Unlike the antioxidant alpha-lipoic acid (ALA), which is unstable and poorly water-soluble, dihydrolipoyl histidinate zinc complex (DHLHZn) was developed for clinical use, and is water-soluble, as well as stable in solution [17, 18]. The aim of this study was to evaluate the effects of DHLHZn administration on the liver fibrosis in the $CCl_4$ rat model, and to examine the expression of fibrogenic factors in an in vitro model of fibrotic activation using surrogate cells to represent HSCs.

## Methods

### Animals

Twenty-four male Wister rats (age 6 weeks, weight 200–250 g) (Kyudo, Fukuoka, Japan) were used for the study. The rats were divided into three groups: the control group was fed a standard laboratory diet, the $CCl_4$ group was fed a standard laboratory diet and orally administered $CCl_4$ and the $CCl_4$+DHLHZn group was fed a 0.5 % DHLHZn-mixed diet along with the orally administered $CCl_4$. The rats were kept in a room under a 12-h light/dark cycle, and were administered $CCl_4$ and olive oil orally (1:1, 2.0 mg/kg) [19]. $CCl_4$ was administered three times per week (Monday, Thursday, and Saturday) for three consecutive weeks. After 3 weeks of oral administration of $CCl_4$, the animals were killed, and blood samples and livers were obtained. The body weights and food intake were measured (Animal Scale; Clare, Tokyo, Japan) weekly in all the groups (at 10:00 am) prior to the killing. This study was approved by the Animal Committee of Oita University and conformed to the Guidelines for Animal Experimentation of Oita University.

### Biochemical examinations

Rat blood samples were obtained, centrifuged, and after the removal of plasma, the samples were stored at −80 °C until all assays were conducted. The total bilirubin (T-bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) levels were estimated using an H7180 automatic biochemical analyzer (Hitachi, Tokyo, Japan).

### Histological examination

Liver samples were fixed in 10 % formalin and embedded in paraffin. The sections were then cut (5 μm) and stained with hematoxylin and eosin for the histopathological examination and with azan to assess the fibrosis. In addition, the liver damage and fibrosis were evaluated by the histological activity index (HAI) score [20]. The pathologist evaluated all histological sections in a blinded fashion, and the sections were examined at high power magnification (200×, 400×).

### Assays for malondialdehyde and glutathione

The tissue MDA and GSH levels were measured from homogenized liver samples. Frozen liver tissue samples were homogenized with a tissue homogenizer (Dremel, Racine, WI, USA), and were subsequently centrifuged at 10,000×$g$ for 10 min at 4 °C. The protein content of the samples was determined with a Quick Start Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The MDA and GSH levels were measured using an assay kit, per the manufacturer's instructions (OxisResearch, Burlingame, CA, USA and Northwest Life Science Specialties LLC, respectively). Absorbance was measured (at 586 nm for MDA and 405 nm for GSH) using an enzyme-linked immunosorbent assay reader (Bio-Rad Laboratories).

### Cell culture and assays

The human HSC line, LI90, was purchased from the Health Science Research Resource Bank (Osaka, Japan). LI90 cells were cultured in Dulbecco's minimal essential medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10 % fetal bovine serum (FBS; Bio-Whittaker, Walkersville, MD, USA), penicillin (50 U/mL, Gibco BRL, Grand Island, NY, USA), and streptomycin (50 μg/mL, Gibco BRL). All cultures were incubated at 37 °C in a humidified atmosphere of 5 % $CO_2$. In the assays, 1 × $10^6$ LI90 cells were incubated in a 25 $cm^2$ angle flask with 25 μM antimycin-A (AMA, Sigma-Aldrich, St. Louis, MO, USA) in the presence or absence of 250 μM DHLHZn for 48 h, as described previously [18].

### Total RNA isolation

The total RNA from each cell was isolated with a BioRobot EZ1 RNA Cell Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized as described previously [21]. Thereafter, total RNA (1.0 μg) was reverse-transcribed in a 25-μL reaction containing 80 pmol random primers (Takara, Otsu, Japan) and 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was used as a template for the subsequent real-time polymerase chain reaction (PCR) analyses.

