	$P = 0.0005$
Direct bilirubin (mg/dL)	$r = 0.728$	$P < 0.0001$†		
AST (IU/L)	$r = -0.481$	$P = 0.0082$		NS
ALT (IU/L)	$r = -0.631$	$P = 0.0002$	$\beta = -0.014$	$P = 0.0138$
ALP (IU/L)		NS		
γ-GTP (IU/L)	$r = -0.334$	$P = 0.0764$		NS
WBC (/μL)		NS		
CRP (mg/dL)		NS		
Diameter of common bile duct (mm)		NS		
R				0.792
Adjusted R^2				0.599
F				21.9
P				$P < 0.0001$

*Mean values of liver stiffness were compared between the patients with benign diseases and those with carcinomas by Student's *t*-test.
†Direct bilirubin levels were not included because of their close correlation with total bilirubin levels.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; γ-GTP, γ-glutamyl transpeptidase; NS, not significant; WBC, white blood cells.

Ultrasound examination

Ultrasound examination was carried out to confirm extrahepatic cholestasis and to measure the diameter of the CBD.

Statistical analysis

Comparison between the patients with carcinomas and those with benign diseases was done by χ^2-test or Student's *t*-test. Factors correlating with LS were estimated by χ^2-test or linear regression analysis. Factors independently correlating with LS were assessed by multiple regression analysis.

RESULTS

LS and laboratory data of extrahepatic cholestasis before biliary drainage

LIVER STIFFNESS WAS higher than the designated normal range of 2.4–5.5 kPa in 25 of 29 patients with extrahepatic cholestasis before biliary drainage, and was 11.4 kPa or higher in 15 patients which is a cut-off value for liver cirrhosis of HCV infection determined in our previous study.[13]

Total and direct bilirubin levels and LS were significantly higher in the patients with carcinomas than those with benign diseases ($P = 0.0008$, =0.0004 and =0.0401, respectively) (Table 1).

Correlation of LS with other laboratory data before biliary drainage

Liver stiffness was positively correlated with total bilirubin levels and direct bilirubin levels ($P < 0.0001$ and <0.0001, respectively), while it was negatively correlated with aspartate aminotransferase (AST) levels and ALT levels ($P = 0.0082$ and =0.0002, respectively) (Table 2 and Fig. 1a,b). Serum bilirubin levels significantly correlated negatively with AST levels ($r = -0.410$, $P = 0.0271$) and with ALT levels ($r = -0.489$, $P = 0.0071$) (Fig. 1c).

Multiple regression analysis for the factors independently affecting LS was done with cause of obstruction, total bilirubin levels, AST levels, ALT levels and γ-glutamyl transpeptidase (γ-GTP) levels. Direct bilirubin levels were not included because of their close correlation with total bilirubin levels. Multiple regression analysis demonstrated that total bilirubin levels and ALT levels independently correlated with LS ($P = 0.0005$ and =0.0138, respectively).

In the patients with benign diseases, LS was positively correlated with total bilirubin levels, direct bilirubin levels and diameters of the CBD ($P = 0.0198$, =0.0068

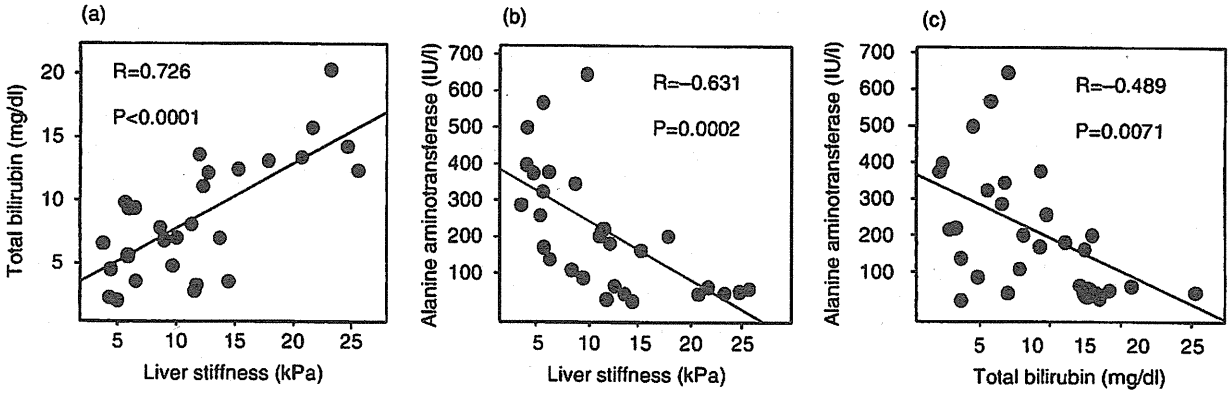

Figure 1 Correlation of liver stiffness with total bilirubin levels and alanine aminotransferase levels before biliary drainage. (a) Liver stiffness was positively correlated with total bilirubin levels ($r = 0.726$, $P < 0.0001$). (b) Liver stiffness was negatively correlated with alanine aminotransferase levels ($r = -0.631$, $P = 0.0002$). (c) Serum bilirubin levels were negatively correlated with alanine aminotransferase levels ($r = -0.489$, $P = 0.0071$).

and =0.0214, respectively), while it was negatively correlated with ALT levels ($P = 0.0153$) (Table 3).

In the patients with carcinomas, LS was positively correlated with total bilirubin levels and direct bilirubin levels ($P = 0.0039$ and =0.0065, respectively), while it was negatively correlated with ALT levels ($P = 0.0065$) (Table 3).

LS after biliary drainage

In 18 patients, LS was measured 5–45 days after biliary drainage by stone extractions in five patients, stent implantations in 11 or PTCD in two. In six of 17 patients whose LS was 5.5 kPa or higher before biliary drainage, LS became lower than 5.5 kPa after biliary drainage. In 10 of 12 patients whose LS was 11.4 kPa or higher before biliary drainage, LS became lower than 11.4 kPa after biliary drainage. LS and laboratory data did not differ significantly between the patients with benign diseases and those with carcinomas after biliary drainage (Table 4).

Decrease of LS after biliary drainage significantly correlated with decrease of total bilirubin levels ($r = 0.524$, $P = 0.0257$) (Table 5).

Table 3 Differences of factors correlating with liver stiffness of the patients with extrahepatic cholestasis between benign diseases and carcinomas

	Benign diseases		Carcinomas	
	r	*P*	*r*	*P*
Sex (female/male)*		NS		NS
Age (year)		NS		NS
Total bilirubin (mg/dL)	$r = 0.559$	$P = 0.0198$	$r = 0.763$	$P = 0.0039$
Direct bilirubin (mg/dL)	$r = 0.629$	$P = 0.0068$	$r = 0.735$	$P = 0.0065$
AST (IU/L)		NS	$r = -0.541$	$P = 0.0695$
ALT (IU/L)	$r = -0.577$	$P = 0.0153$	$r = -0.735$	$P = 0.0065$
ALP (IU/L)	$P = 0.438$	$P = 0.0785$		NS
γ-GTP (IU/L)		NS		NS
WBC (/μL)		NS		NS
CRP (mg/dL)	$r = 0.455$	$P = 0.0666$		NS
Diameter of common bile duct (mm)	$r = 0.569$	$P = 0.0214$	$r = -0.565$	$P = 0.0702$

*Mean values of liver stiffness were compared between female patients and male patients by Student's *t*-test.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; γ-GTP, γ-glutamyl transpeptidase; NS, not significant; WBC, white blood cells.

Table 4 Characteristics of the patients with extrahepatic cholestasis after drainage

	All	Causes of cholestasis		*P*-value in comparison between benign diseases and carcinomas
		Benign diseases	Carcinomas	
Sex (female/male)	9/9	4/6	5/3	NS
Age (year)	72 ± 11	71 ± 13	74 ± 9	NS
Interval between liver stiffness measurements (days)	23.5 ± 12.4	19.5 ± 12.4	28.5 ± 11.2	NS
Total bilirubin (mg/dL)	2.5 ± 1.7	2.3 ± 1.7	2.7 ± 1.9	NS
Direct bilirubin (mg/dL)	1.0 ± 1.1	0.9 ± 1.0	1.1 ± 1.4	NS
AST (IU/L)	52 ± 47	39 ± 35	67 ± 57	NS
ALT (IU/L)	81 ± 72	61 ± 50	107 ± 90	NS
ALP (IU/L)	667 ± 576	642 ± 537	698 ± 659	NS
γ-GTP (IU/L)	277 ± 265	346 ± 320	190 ± 154	NS
WBC (/μL)	6438 ± 2804	6570 ± 2294	6275 ± 3505	NS
CRP (mg/dL)	1.0 ± 1.4	0.7 ± 0.4	1.4 ± 2.0	NS
Liver stiffness (kPa)	7.9 ± 4.0	7.7 ± 5.0	8.3 ± 2.8	NS

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; γ-GTP, γ-glutamyl transpeptidase; NS, not significant; WBC, white blood cells.

Persistent elevation of LS values despite successful biliary drainage

In two of 12 patients whose LS was 11.4 kPa or higher before biliary drainage, LS remained 11.4 kPa or higher after biliary drainage. In a patient with stones in the CBD whose LS was 21.8 kPa before biliary drainage, LS became 20.4 kPa 14 days after biliary drainage and total bilirubin reduced from 15.9 mg/dL to 4.8 mg/dL. Computed tomography showed no clues of the presence of cirrhosis 11 days after biliary drainage. In the other patient with a carcinoma in the lower CBD whose LS was 24.8 kPa before biliary drainage, LS became 12.0 kPa 36 days after biliary drainage and total bilirubin reduced from 14.4 mg/dL to 2.2 mg/dL. When pancreaticoduodenectomy was done 47 days after biliary drainage, the liver showed no appearance indicating cirrhosis. Neither patients had any etiological factors such as infection of HCV or hepatitis B virus, autoimmune hepatitis, excessive alcohol consumption, diabetes mellitus or metabolic syndrome which cause elevation of LS.