### Real-time PCR for mRNA quantification of MMP-2 and collagen α1 (I)

Quantitative real-time PCR was performed using the Light Cycler System (Roche Diagnostics, Lewes, East Sussex,

UK). The primers used for transforming growth factor (TGF)-β1 were: 5′-ACTACTACGCCAAGGAGGTCAC-3′ (forward) and 5′-TGCTTGAACTTGTCATAGATTTCG-3′ (reverse) (Nihon Gene Research Laboratories, Sendai, Japan) and for collagen α1 were (I): 5′-GCGTTAAGGGGG AAAAAGG-3′ (forward) and 5′-CAGCCAGGCATGGG TAAG-3′ (reverse) (Nihon Gene Research Laboratories). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH primer set; Search LC, Heidelberg, Germany) was amplified according to the manufacturer's protocol as an internal control to allow for the quantitation of the TGF-β1 and collagen α1 (I) amplification products. A fresh standard dilution series was prepared prior to experiments. The PCR mix contained 8.6 μL PCR-grade water, 0.6 μL each of TGF-β1 and collagen α1 (I) primers (0.3 μL), 3.2 μL of $2.5 \times 10^{-2}$ mol/L $MgCl_2$, and 2 μL LightCycler-FastStart DNA Master SYBR Green I dye, and was added to a 1.5-mL light-protected reaction tube on ice. The PCR mix was pipetted into a precooled LightCycler capillary, and 5 μL cDNA template (diluted 20×) was added. Thereafter, 15 μL of the PCR mix was pipetted into four precooled LightCycler capillary tubes, and 5 μL undiluted and 5 μL freshly diluted standards were added to each capillary. After each capillary was sealed with a stopper and centrifuged at 700*g* for 15 s, the samples were amplified. The PCR amplification conditions were set for one cycle of pre-denaturation at 95 °C for 10 s, annealing at 62 °C for 10 s, and extension at 72 °C for 7 s. PCR cycles were monitored continuously with SYBR Green I dye. After amplification, the melting curve analysis permitted accurate identification of the PCR amplicons. The data were analyzed using the LightCycler analysis software program (Roche Diagnostics, Lewes, East Sussex, UK), and a standard curve that correlated the cycle number with the amount of formed products was plotted for each sequence of interest. Subsequently, the TGF-β1 and collagen α1 (I) mRNA expression were then normalized to that of GAPDH.

### Statistical analysis

All data are expressed as the mean ± standard deviation. All data were evaluated using Student *t* test or a one-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant. The statistical analyses were performed using the SPSS II software program (SPSS, Inc, Chicago, IL, USA).

## Results

### Food intake, body weight and biochemical findings

The rats were monitored for food intake and for their plasma enzyme levels. The mean food intake and plasma levels of T-bil, AST, ALT, ALP, and LDH are summarized in Table 1. There were no significant differences in the food intake among the three groups. The levels of T-bil, AST, ALT, ALP, and LDH in the $CCl_4$ and $CCl_4$+DHLHZn groups were significantly higher than those in the control group. Compared with the $CCl_4$ group, the levels of T-bil, AST, ALT, and ALP were significantly lower in the $CCl_4$+DHLHZn group. The body weights 2 and 3 weeks after $CCl_4$ administration in the $CCl_4$ and $CCl_4$+DHLHZn groups were significantly lower than that in the control group. However, there were no significant differences in the body weight between the $CCl_4$ and $CCl_4$+DHLHZn groups (Fig. 1).

**Table 1** The food intake and serum biochemical parameters of liver function in rats with $CCl_4$-induced liver fibrosis

| | Control | $CCl_4$ | $CCl_4$+DHLHZn |
|---|---|---|---|
| Food intake (g/week) | 96.2 ± 3.2 | 88.1 ± 26.2 | 75.2 ± 26.0 |
| T-bil (mg/dL) | 0.2 ± 0.1 | 2.2 ± 0.8* | 0.9 ± 0.3**, # |
| AST (IU/L) | 64.8 ± 20.9 | 4085.6 ± 1992.9* | 1832.2 ± 679.7**, # |
| ALT (IU/L) | 31.5 ± 3.9 | 2604.9 ± 1365.6* | 1379.4 ± 680.2**, ## |
| ALP (IU/L) | 327.8 ± 6.9 | 3171.0 ± 809.6* | 1846.1 ± 677.3*, # |
| LDH (IU/L) | 173.3 ± 83.0 | 3750.4 ± 2093.7* | 968.6 ± 446.8# |

*T-bil* total bilirubin, *AST* aspartate amino transferase, *ALT* alanine amino transaminase, *ALP* alkaline phosphatase, *LDH* lactate dehydrogenase

* $P < 0.01$, ** $P < 0.05$ versus the control group

# $P < 0.01$, ## $P < 0.05$ versus the $CCl_4$ group

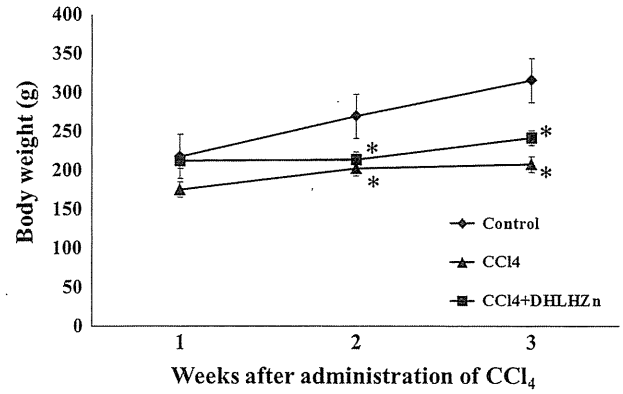


**Fig. 1** The changes in body weight. The changes in body weight after the administration of $CCl_4$ in the control, $CCl_4$ and $CCl_4$+DHLHZn groups. *$P < 0.01$ versus the control group. Each group consisted of eight rats

### Histological changes of the liver

We assessed the effects of DHLHZn administration on liver the pathology through a histological analysis. The liver samples from the $CCl_4$ group revealed reduced hepatocytes, cirrhotic nodules and marked fibrosis (Fig. 2b). In the $CCl_4$+DHLHZn group, the hepatocyte damage, fibrosis and