Table 5 Factors correlating with the decrease of liver stiffness of the patients with extrahepatic cholestasis

	r	*P*
Interval between liver stiffness measurements (days)		NS
Decrease of total bilirubin (mg/dL)	$r = 0.524$	$P = 0.0257$
Decrease of direct bilirubin (mg/dL)	$r = 0.461$	$P = 0.0543$
Decrease of AST (IU/L)	$r = -0.432$	$P = 0.0734$
Decrease of ALT (IU/L)	$r = -0.464$	$P = 0.0525$
Decrease of ALP (IU/L)		NS
Decrease of γ-GTP (IU/L)		NS
Decrease of WBC (/μL)		NS
Decrease of CRP (mg/dL)		NS

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; γ-GTP, γ-glutamyl transpeptidase; NS, not significant; WBC, white blood cells.

DISCUSSION

THE PRESENT STUDY demonstrated that LS of patients with extrahepatic cholestasis was higher than the designated normal upper range of 5.5 kPa in 25 of 29 patients and was 11.4 kPa or higher in 15 patients which is a cut-off value for liver cirrhosis of HCV infection determined in our previous study.[13] In six of 17 patients whose LS was 5.5 kPa or higher before biliary drainage, LS became lower than 5.5 kPa after biliary drainage. In 10 of 12 patients whose LS was 11.4 kPa or higher, LS became lower than 11.4 kPa after biliary drainage. Thus, extrahepatic cholestasis caused the elevation of LS, which was reversible when biliary drainage was done in a short period. It is suggested that elevation of LS does not necessarily reflect liver fibrosis, although fibrosis in liver histology was not examined in the present study.

Millonig *et al.* reported that the experimental bile duct ligation of pigs led to a swelling of the liver and LS elevated to the values suggesting F3 fibrosis.[12] After removal of the ligation, LS returned to almost normal values. This experiment indicates that bile duct ligation causes elevation of LS which is probably because of the increased hepatic hydrostatic pressure in the main and is reversible when the bile duct is reopened and the hydrostatic pressure is reduced. Millonig *et al.* also reported that LS is directly influenced by central venous pressure.[14]

The present study demonstrated that the elevation of LS significantly correlated positively with total bilirubin levels and negatively with ALT levels. These correlations were also noted when the patients with carcinomas and those with benign diseases were separately analyzed. Decrease of LS after biliary drainage significantly correlated with decrease of total bilirubin levels. This is the first report describing these correlations. Millonig *et al.* reported only the correlation of decrease of LS after biliary drainage with decrease of bilirubin.[12] The positive correlation between bilirubin levels and LS probably indicates that the higher intraductular pressure causes the higher retention of bilirubin and the higher bilirubin levels. In acute hepatitis, the positive correlation of LS with ALT levels[11,15] and bilirubin levels[10,16,17] has been reported. These positive correlations in acute hepatitis may be attributed to the dominance of inflammatory liver injury instead of increased hepatic hydrostatic pressure.

The negative correlation between ALT levels and LS probably indicates the negative correlation between ALT levels and the hepatic hydrostatic pressure. The leakage of bile fluid from the bile canalicular lumen into the lateral intracellular space and the perisinusoidal space causes the damage of hepatocytes and may also reduce the hydrostatic pressure in the bile duct.[18] The time of admission may also contribute to the negative correlation of ALT levels and LS. The fatigue or abdominal discomfort caused by severe hepatic damage causes the presentation of many patients to hospital before the hepatic hydrostatic pressure raises to the high levels and causes retention of bilirubin. Further studies on the mechanism underlying the elevation of LS, bilirubin levels and ALT levels is needed to elucidate the pathogenesis of jaundice and liver injury in extrahepatic cholestasis.

In the present study, LS did not reduce to the normal levels in more than half of the patients after drainage which should normalize the hydrostatic pressure of the liver. Therefore, not only the increased hydrostatic pressure, but the other features due to impaired bile flow also could be related to the elevation of LS in cholestasis. Extrahepatic cholestasis causes inflammatory features including edema, neutrophil infiltration, proliferation of the biliary epithelial cells and fibrosis.[19–21] This inflammation and fibrosis may also contribute to the elevation of LS, and cause the delay of reduction of LS after drainage.

Fibroscan measures LS without B-mode imaging, while other non-invasive methods such as acoustic radiation force impulse (ARFI)[22] and real-time tissue elastography[23] could measure LS with B-mode imaging. The LS measurement by Fibroscan may be affected by dilated intrahepatic bile ducts in extrahepatic cholestasis, while this effect can be avoided by ARFI or real-time tissue elastography. In the present study, only procedures with 10 validated measurements and a success rate of at least 60% (ratio of the number of successful acquisitions over the total number of acquisitions) were considered reliable. The median value was considered representative of the liver elastic modulus. With this protocol, LS was successfully measured in all the 29 patients examined in the present study. No extreme scattering of the 10 values which might be caused by dilated bile ducts was noted. The measurement depth of Fibroscan was between 25 mm and 65 mm under skin. In this measurement depth of the right lobe, there may be no extremely dilated bile duct. It may be the reason why LS was measured in the present study without any difficulty, although Fibroscan has the weak point of measuring LS without B-mode imaging. The study on LS by ARFI in extrahepatic cholestasis is now under way in Fujita Health University Hospital.

In conclusion, the elevation of LS in extrahepatic cholestasis can be mainly attributed to the increased hydrostatic pressure of the liver. The present study demonstrated that the elevation of LS in extrahepatic cholestasis correlates positively with the accumulation of bilirubin but negatively with damage of hepatocytes indicated by ALT levels. Further studies on the mechanism underlying the elevation of LS are needed to elucidate the pathogenesis of jaundice and liver injury in extrahepatic cholestasis.

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J Gastroenterol (2011) 46:1324–1334
DOI 10.1007/s00535-011-0444-4

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Reduction of liver stiffness by antiviral therapy in chronic hepatitis B

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Received: 4 April 2011 / Accepted: 7 July 2011 / Published online: 6 August 2011

Abstract
Background Liver stiffness (LS) has been reported to correlate with fibrosis stage (F). The correlation between LS and fibrosis stage and the reduction of LS by antiviral therapy were examined in patients with hepatitis B infection.
Methods LS was measured by FibroScan in 212 patients infected with hepatitis B virus. Liver biopsies were done in 51 patients. Changes of LS were assessed in 29 patients treated with nucleotide or nucleoside analogs and 52 patients without antiviral therapy.
Results LS was significantly correlated with fibrosis stage ($\rho = 0.686$, $P < 0.0001$). The optimal cut-off values of LS were 7.1 kPa for F $\geq$ 2, 10.7 kPa for F $\geq$ 3, and 16.0 kPa for F4. LS was significantly reduced by antiviral therapy, from 12.9 (range 6.2–17.9) kPa to 6.6 (4.4–10.3) kPa measured at an interval of 512 (range 366–728) days ($P < 0.0001$). Eleven of 19 (58%) patients with baseline fibrosis stages of F3-4 deduced from LS had 2-point or greater reductions of deduced stage at the last LS measurement. The change ratio of hyaluronic acid ($P = 0.0390$) was associated with a 2-point or greater reduction of deduced fibrosis stage. Without antiviral therapy, LS tended to increase, increasing from 6.1 (range 3.9–8.5) kPa to 6.3 (range 4.4–9.7) kPa at an interval of 422 (range 358–709) days ($P = 0.0682$).
Conclusions LS was significantly correlated with fibrosis stage in patients with chronic hepatitis B. The reduction of LS by antiviral therapy was significantly correlated with the reduction of hyaluronic acid. Thus, we conclude that LS can be useful to assess the progression and regression of liver fibrosis stage noninvasively.

Keywords Hepatitis B · Antiviral therapy · Liver stiffness · Transient elastography · Fibrosis stage

Abbreviations

LS	Liver stiffness
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
TE	Transient elastography
kPa	Kilopascals
ROC	Receiver operating characteristics
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
APRI	Aminotransferase-to-platelet ratio index

Introduction

Chronic infection with hepatitis B virus (HBV) currently affects about 400 million people, with particularly high prevalence in developing countries, and it is estimated that worldwide more than 200,000 and more than 300,000

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chronic HBV carriers die each year from cirrhosis and hepatocellular carcinoma (HCC), respectively. The most important predictors of cirrhosis or HCC in patients with chronic hepatitis B are persistently elevated HBV DNA and alanine aminotransferase (ALT) levels [1, 2]. It has been estimated that up to 60% of HBV-related HCC occurs in patients with cirrhosis, while almost all HCV-related HCC occurs in the setting of cirrhosis [3]. Thus, the diagnosis of fibrosis stages is important not only for assessing prognosis and the need for antiviral therapy but also for identifying patients with cirrhosis who are likely to develop HCC.

Liver biopsy is currently considered the gold standard for assessing fibrosis stage in chronic liver disease. However, it is an invasive procedure, with rare but potentially life-threatening complications. In addition, the accuracy of liver biopsy in assessing fibrosis has limitations because of sampling errors and interobserver variability [4–6].

Over the past several years, many researchers have shown an interest in the estimation of liver fibrosis in liver diseases by transient elastography. Transient elastography is a rapid, noninvasive, and reproducible method for measuring liver stiffness (LS). The LS measurement can be performed in about 95% of patients but is problematic in those with ascites or a body mass index above 28 kg/m^2. However the intra- and interoperator reproducibility of LS measurement has been proven by a previous study [7].

LS has been reported to correlate with the stage of liver fibrosis in various liver diseases [8–20]. In addition, serial measurements of LS were shown to be useful to follow up patients with liver disease [8, 9]. However, there have been only a few studies of LS in patients with HBV infection [21–25].