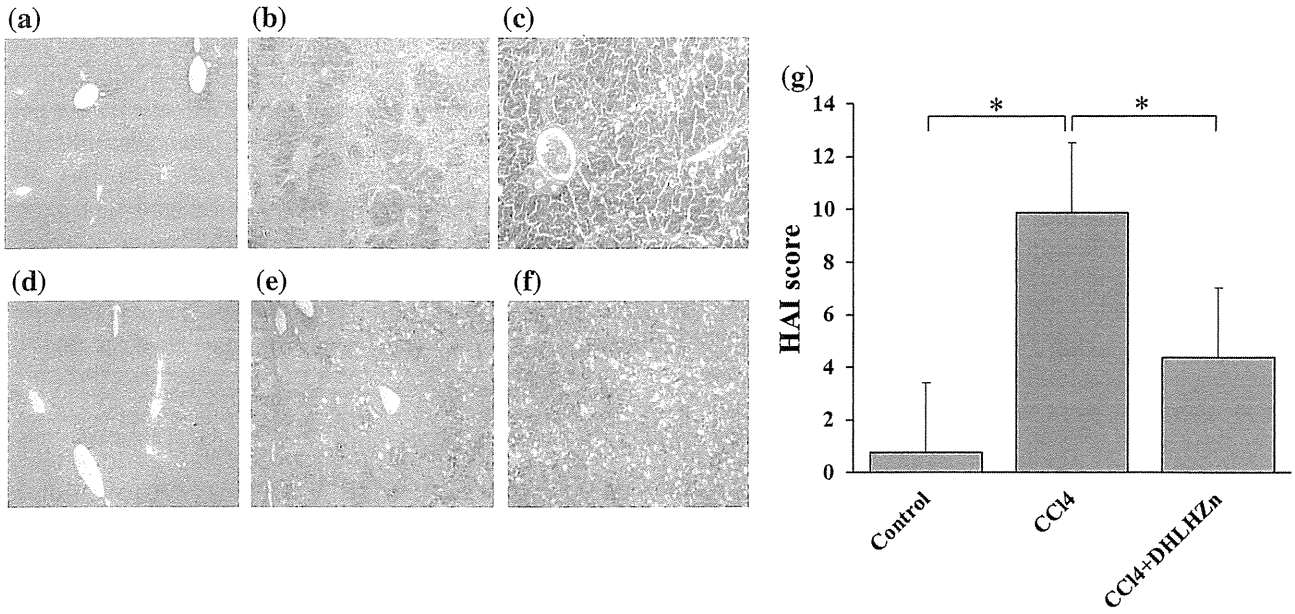


**Fig. 2** Photomicrographs of liver sections stained with hematoxylin and eosin (HE) and with azan 3 weeks after carbon tetrachloride ($CCl_4$) administration with or without dihydrolipoyl histidinate zinc complex (DHLHZn) treatment. In the control group, normal collagen fibers were recognized in the areas of the portal and central veins (**a** HE), while collagen fibers were not observed (**d** azan). The $CCl_4$ group showed isolated hepatocytes and islands of some hepatocytes due to dense fibrous tissues (**b** HE), and fibrosis with fiber extension and collagen accumulation was revealed (**e** azan). In the $CCl_4$+DHLHZn group, the fibrotic changes were attenuated (**c** HE, **f** azan). The HAI score in the $CCl_4$+DHLHZn group was significantly lower than that in the $CCl_4$ group. * $P < 0.01$. Each group consisted of eight samples

cirrhotic changes were attenuated (Fig. 2c). In addition, azan staining clearly revealed that livers from the $CCl_4$ group had remarkable fibrosis, with fiber extension and collagen accumulation (Fig. 2e), and these pathological changes were attenuated in the $CCl_4$+DHLHZn group (Fig. 2f). The degree of liver damage and fibrosis revealed by the HAI score in the $CCl_4$+DHLHZn group was significantly lower than that in the $CCl_4$ group (Fig. 2g).

## MDA and GSH levels in the liver

We determined the levels of oxidative stress and natural antioxidants present in the three rat groups. The liver MDA levels in both the control and $CCl_4$+DHLHZn groups were significantly lower than that in the $CCl_4$ group (Fig. 3a). On the other hand, the GSH levels in the livers of the control and $CCl_4$+DHLHZn groups were significantly higher than that in the $CCl_4$ group (Fig. 3b).

## Expression of TGF-β1 and collagen α1 (I)

We utilized an in vitro model of fibrotic activation to study the effects of DHLHZn on the expression of TGF-β1 and collagen α1 (I). The mRNA expression levels of both molecules were significantly higher in the AMA group than in the control and DHLHZn groups (Fig. 4); however,

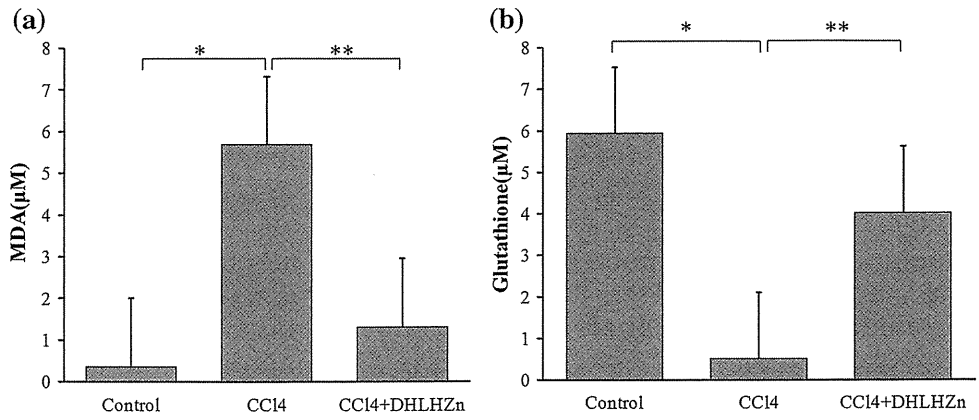


**Fig. 3** The malondialdehyde (MDA) and glutathione levels in the liver of rats 3 weeks after carbon tetrachloride ($CCl_4$) administration with or without dihydrolipoyl histidinate zinc complex (DHLHZn) treatment. *$P < 0.01$, **$P < 0.05$. Each group consisted of eight animals