In the present study, we evaluated the correlation of LS with biological parameters in 212 patients with chronic hepatitis B, and we evaluated the correlation of LS with the fibrosis stage in 51 patients. In addition, we evaluated changes in LS in 81 patients, 29 of whom received antiviral therapy.

Patients and methods

Patients

Two hundred and twelve patients with chronic HBV infection diagnosed consecutively at Fujita Health University Hospital from November 2005 to December 2009 were examined for LS. We evaluated the correlation of LS with biological, serological, and virological parameters in these 212 patients who had not received antiviral therapy. Liver biopsy was performed in 51 patients. In 81 patients, LS was measured more than twice. Twenty nine of the 81 patients were subsequently treated with nucleotide or nucleoside analogs and 52 patients did not receive antiviral therapy (Fig. 1). Eight patients were treated with lamivudine and 21 with entecavir (Table 1).

The biochemical, serological, and virological examinations were performed within 2 days of the LS measurements. Aminotransferase-to-platelet ratio index (APRI) values were calculated using the formula: aspartate aminotransferase (AST) [IU/L]/platelets [10^9/L] × 100 [26]. FIB-4 values were calculated using the formula: age (years) × AST [IU/L]/(platelets [10^9/L] × (ALT [IU/L])$^{1/2}$) [27].

According to the guidelines for the treatment of chronic hepatitis and cirrhosis due to HBV infection, patients younger than 35 years did not receive antiviral therapy, except for those who were hepatitis B e antigen (HBeAg)-negative with a platelet count of less than 150 × 10^3/μL or fibrosis stage F2 or higher. Patients aged 35 years or older with ALT of ≥ 31 IU/L received antiviral therapy [28].

Patients were recommended to have a liver biopsy before making the decision about starting antiviral therapy,

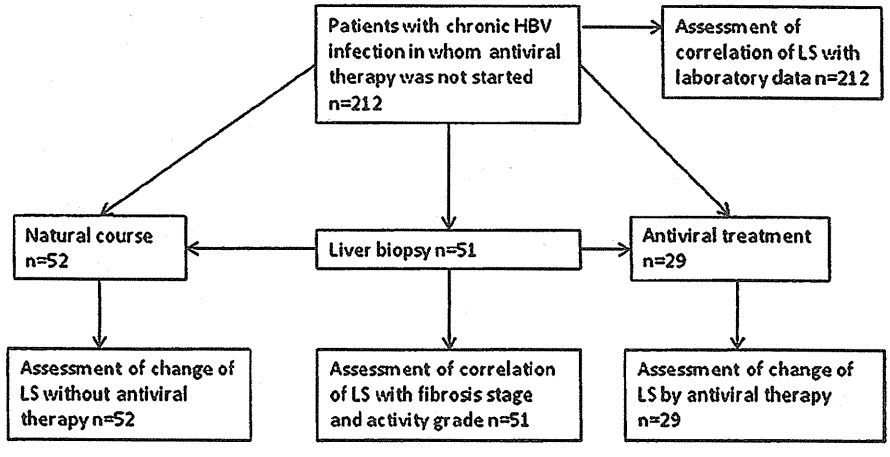

Fig. 1 Flow chart of the study patients. *HBV* hepatitis B virus, *LS* liver stiffness

Table 1 Clinical and biological characteristics of the patients

	Patients for baseline study of correlation of liver stiffness and laboratory data	Subjects of follow-up study		*P* value
		Antiviral therapy	Without treatment	
Number of patients	212	29	52	
Age (years)	51 (39–60)	49 (41–62)	45 (31–59)	0.0438
Gender (female/male)	78/134	11/18	20/32	NS
AST (IU/L)	29.0 (21.0–57.0)	51.0 (39.0–93.5)	28.0 (21.0–56.8)	0.0011
ALT (IU/L)	34.0 (20.3–80.0)	66.0 (33.5–116.0)	39.5 (19.3–84.5)	0.0294
Total bilirubin (mg/dL)	0.9 (0.7–1.2)	1.0 (0.7–1.7)	1.0 (0.8–1.2)	NS
Total protein (g/dL)	7.5 (7.1–7.9)	7.4 (7.2–7.9)	7.6 (7.3–8.0)	NS
Albumin (g/dL)	4.4 (4.0–4.6)	4.3 (3.8–4.5)	4.4 (4.1–4.6)	NS
γ-Globulin (%)	17.7 (15.2–20.9)	21.4 (15.3–26.8)	16.9 (14.0–19.3)	NS
Platelet count ($\times 10^4/\mu L$)	17.6 (13.5–20.8)	12.2 (9.1–17.0)	18.8 (14.3–24.1)	<0.0001
Prothrombin time (%)	92 (81–100)	86 (77–97)	89 (82–98)	NS
Hyaluronic acid (ng/mL)	33.0 (20.5–98.0)	133.0 (34.3–170.8)	29.0 (21.0–83.0)	0.0029
APRI	0.54 (0.33–1.09)	1.16 (0.68–2.31)	0.45 (0.34–0.95)	<0.0001
FIB-4	1.57 (1.00–2.43)	2.48 (1.43–5.25)	1.35 (0.80–1.80)	<0.0001
HBeAg (+/−)	69/109	17/10	24/19	NS
HBV DNA (log copy/mL)	4.6 (3.0–7.0)	6.7 (4.1–7.2)	4.5 (3.1–7.6)	NS
HBV genotype (A/B/C/F)	2/9/142/1	0/1/20/0	0/1/28/0	NS
Antiviral therapy (LAM/ETV)	8/21	8/21	–	–
Liver stiffness (kPa)	6.1 (4.4–9.5)	12.9 (6.2–17.9)	6.1 (3.9–8.5)	<0.0001

Values are medians (interquartile ranges)

P value of Mann–Whitney *U*-test between patients at the beginning of antiviral therapy and those without treatment

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *LAM* lamivudine, *ETV* entecavir, *APRI* aminotransferase-to-platelet ratio index, *NS* not significant, *HBeAg* hepatitis B e antigen

Differences in proportions of patients according to gender, HBe Ag, and hepatitis B virus (HBV) genotype were assessed by χ^2 test between patients at the beginning of antiviral therapy and those without treatment

to assess the diagnosis and prognosis of hepatitis and to confirm the necessity for antiviral therapy.

The study was performed in accordance with the principles of good clinical practice, the principles of the Declaration of Helsinki and its appendices, and local and national laws. Approval for the present study was obtained from the review board of Fujita Health University.

Liver stiffness measurement

LS measurement by transient elastography was performed with a FibroScan® (EchoSens, Paris, France).The FibroScan® is equipped with a probe including an ultrasonic transducer and a vibrator. A vibration of mild amplitude and low frequency is transmitted from the vibrator placed on the body surface toward the liver through the intercostal space. The vibration induces an elastic shear wave that propagates through the liver tissue. Then pulse-echo ultrasound acquisitions follow the propagation of the shear wave and determine its velocity. The velocity is directly related to tissue stiffness; the harder the tissue, the faster the shear wave propagates. LS is calculated from the velocity and is expressed in kilopascals (kPa). LS measurement was performed after an overnight fast. Ten successful acquisitions were performed for each measurement, and the median value was adopted as representative of LS. LS was measured within a month of liver biopsy.

The procedures were performed by two investigators (T.N. and H.S.) who were blind to the clinical, serological, and histological data. These two investigators have 15 and 10 years' experience, respectively, in ultrasound diagnosis and had carried out more than 30 LS measurements before the present study. The agreement rate for LS values by the two investigators was assessed in 10 patients; as well, LS values measured on two different occasions by the same investigator were assessed in these 10 patients. LS values measured by the two investigators did not significantly differ and the coefficient of variation was 5.3% (range 2 to 9%). LS values measured on two different occasions by the same investigator did not significantly differ and the coefficient of variation was 4.6% (range 2 to 7%).

In our follow-up study, LS was measured at pretreatment or the beginning of the study, and at 1 year (range 7 to 18 months), 2 years (range 19 to 30 months), and 3 years after the beginning of the treatment or the study (range 31 to 42 months).

Liver biopsy

Liver biopsies were done in 51 patients before antiviral treatment was initiated or before the start of the study. Liver biopsy was performed using a 14G disposable Tru-cut needle (Tru-Core Biopsy Instrument, Medical Device Technologies, Gainesville, FL) under ultrasonographic guidance.

Liver specimens of at least 1.5-cm length with more than 8 portal tracts were assessed. All biopsy specimens were analyzed by two hepatologists (K.Y. and N.K.) of 30 and 15 years' experience, respectively. There were 5 and 4 specimens where the stage and grade, respectively, evaluated by the 2 hepatologists differed, and the higher stage and grade were adopted. Fibrosis was staged as: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Inflammatory activity was graded as: A0, none; A1, mild; A2, moderate; and A3, severe activity.

Statistical analysis

The correlation between LS and serum biological parameters and fibrosis stages was estimated by the Spearman's rank correlation test. The χ^2 test was used for categorical variables and Fisher's test was used where appropriate. Differences of LS between fibrosis stages were estimated by the Tukey–Kramer test.

The significance of changes in LS values at two points were compared by the Wilcoxon signed-rank test. Analyses of unpaired data were evaluated by the Mann–Whitney *U*-test.

Data values are expressed as medians and interquartile ranges.

The change ratio of a value was calculated by the formula below:

(Value after treatment)/(pretreatment value) × 100 − 100.

The change ratio of a value in patients without antiviral therapy was calculated by the formula below:

(Value at end of observation)/(baseline value) × 100 − 100.

The diagnostic performance of LS was determined in terms of sensitivity, specificity, positive and negative predictive values, diagnostic accuracy, and area under receiver operating characteristic curve (AUROC). Optimal cut-off values for fibrosis stages were determined at the maximum total of sensitivity and specificity.

Statistical analysis was performed with JMP® (SAS Institute, Cary, NC, USA).

Results

Correlation of liver stiffness with biological parameters

LS values were significantly correlated with AST ($\rho = 0.536$, $P < 0.0001$), ALT ($\rho = 0.493$, $P < 0.0001$), total bilirubin ($\rho = 0.248$, $P < 0.0001$), albumin ($\rho = -0.413$, $P < 0.0001$), γ-globulin ($\rho = 0.466$, $P < 0.0001$), platelet count ($\rho = -0.381$, $P < 0.0001$), prothrombin time ($\rho = -0.540$, $P < 0.0001$), HBV DNA ($\rho = 0.315$, $P < 0.0001$), hyaluronic acid ($\rho = 0.578$, $P < 0.0001$), APRI ($\rho = 0.601$, $P < 0.0001$), and FIB-4 ($\rho = 0.390$, $P < 0.0001$) (Table 2). Male gender and HBeAg positivity were associated with a higher LS ($P = 0.0041$ and $P = 0.0003$).

AST ($P = 0.0334$), prothrombin time ($P = 0.0210$), and hyaluronic acid ($P = 0.0381$) were selected as factors independently associated with LS by multiple regression analysis (Table 2).

Liver stiffness and fibrosis stage in the liver biopsy specimens

The liver biopsies of 51 patients were assessed by the METAVIR system. Fibrosis stage was F0 in 2 patients, F1 in 4, F2 in 18, F3 in 13, and F4 in 14. LS was 5.8 (4.9–6.4) kPa for F0-1, 7.0 (5.5–9.5) kPa for F2, 9.5 (6.7–12.5) kPa for F3, and 17.5 (15.3–19.7) kPa for F4 (Fig. 2a). LS significantly differed between F0-1 and F4 ($P < 0.0001$), between F2 and F4 ($P < 0.0001$), and between F3 and F4 ($P = 0.0001$). LS was significantly correlated with fibrosis stage ($\rho = 0.689$, $P < 0.0001$)

The inflammatory grade was A0 in 2 patients, A1 in 12, A2 in 28, and A3 in 9. LS was 7.0 (5.3–10.1) kPa for A0-1, 9.5 (6.2–16.4) kPa for A2, and 14.5 (8.8–17.7) kPa for A3 (Fig. 2b). LS significantly differed between A0-1 and A3 ($P = 0.033$). LS was significantly correlated with the inflammatory grade ($\rho = 0.359$, $P = 0.0098$).

ROC analysis was done to assess the diagnostic value of LS for different fibrosis stages. AUROC values were 0.844 for F ≥ 2, 0.839 for F ≥ 3, and 0.930 for F4. Based on the ROC curves, the optimal discriminant cut-off values were determined at the maximum total of sensitivity and specificity. The cut-off values were 7.1 kPa for F ≥ 2, 10.7 kPa for F ≥ 3, and 16.0 kPa for F4 (Table 3).

Table 2 Factors correlated with liver stiffness in all patients as assessed by Spearman's rank correlation test and multiple regression analysis

	Spearman's rank correlation test		Multiple regression analysis	
	ρ	P value	β	P value
Age (years)	−0.005	NS	–	–
Gender[a]		0.0041	–	–
Male	6.6 (4.7–12.0)			
Female	5.4 (4.0–6.9)			
AST (IU/L)	0.536	<0.0001	−0.856	0.0334
ALT (IU/L)	0.493	<0.0001	0.407	NS
Total bilirubin (mg/dL)	0.248	<0.0001	−0.106	NS
Total protein (g/dL)	−0.096	NS	–	–
Albumin (g/dL)	−0.413	<0.0001	−0.005	NS
γ-Globulin (%)	0.466	<0.0001	0.025	NS
Platelet count ($\times 10^4/\mu L$)	−0.381	<0.0001	0.011	NS
Prothrombin time (%)	−0.540	<0.0001	−0.271	0.0210
HBeAg (+ vs. −)*		0.0003	–	–
Positive	7.2 (5.8–13.7)			
Negative	5.6 (4.1–8.1)			
HBV DNA (log copy/mL)	0.315	<0.0001	0.037	NS
Hyaluronic acid (ng/mL)	0.578	<0.0001	0.299	0.0381
APRI	0.601	<0.0001	0.578	NS
FIB-4	0.390	<0.0001	0.331	NS

Values are medians (interquartile ranges)

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *APRI* aminotransferase-to-platelet ratio index, *NS* not significant

[a] Mann–Whitney *U*-test

Changes in liver stiffness and biochemical and serological parameters

In the patients with antiviral therapy, LS values at pretreatment and at 1, 2, and 3 years after the beginning of treatment were 12.9 (6.2–17.9) kPa, 7.5 (5.4–11.7) kPa, 6.5 (5.1–10.6) kPa, and 4.7 (3.1–7.9) kPa, respectively.

LS was significantly decreased at 1 year ($P < 0.0001$), 2 years ($P = 0.0001$), and 3 years after the beginning of treatment ($P = 0.0060$) compared with LS at pretreatment. LS was significantly decreased at 2 years ($P = 0.0210$) compared with that at 1 year after the beginning of treatment (Fig. 3a).

LS was 12.9 (6.2–17.9) kPa at pretreatment and was significantly reduced, to 6.6 (4.4–10.3) kPa at the last measurement ($P < 0.0001$). The change ratio of LS was −37.5 (−57.0 to −19.0) %. The interval between the pretreatment measurement and the last measurement of LS was 512 (366–728) days.

In the patients without antiviral treatment, LS values at the 1st measurement and at 1, 2, and 3 years after the beginning of the study were 6.1 (3.9–8.5) kPa, 5.4 (3.7–8.7) kPa, 6.3 (5.2–9.8) kPa, and 7.6 (4.1–11.4) kPa, respectively (Fig. 3b). No significant difference was observed between any of these values. In these patients, LS tended to increase, increasing from 6.1 (3.9–8.5) kPa at the 1st measurement to 6.3 (4.4–9.7) kPa at the last measurement ($P = 0.0682$). The change ratio of LS was 8.7 (−16.6 to 43.7) %. The interval between the 1st measurement and the last measurement was 422 (358–709) days.

The change ratio of LS in the patients with antiviral therapy was significantly higher than that in the patients without antiviral therapy ($P < 0.0001$). The intervals between the 1st measurement and the last measurement did not differ significantly between these two patient groups ($P = 0.5721$).

AST levels were significantly lower at 1 year, at 2 years, and at 3 years after the beginning of treatment than at pretreatment ($P = 0.0002$, $P = 0.0039$, and $P = 0.0313$) (Table 4). The AST level was significantly lower at 2 years after the beginning of treatment than at 1 year after the beginning of treatment ($P = 0.0024$). ALT levels were significantly lower at 1 year, at 2 years, and at 3 years after the beginning of treatment than at pretreatment ($P = 0.0001$, $P = 0.0078$, and $P = 0.0313$). The ALT level was significantly lower at 2 years after the beginning of treatment than at 1 year after the beginning of treatment ($P = 0.0081$). The platelet count was significantly higher at

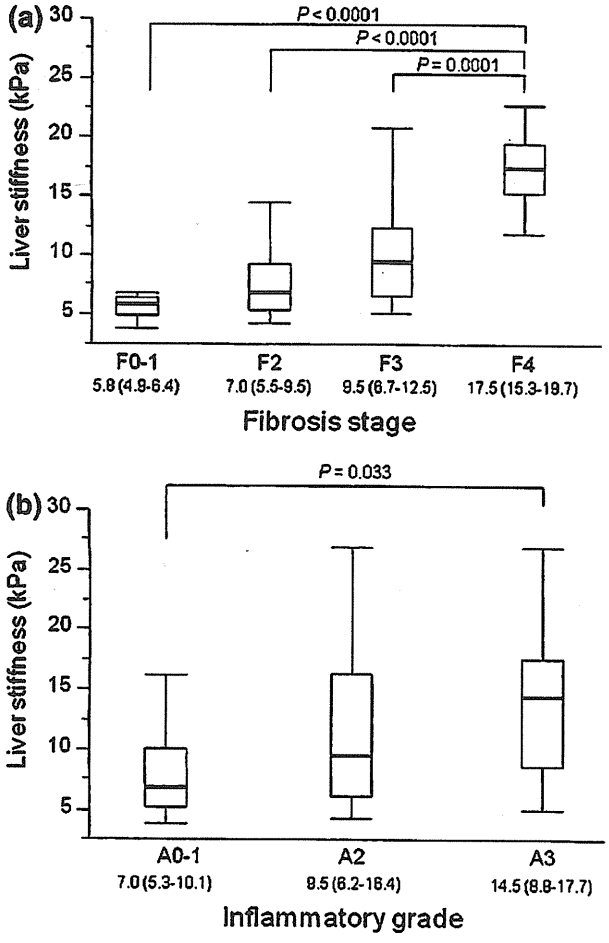

Fig. 2 Liver stiffness (LS) was significantly correlated with fibrosis stage (a) and inflammatory grade (b). LS significantly differed between F0–1 and F4 ($P < 0.0001$); between F2 and F4 ($P < 0.0001$); and between F3 and F4 ($P = 0.0001$). LS was significantly correlated with fibrosis stage ($\rho = 0.703$, $P < 0.0001$). Inflammatory grade significantly differed between A0–1 and A3 ($P = 0.042$). LS was significantly correlated with inflammatory grade ($\rho = 0.359$, $P = 0.0121$). *Bold lines* are medians; *tops and bottoms of the boxes* are the 25th and 75th percentiles; *horizontal lines* at the ends of the *vertical lines* are the minimum and maximum values observed. *Figures* on the horizontal axis are medians (interquartile range)

Table 3 Cut-off values of liver stiffness for each fibrosis stage (ROC analysis)

	F ≥ 2	F ≥ 3	F = 4
Cut-off value (kPa)	7.1	10.7	16.0
Positive predictive value (%)	100	86.4	91.7
Negative predictive value (%)	33.3	72.4	92.3
Sensitivity (%)	73.3	70.4	78.6
Specificity (%)	100	87.5	97.3
Diagnostic accuracy (%)	76.5	78.4	92.2
Area under ROC curve value	0.844	0.839	0.930

ROC receiver operating characteristics

2 years after the beginning of treatment than at 1 year after the beginning of treatment ($P = 0.0410$). HBV DNA levels were significantly lower at 1 year and at 2 years after the beginning of treatment than at pretreatment ($P < 0.0001$ and $P = 0.0039$).

Changes in fibrosis stages deduced from LS according to cut-off values for fibrosis stages

Fibrosis stages were deduced from LS according to the cut-off LS values for each fibrosis stage. The deduced fibrosis stages of the 1st (pretreatment) and last measurements were compared (Fig. 4).

Seventeen of 21 (81%) patients with deduced fibrosis stages of F2-4 at the 1st measurement had a reduction of deduced stage at the last measurement by antiviral therapy, while none had an increase of the deduced stage. Eleven of 19 (58%) patients with deduced fibrosis stages of F3-4 at the 1st measurement had a 2-point or greater reduction of deduced stage at the last measurement.

The factors associated with a 2-point or greater reduction of deduced fibrosis stage were examined in 19 patients with pretreatment deduced fibrosis stage of F3-4. The change ratio of hyaluronic acid significantly differed between the patients with a 2-point or greater reduction of deduced fibrosis stage and those without such a reduction ($P = 0.0390$) (Table 5).

In patients without antiviral therapy, 11 of 50 (22%) patients with deduced fibrosis stages of F0-3 at the 1st measurement had an increase of the deduced stage, while 8 of 20 (40%) patients with deduced fibrosis stages of F2-4 at the 1st measurement had a reduction of the deduced stage. The factors associated with a 1-point or greater increase in the deduced fibrosis stage were examined in 50 patients with a deduced fibrosis stage of F0-3 at the 1st measurement. Lower baseline albumin levels were associated with a 1-point or greater increase of deduced fibrosis stage ($P = 0.0092$) (Table 6).

Discussion

In the present study, LS was shown to be correlated with fibrosis stage in patients with HBV infection, as has been previously reported. Optimal LS cut-off values with reasonably high sensitivity and specificity were determined to be 7.1 kPa for F ≥ 2, 10.7 kPa for F ≥ 3, and 16.0 kPa for F4, although the negative predictive value for F ≥ 2 was low. In patients with HBV infection, optimal cut-off values of LS have been reported to be 7.2–8.1 kPa for F 2 ≥ and 10.3–13.4 kPa for F4 [21–24]. The cut-off values

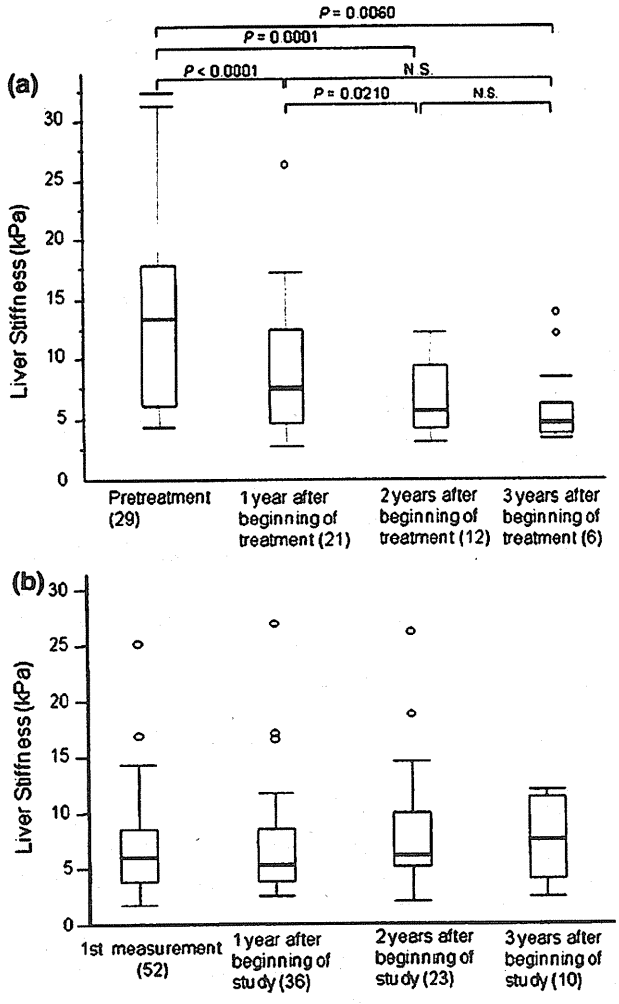

Fig. 3 Changes of liver stiffness (LS) in 29 patients with antiviral therapy (a) and 52 patients without antiviral therapy (b). a In the patients with antiviral therapy, LS values at pretreatment and at 1, 2, and 3 years after the beginning of treatment were 12.9 (6.2–17.9) kPa, 7.5 (5.4–11.7) kPa, 6.5 (5.1–10.6) kPa, and 4.7 (3.1–7.9) kPa, respectively. LS was significantly decreased at 1 year ($P < 0.0001$), 2 years ($P = 0.0001$), and 3 years after the beginning of treatment ($P = 0.0060$) compared with pretreatment values. In addition, LS was significantly decreased at 2 years ($P = 0.0210$) compared with 1 year after the beginning of treatment. b In patients without antiviral therapy, LS values of at the 1st measurement and at 1, 2, and 3 years after the beginning of the study were 6.1 (3.9–8.5) kPa, 5.4 (3.7–8.7) kPa, 6.3 (5.2–9.8) kPa, and 7.6 (4.1–11.4) kPa, respectively. No significant difference was observed between any of these values. *Bold lines* are medians; *tops and bottoms of the boxes* are the 25th and 75th percentiles; and *horizontal lines* at the ends of the *vertical lines* are the minimum and maximum values observed

determined in the present study were slightly lower for $F \geq 2$ and slightly higher for F4 compared with those reported previously. Because the number of patients examined in the present study was small, larger studies are needed to confirm the optimal cut-off values of LS for correlating with fibrosis stage in patients with chronic hepatitis B.

The present study showed that LS was correlated with several biological and virological parameters in patients with HBV infection; these parameters included gender, AST, ALT, γ-globulin, total bilirubin, albumin, platelet count, prothrombin time, hyaluronic acid, APRI, FIB-4, HBeAg positivity, and HBV DNA. Mutivariate analysis demonstrated that AST levels, prothrombin time, and hyaluronic acid were independently correlated with LS. Prothrombin time is associated with liver fibrosis and hyaluronic acid is a marker of liver fibrosis. Thus, it is suggested that LS is associated with liver fibrosis. Oliveri et al. [21] reported that, in a multivariate analysis of 171 chronic HBV carriers, fibrosis stage, activity of HBV infection, ALT, and HBV DNA were independently associated with LS. In the present study, the association of LS with AST levels was noted, and this finding indicates the association of LS with inflammatory activity. However, multivariate analysis did not show an association of LS with ALT levels or HBV DNA. The difference between our results and the findings of Oliveri et al. [21] may be attributed to a difference in the population studied, because patients with chronic HBV infection show a variety of pathological states, such as healthy carriers with a high viral load, people with chronic hepatitis, and healthy carriers with a low viral load. Further studies are needed to elucidate the association of LS with ALT and HBV DNA.

The present study showed that antiviral treatment reduced LS. LS was reduced at 1, 2, and 3 years after the beginning of antiviral treatment compared with the pretreatment values. In addition, it was reduced at 2 years after the beginning of antiviral treatment compared with the values at 1 year after the beginning of treatment. Enomoto et al. [25] reported that LS was significantly decreased in patients with chronic hepatitis B by 1 year of therapy with entecavir. The present results indicate that LS continues to reduce from 1 year after the beginning of treatment.

Attenuation of liver fibrosis has been reported in 35–38% of patients with chronic hepatitis B with lamivudine and in 36–39% with entecavir after 1 year of treatment [29, 30]. Attenuation of necroinflammatory activity was also noted in 61–62% of patients with chronic hepatitis B with lamivudine and in 70–72% with entecavir [31, 32]. Long-term treatment (more than 3 years) with entecavir was reported to attenuate necroinflammatory activity in all the patients and to attenuate liver fibrosis in 57–100% of the patients [31, 32]. In the present study, 17 of 21 (81%) patients with deduced fibrosis stages of F2-4 had a reduction of the deduced stage by antiviral therapy after 1.5 years, while none had an increase of the deduced stage. Eleven of 19 (58%) patients with deduced fibrosis stages of F3-4 had a 2-point or greater reduction of the deduced stage. The proportion of patients with a reduction of

Table 4 Changes in biochemical and serological parameters during antiviral therapy

	Pretreatment	1 year after beginning of treatment	2 years after beginning of treatment	3 years after beginning of treatment
AST (IU/L)	50.0 (38.5–77.0)[a]	24.5 (19.8–36.3)[a]	21.0 (18.0–26.0)[a]	20.0 (18.0–25.0)[a]
ALT (IU/L)	62.0 (33.0–109.0)[b]	23.0 (14.0–30.8)[b]	18.0 (13.0–20.0)[b]	18.0 (14.0–26.0)[b]
Platelet count ($\times 10^4/\mu L$)	12.2 (9.1–17.0)	12.3 (10.1–17.8)[c]	13.5 (10.4–17.0)[c]	12.6 (10.0–20.9)
HBV DNA (log copy/mL)	6.7 (4.1–7.2)[d]	2.6[d]	2.6[d]	2.6 (2.6–2.9)

Values are medians (interquartile ranges)

AST aspartate aminotransferase, *ALT* alanine aminotransferase

[a] AST levels were significantly lower at 1 year, at 2 years, and at 3 years after the beginning of treatment than at pretreatment ($P = 0.0002$, $P = 0.0039$, and $P = 0.0313$). AST level was significantly lower at 2 years after the beginning of treatment than at 1 year after the beginning of treatment, ($P = 0.0024$)

[b] ALT levels were significantly lower at 1 year, at 2 years, and at 3 years after the beginning of treatment than at pretreatment ($P = 0.0001$, $P = 0.0078$ and $P = 0.0313$). ALT level was significantly lower at 2 years after the beginning of treatment than at 1 year after the beginning of treatment, ($P = 0.0081$)

[c] Platelet count was significantly higher at 2 years after the beginning of treatment than at 1 year after the beginning of treatment ($P = 0.0410$)

[d] HBV DNA levels were significantly lower at 1 year and at 2 years after the beginning of treatment than at pretreatment ($P < 0.0001$ and $P = 0.0039$)

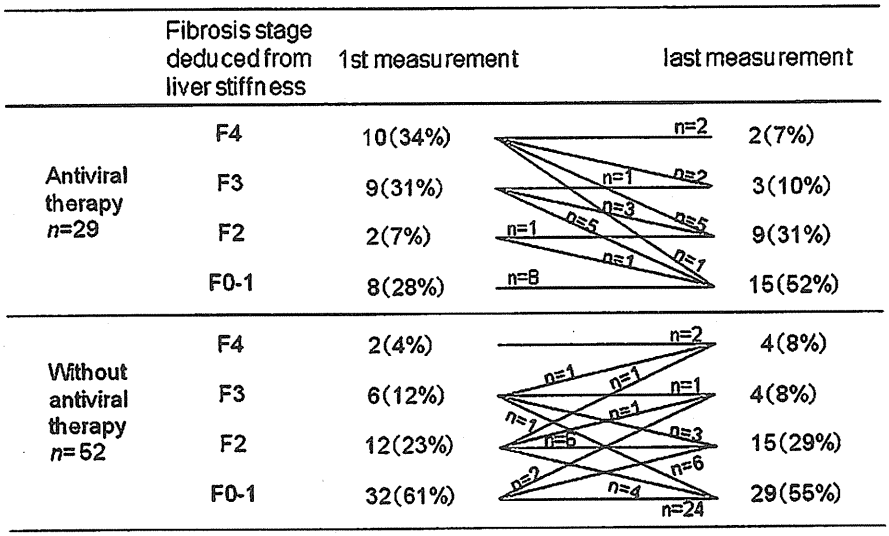

Fig. 4 Changes of fibrosis stages deduced from liver stiffness (LS) according to cut-off values for fibrosis stages. Seventeen of 21 (81%) patients with deduced fibrosis stages of F2-4 at the 1st measurement had a reduction of deduced stage by antiviral therapy, while none of these patients had an increase of the deduced stage at the last LS measurement. Eleven of 19 (58%) patients with deduced fibrosis stages of F3-4 at the 1st measurement had a 2-point or greater reduction of deduced stage at the last LS measurement. The interval between the pretreatment measurement and the last measurement of LS was 512 (range 366–728) days. In patients without antiviral therapy, 11 of 50 (22%) patients with deduced fibrosis stages of F0-3 at the 1st LS measurement had an increase of the deduced stage, while 8 of 20 (40%) patients with deduced fibrosis stages of F2-4 at the 1st measurement had a reduction of the deduced stage. The interval between the 1st LS measurement and the last measurement was 422 (range 358–709) days

deduced fibrosis stage in the present study is similar to that in previous reports based on biopsy. Thus, it seems that LS measurement is useful to monitor the regression of fibrosis by antiviral treatment.

We found that a 2-point or greater reduction of deduced fibrosis stage was significantly associated with a reduction of hyaluronic acid. In the present study, no liver biopsy was done after antiviral therapy. A reduction of deduced fibrosis stage can be attributed not only to reduction of fibrosis but also to a reduction of necroinflammatory activity. However, the finding that the reduction of deduced fibrosis stage was correlated with a reduction of hyaluronic acid but not with a reduction of ALT levels indicates that reduction of the deduced fibrosis stage can be attributed to

Table 5 Factors correlated with reduction of deduced fibrosis stage by antiviral therapy

	2-point or greater reduction of deduced fibrosis stage	1-point reduction or no change of deduced fibrosis stage	*P* value
Number of patients	11	8	
Age (years)	55 (43 to 62)	49 (42 to 61)	NS
Gender (female/male)	3/8	4/4	NS
AST (IU/L)	71.0 (41.0 to 218.0)	51.5 (41.5 to 115.0)	NS
ALT (IU/L)	109.0 (53.0 to 332.0)	63.5 (35.0 to 109.5)	NS
Total bilirubin (mg/dL)	1.0 (0.7 to 1.4)	1.2 (0.8 to 1.7)	NS
Total protein (g/dL)	7.4 (7.1 to 7.8)	7.4 (6.9 to 8.0)	NS
Albumin (g/dL)	3.9 (3.7 to 4.3)	4.3 (3.6 to 4.5)	NS
Platelet count ($\times 10^4/\mu L$)	10.8 (8.5 to 13.8)	9.8 (7.4 to 15.7)	NS
Prothrombin time (%)	82 (79 to 86)	76 (74 to 100)	NS
Hyaluronic acid (ng/mL)	135.0 (84.5 to 162.3)	161.5 (57.3 to 362.0)	NS
HBeAg (+/−)	7/3	3/5	NS
Seroconversion HBeAg (+ → −)	3/7	0/3	NS
HBV DNA (log copy/mL)	6.8 (5.6 to 7.4)	6.8 (5.0 to 7.5)	NS
Antiviral therapy (LAM/LAM + ADV/ETV)	3/1/7	1/7	NS
Liver stiffness (kPa)	16.3 (11.8 to 17.9)	17.4 (13.8 to 25.4)	NS
Change ratio of AST (%)	−63.4 (−89.9 to −31.7)	−47.6 (−75.0 to −32.9)	NS
Change ratio of ALT (%)	−79.9 (−94.4 to −47.5)	−51.0 (−83.9 to −29.3)	NS
Change ratio of total bilirubin (%)	5.0 (−35.7 to 21.4)	1.4 (−30.6 to 57.1)	NS
Change ratio of total protein (%)	0.6 (−4.2 to 3.3)	−1.5 (−6.5 to 5.4)	NS
Change ratio of albumin (%)	13.0 (8.3 to 15.0)	5.8 (−1.2 to 15.7)	NS
Change ratio of platelet count (%)	10.1 (8.2 to 26.1)	9.5 (−1.9 to 17.9)	NS
Change ratio of prothrombin time (%)	8.8 (5.3 to 22.1)	4.0 (0.0 to 11.5)	NS
Change ratio of hyaluronic acid (%)	−72.2 (−84.6 to −28.8)	−27.7 (−59.4 to 23.3)	0.0390
Change ratio of HBV DNA ratio (%)	−61.5 (−64.5 to −21.4)	−61.8 (−65.2 to −47.9)	NS
Change ratio of liver stiffness (%)	−58.8 (−70.3 to −47.8)	−30.9 (−43.4 to −19.8)	0.0034
Interval between 1st and last liver stiffness measurement (days)	698 (615 to 1120)	431 (227 to 677)	NS

Values are medians (interquartile ranges)

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *LAM* lamivudine, *ADV* adefovir dipivoxil, *ETV* entecavir, *NS* not significant

Differences in the proportions of patients according to gender, HBe-Ag, seroconversion of HBeAg, and antiviral therapy were assessed by the χ^2 test between the patients with a 2-point or greater reduction of deduced fibrosis stage and those without it

a reduction of fibrosis but not to attenuation of necroinflammatory activity, because hyaluronic acid has been considered to correlate with liver fibrosis [33]. Our previous study demonstrated, in patients with chronic hepatitis C, that interferon (IFN) treatment significantly reduced LS in patients with a sustained virological response (SVR) and in relapsers [8].

In the present study, in patients without antiviral therapy, a 1-point or greater increase of deduced fibrosis stage was observed in 11 patients, a 1-point or greater reduction was seen in 8, and 27 patients showed no change. A 1-point or greater increase of deduced fibrosis stage was significantly associated with lower baseline albumin levels. This may suggest that patients with lower albumin levels will have progression of liver fibrosis. The elevation of AST and ALT values is generally considered to be important in the exacerbation of liver fibrosis. The present study demonstrated that the baseline AST and ALT levels were not significant factors for progression of liver fibrosis in the patients without antiviral therapy. The patients without antiviral therapy had normal or slightly elevated AST and ALT levels during the study period, even if baseline levels were elevated. Thus, the baseline AST and ALT levels probably did not correlate with the progression of fibrosis. Further studies are needed to elucidate the factors associated with the progression of liver fibrosis.

Factors other than fibrosis, including necroinflammatory activity and extrahepatic cholestasis, can affect the results of LS measurement [10, 34–38]. Patients with chronic HBV infection sometimes suffer transient exacerbation of

Table 6 Factors correlated with increase of deduced fibrosis stage in the natural disease course

	Reduction or no change of deduced fibrosis stage	1-point or greater increase of deduced fibrosis stage	*P* value
Number of patients	39	11	
Age (years)	45 (31 to 59)	36 (31 to 62)	NS
Gender (female/male)	25/14	6/5	NS
AST (IU/L)	25.0 (21.0 to 42.0)	37.0 (27.0 to 62.0)	NS
ALT (IU/L)	28.0 (19.0 to 74.0)	52.0 (26.0 to 168.0)	NS
Total bilirubin (mg/dL)	0.9 (0.7 to 1.2)	1.0 (0.9 to 1.2)	NS
Total protein (g/dL)	7.7 (7.3 to 7.9)	7.5 (7.1 to 7.8)	NS
Albumin (g/dL)	4.5 (4.3 to 4.7)	4.2 (4.0 to 4.4)	0.0092
Platelet count ($\times 10^4/\mu L$)	18.1 (14.2 to 24.3)	19.7 (13.5 to 24.4)	NS
Prothrombin time (%)	90.5 (81.5 to 98.8)	93.5 (85.0 to 102.0)	NS
Hyaluronic acid (ng/mL)	28.0 (12.0-63.0)	67.0 (28.5 to 125.0)	NS
HBeAg (+/−)	12/20	5/5	NS
Seroconversion HBeAg (+ → −)	2/12	2/5	NS
HBV DNA (log copy/mL)	4.4 (2.8 to 7.6)	3.9 (3.1 to 7.7)	NS
Liver stiffness (kPa)	5.9 (3.9 to 8.3)	6.0 (3.8 to 7.6)	NS
Change ratio of AST (%)	−10.6 (−25.4 to 10.8)	−5.3 (−15.3 to 34.8)	NS
Change ratio of ALT (%)	−16.0 (−35.9 to 15.7)	−2.3 (−23.8 to 25.0)	NS
Change ratio of total bilirubin (%)	0.0 (−20.4 to 19.2)	0.0 (−9.1 to 22.2)	NS
Change ratio of total protein (%)	0.0 (−5.1 to 2.8)	−1.4 (−3.8 to 4)	NS
Change ratio of albumin (%)	0.0 (−4.2 to 4.7)	0.0 (−2.3 to 4.9)	NS
Change ratio of platelet count (%)	−2.7 (−8.5 to 9.4)	−9.8 (−13.8 to 7.4)	NS
Change ratio of prothrombin time (%)	2.5 (−5.3 to 19.1)	−3.3 (−4.6 to −2.0)	NS
Change ratio of hyaluronic acid (%)	13.9 (−48.2 to 142.7)	33.9 (−10.9 to 79.5)	NS
Change ratio of HBV DNA ratio (%)	0.0 (−14.0 to 5.2)	0.0 (−6.5 to 0.0)	NS
Change ratio of liver stiffness (%)	−5.0 (−21.2 to 25.7)	54.5 (36.0 to 192.7)	<0.0001
Interval between 1st and last liver stiffness measurement (days)	401 (351 to 704)	560 (372 to 958)	NS

Values are medians (interquartile ranges)

Differences in the proportions of patients according to gender, HBe-Ag, and seroconversion of HBeAg were assessed by the χ^2 test between the patients with a reduction or no change of deduced fibrosis stage and those with a 1-point or greater increase of deduced fibrosis stage

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *NS* not significant

hepatitis, which has been reported to increase LS values. The present study showed the association of LS with hyaluronic acid both in the baseline study and the follow-up study, although AST levels were not associated with LS in the baseline study. Thus, LS values can be considered as a reliable marker of fibrosis stage, although LS may be affected by necroinflammatory activity.

Liver biopsy is the gold standard for assessing fibrosis stage and monitoring progress. However, liver biopsy is invasive, costly, and associated with possible complications. Thus, it is not suitable for monitoring progression and regression of the fibrosis stage. The present study demonstrated that LS was significantly correlated with fibrosis stage in patients with chronic hepatitis B. Thus, LS measurement can be useful to assess the progression and regression of liver fibrosis stage noninvasively.

Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Association between perceived happiness levels and peripheral circulating pro-inflammatory cytokine levels in middle-aged adults in Japan

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Submitted: 2011-07-06 *Accepted: 2011-07-21* *Published online: 2011-08-29*

Key words: **happiness; health-related quality of life; pro-inflammatory cytokine; interferon-γ; warm physical contact**

Neuroendocrinol Lett 2011; 32(4):458–463 **PMID:** 21876513 NEL320411A11

Abstract

OBJECTIVES: The idea that perceived happiness may be associated with health and well-being is a recent topic of focus. However, the neurobiological mechanisms underlying the positive effects of happiness on psychological and physiological wellness remain obscure. In this study, we attempted to clarify the association between systemic inflammation and happiness.
METHODS: We recruited 160 healthy volunteers for experiment 1 and compared peripheral inflammatory markers, namely the concentrations of pro-inflammatory cytokines in the serum, between perceived high-happiness and low-happiness groups. Subsequently, we recruited 7 romantic couples for experiment 2 and investigated changes in peripheral pro-inflammatory cytokine levels after the evocation of happiness, which was induced by warm physical contact with the partner.
RESULTS: We found that circulating levels of interferon-γ (IFN-γ), which can affect brain functions and induce depressive symptoms, were lower in the high-happiness group than in the low-happiness group. A negative correlation between the levels of perceived happiness and IFN-γ concentrations was also observed. Furthermore, we also found that experimentally induced happiness could reduce peripheral IFN-γ levels.
CONCLUSIONS: These results revealed an association between the perception of happiness and systemic inflammation. Increased happiness may suppress the peripheral circulation of pro-inflammatory cytokines.

To cite this article: **Neuroendocrinol Lett 2011; 32(4):458–463**

INTRODUCTION

It is well known that stress and anxiety can lead to depression (Magalhaes *et al.* 2010). One of the biological explanations why such psychosocial stressors induce depression is the mediation of pro-inflammatory cytokines, the immune signaling molecules that promote systemic inflammation, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Maes *et al.* 2011). The central nervous and immune systems are interrelated via complex biochemical pathways (Ader, 2000), and psychoneuroimmunologic studies have demonstrated that such psychosocial stressors can induce the active secretion of pro-inflammatory cytokines from immune cells stimulated by the sympathetic nervous system. Peripheral circulating pro-inflammatory cytokines can reach the brain via leaky regions in the blood-brain barrier, active transport molecules, and afferent nerve fibers (Raison *et al.* 2006; Dantzer *et al.* 2008). The enzyme indoleamine 2,3-dioxygenase (IDO), which degrades tryptophan along the kynurenine pathway, is expressed in all organs including the brain, and the enzymatic activity of IDO can be potently activated by a number of cytokines, including TNF-α and IFN-γ. Activation of IDO results in decreased levels of tryptophan and other tryptophan-derived metabolites, such as 5-hydroxytryptamine (serotonin). Serotonin in the brain has an antidepressant effect; therefore, the deceleration of serotonin in the brain by pro-inflammatory cytokines may induce depression. In fact, previous studies indicated that circulating pro-inflammatory cytokine levels in individuals with depressive symptoms were higher than those in individuals with no depressive symptoms (Pan *et al.* 2008).

The idea that perceived happiness may be associated with health and well-being has recently been investigated intensely. Individuals with high levels of perceived happiness have reduced sympathetic activation after exposure to psychological stressors compared to those with low levels of perceived happiness (Horiuchi *et al.* 2008). A recent study indicated that the level of perceived happiness is negatively correlated with depression and anxiety (Shimai *et al.* 2004). The biological mechanism underlying the positive effect of happiness remains obscure; however, it is suggested that circulating pro-inflammatory cytokine levels may be lower in individuals with high-perceived happiness than in individuals with low-perceived happiness, although this has yet to be demonstrated. In addition, it is still unclear whether peripheral circulating pro-inflammatory cytokine levels are decreased if happiness is experimentally evoked. We have recently demonstrated that warm physical contact with the partner can increase subjective happiness (Matsunaga *et al.* 2009a). Accompanying feelings of happiness, serum levels of albumin were also increased (Matsunaga *et al.* 2009a). A previous study demonstrated a negative correlation between peripheral albumin levels and pro-inflammatory cytokine levels (Odamaki *et al.* 2004), suggesting that pro-inflammatory cytokine levels may also be decreased by warm partner contact, accompanied by decrease in albumin levels.

Based on these previous observations, we first compared the serum concentrations of pro-inflammatory cytokines (interleukin-6 (IL-6), TNF-α, and IFN-γ) and health-related quality of life (QOL) between individuals with high-perceived happiness and those with low-perceived happiness (Experiment 1). Subsequently, we investigated whether warm partner contact could decrease the serum concentrations of pro-inflammatory cytokines (IL-6, TNF-α, and IFN-γ) (Experiment 2). Some reports have noted gender-related differences regarding the levels of perceived happiness and physiological reactivity (Shimai *et al.* 2004; Bosch *et al.* 2005). Further, obesity is strongly associated with circulating pro-inflammatory cytokine levels, as adipose tissue is a major production site of inflammatory markers (Hamer & Stamatakis 2008). Thus, we compared the levels of inflammatory markers by using age-, gender-, and BMI-matched groups.

METHODS

Experiment 1

Participants

We recruited 160 healthy volunteers (77 males and 83 females; age range: 19–40 years) for experiment 1. The participants were Japanese undergraduate and graduate students at Nagoya University and Mie University and technical staff at Aichi Medical University and Fujita Health University. Participants were instructed not to eat 2 h before blood sampling, but they were allowed to consume non-alcoholic and caffeine-free fluids. All participants provided written informed consent in accordance with the Declaration of Helsinki. Participants were excluded if they had any chronic or oral illness, if they had taken medication known to influence immunity such as a steroid during the 3-month period before the experiment, or if they used oral contraceptives. In addition, participants who contracted an infectious illness within 3 weeks before the experiment were rescheduled. This study was approved by the Ethics Committee of Fujita Health University. To screen individuals with higher or lower levels of perceived happiness, we requested that they use the Japanese version of the subjective happiness scale (JSHS) (Shimai *et al.* 2004). The JSHS is a 4-item scale that measures relatively stable perceived happiness. The internal consistency, test-retest reliability, convergent validity, and discriminant validity of the JSHS have been confirmed (Shimai *et al.* 2004). The results of this screening investigation indicated that the mean JSHS score for the participants was 4.83 with a standard deviation (SD) of 0.9, which was similar to that reported previously (Shimai *et al.* 2004). According to the mean JSHS score of the participants, we classified respondents with scores exceed-

ing 5.50 as high-happiness respondents (n=48) and those with scores below 4.25 as low-happiness respondents (n=48).

Blood sampling and measurements of cytokine concentrations

Blood sampling was performed between 1400 and 1700 h to minimize the influence of circadian rhythms on cytokines. Furthermore, women were examined during the late luteal and early follicular phases of the menstrual cycle when the secretion of female sex hormones is low, thus minimizing the influences of these hormones on the immune system. Blood samples were collected in serum-separator tubes and centrifuged at 3000 × *g* for 10 min; the serum was separated and then stored at −80 °C until analysis. The levels of several pro-inflammatory serum cytokines (IL-6, TNF-α, and IFN-γ) were determined by a BD™ Cytometric Bead Array (Human Th1/Th2 Cytokine Kit II; BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Measurement of health-related QOL

The Japanese-translated version of the Short-Form 36 Health Survey (SF-36) was used to assess the impact of 8 QOL dimensions (physical functioning, physical role, bodily pain, general health, vitality, social functioning, emotional role, and mental health) on health-related QOL. This translated version has been studied extensively for its reliability and validity (Fukuhara *et al.* 1998a, b). We displayed the 8 dimension scores with the raw values.

Statistical analyses of self-reported and physiological data

The results are expressed as means ± standard error of the mean (SEM). We compared age, BMI, health-related QOL, and circulating levels of IL-6, TNF-α, and IFN-γ by Student's *t* test between the high-happiness and low-happiness groups. Pearson's correlation coefficient between the levels of perceived happiness and IFN-γ concentrations was computed using the entire sample (n=160).

Experiment 2

Participants

Fourteen healthy volunteers (7 romantic couples; 7 males and 7 females) participated in experiment 2. Patient ages ranged from 21 to 38 years. All participants provided written informed consent in accordance with the Declaration of Helsinki. The participants received no medication during the experimental period. They were requested to evaluate their feelings of romantic love for their partners by using the passionate love scale (PLS) (Hatfield & Sprecher, 1986) (example items: "Sometimes I can't control my thoughts; they are obsessively focused on _____;" I would rather be with _____ than anyone else"). Four participants were evaluated as "extremely passionate," 7 as "passionate," and 3 as "average"; therefore, all couples may be considered to have relatively passionate love. This study was approved by the Human Studies Committee of Aichi Medical University.

Experimental procedure

Each couple entered an experimental room, following which they were given instructions prior to the commencement of the experiment. The couple was instructed not to eat and drink during the experimental session. In the warm contact condition, participants were first requested to evaluate their present happiness levels, and the first blood sample was obtained. They then freely kissed and hugged their romantic partner, but did not have intercourse, for 1 h in a room with closed doors. After the warm contact session, a second blood sample was obtained, and the present happiness level of each participant was evaluated. To assess the level of physical contact, the participants were requested to subjectively rate the following 3 questions on a scale of 1 (not at all) to 7 (Yes, extremely): Did you kiss and hug your partner very much? (contact); Did you evoke much love? (love); Did you sense your partner's love? (love). The average value of the rating score of contact was 5.64 ± 0.29, and the average value of the rating score of love was 11.28 ± 0.58. Because both values were higher than the neutral values (4 (contact) and 8 (love)), the participants were believed to have engaged in extensive warm partner contact during the warm contact session.

In the control condition, participants were first requested to evaluate their present happiness levels, and the first blood samples were obtained. Then, 1 partner remained in the experimental room, while the other partner was moved to another experimental room. The partners then read a book separately for 1 h in a room with closed doors. The content of the books did not include romance. After the reading session, a second blood sample was obtained from each participant, and his or her present happiness level was evaluated. The order of the 2 conditions was counterbalanced across the couples, and there was at least a 2-week interval between the 2 conditions.

Measurement of happiness feeling

To evaluate the feelings of happiness of the participants, they were asked to subjectively evaluate their present emotions by rating the following question on a scale of 1 (not at all) to 7 (Yes, extremely): Do you feel happy at present? (happiness).

Measurements of cytokine concentrations

Blood samples were collected in serum-separator tubes and centrifuged at 3000 × *g* for 10 min; the serum was separated and then stored at −80 °C until analysis. Cytokine levels were assessed as described in a previous section.

Statistical analyses of self-reported and physiological data

The results were expressed as mean ± SEM. The psychological and physiological indices were compared using 2-way repeated-measures analysis of variance (ANOVA) [condition (control vs. warm contact) × period (before vs. after)] followed by paired *t* tests.

RESULTS

EXPERIMENT 1: *Association between the level of perceived happiness and those of circulating pro-inflammatory cytokines*

As shown in Table 1, there were no significant differences in gender distribution, age, and BMI between the high-happiness and low-happiness groups. Using these gender-, age-, and BMI-matched groups, to assess whether the level of perceived happiness is associated with health-related QOL and the peripheral pro-inflammatory cytokine levels, we compared the scores of the SF-36 subscales and serum concentrations of IL-6, TNF-α, and IFN-γ between the high-happiness and low-happiness groups. Statistical analysis revealed that the SF-36 subscale scores of general health ($df=94$, $t=-3.15$, $p<0.01$), vitality ($df=94$, $t=-6.36$, $p<0.01$), emotional role ($df=94$, $t=-2.22$, $p<0.05$), and mental health ($df=94$, $t=-5.03$, $p<0.01$) were significantly higher in the high-happiness group than in the low-happiness group. Interestingly, serum concentrations of IFN-γ were significantly lower in the high-happiness group ($df=94$, $t=-2.12$, $p<0.05$) than in the low-happiness group. No significant differences were observed between the IL-6 and TNF-α levels between the 2 groups. In addition, Pearson's correlation coefficient between the level of perceived happiness and IFN-γ concentrations was computed, and a significant negative correlation was observed ($r(160)=-0.23$, $p<0.01$; Figure 1A).

Tab. 1. Comparisons of gender, age, BMI, SF-36 subscale scores, and serum cytokine concentrations between the high-happiness and low-happiness groups.

	Low (<4.25)	High (> 5.50)
Gender (male female)	25/23	24/24
Age	22.64 ± 0.64	23.46 ± 0.73
BMI	20.81 ± 0.53	20.77 ± 0.43
SF-36 subscales		
Physical functioning	29.39 ± 0.27	29.43 ± 0.14
Physical role	18.83 ± 0.31	18.96 ± 0.39
Bodily pain	3.93 ± 0.25	4.02 ± 0.27
General health	17.42 ± 0.54	19.79 ± 0.49**
Vitality	11.84 ± 0.38	15.05 ± 0.35**
Social functioning	8.67 ± 0.24	9.00 ± 0.17
Emotional role	12.06 ± 0.43	13.33 ± 0.38*
Mental health	17.11 ± 0.52	20.32 ± 0.36**
Cytokines (pg/ml)		
IL-6	2.63 ± 0.52	1.83 ± 0.23
TNF-α	2.35 ± 0.24	2.23 ± 0.18
IFN-γ	7.58 ± 0.88	5.45 ± 0.47*

Each result represents the mean ± SEM concentration or rating score. **$p<0.01$ and *$p<0.05$ vs. low-happiness group by Student's *t* test.

EXPERIMENT 2: *Effect of happiness on circulating pro-inflammatory cytokine level*

To assess changes in the levels of happiness and those of peripheral circulating pro-inflammatory cytokine after warm partner contact, the participants were asked

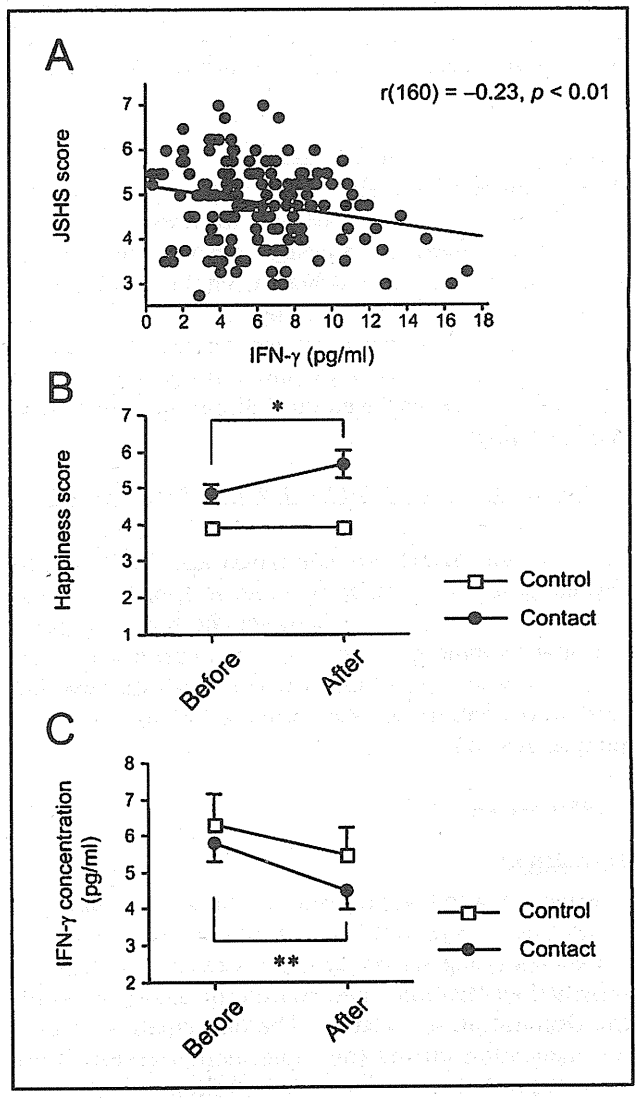

Fig. 1. (A) Scatter plot showing the negative correlation between the level of perceived happiness and serum IFN-γ concentrations ($r(160)=-0.23$, $p<0.01$). (B) Change in happiness feelings after warm partner contact. *$p<0.05$ vs. before contact by paired *t* test. (C) Changes in serum IFN-γ concentrations after warm partner contact. **$p<0.01$ vs. before contact by paired *t* test.