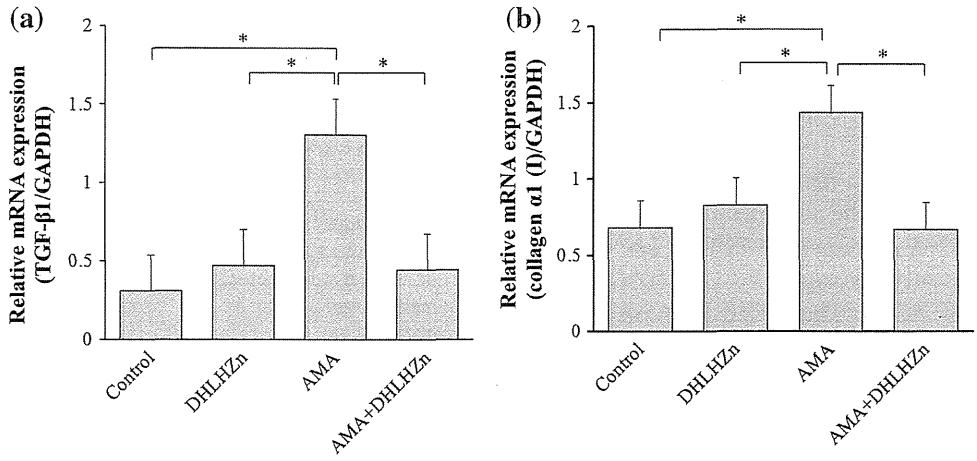


**Fig. 4** The effects of dihydrolipoyl histidinate zinc complex (DHLHZn) on the antimycin-A (AMA)-induced transforming growth factor (TGF)-β1 and collagen α1(I) mRNA expression in the LI90 cells. The mRNA expression of TGF-β1 and collagen α1 (I) were measured in the cells treated with saline (control), 250 μM DHLHZn, 25 μM AMA or 25 μM AMA and 250 μM DHLHZn (AMA+DHLHZn). The mRNA expression of TGF-β1 and collagen α1 (I) was significantly decreased in the AMA+DHLHZn group compared to the AMA group. $^*P < 0.01$. Each group consisted of six samples

DHLHZn administration significantly decreased the levels of both TGF-β1 and collagen α1 (I) mRNA expression in comparison with the AMA group (Fig. 4).

## Discussion

Hepatic fibrosis is a physiological process of wound healing in the liver; but chronic fibrosis can lead to hepatocellular carcinoma [22]. Chronic fibrosis is induced by the interactions with HSC, which are activated by ROS [23]. In fact, fibrogenesis and hepatocarcinogenesis in the cirrhotic liver are associated with severe oxidative stress [24]. The $CCl_4$-induced fibrosis model is widely used to evaluate the hepatoprotective effects of various pharmacological therapies. $CCl_4$ is metabolized into trichloromethyl ($-CCl_3$) and trichloromethyl peroxyl (–OOCCl) by cytochrome P450 enzymes in the liver, and generates free radicals in the process. These free radicals are responsible for the oxidative stress-induced hepatic damage and fibrosis [4, 25]. Therefore, antioxidant drug therapies may be effective for preventing $CCl_4$-induced hepatic fibrosis.

Previous reports demonstrated that several drugs can attenuate $CCl_4$-induced hepatotoxicity [3, 26–28]. The administration of *N*-acetylcysteine decreases the liver fibrosis by increasing the GSH levels and decreasing the expression of induced nitric oxide synthase (iNOS) [3]. Moreover, treatment with insulin-like growth factor-I reduced the myeloperoxidase levels and the expression of iNOS in the $CCl_4$ model rats [27]. The levels of endogenous antioxidants, such as GSH, have been thought to be decreased under conditions of excess oxidative stress [29–31]. The administration of antioxidant drugs can increase the levels of endogenous antioxidants [30, 31]. In the present study, DHLHZn administration attenuated the $CCl_4$-induced fibrosis. In addition, the MDA levels were significantly decreased and the GSH levels were significantly increased in the $CCl_4$+DHLHZn group compared with the $CCl_4$ group. Therefore, DHLHZn might attenuate $CCl_4$-induced hepatic fibrosis by reducing the oxidative stress.

HSCs play a major role in the progression of liver fibrosis [12]. Continuation of chronic liver injury activates HSCs, which transform into myoblastic cells. As a result, they induce the accumulation of collagen in the liver [13]. Increased ROS generation leads to the upregulation of fibrogenic genes, such as α-smooth muscle actin, TGF-β1 and collagen Iα2 in LI90 cells [32]. Furthermore, LI90 cells cultured with platelet-derived growth factor induced higher mRNA expression levels of collagen α1 (I) and (IV) [33]. In the present study, the mRNA expression of TGF-β1 and collagen α1 (I) was significantly decreased in the LI90 cells treated with AMA and DHLHZn compared with those treated with AMA alone. This attenuation of hepatic fibrogenic factors in LI90 cells might be result of the potent antioxidant activity of DHLHZn.

DHLHZn, composed of dihydrolipoyl histidine and zinc, is water-soluble and stable in aqueous solution. Several reports have indicated that DHLHZn improved various disease conditions [34–36]. For example, DHLHZn administration attenuated the histological changes of renal ischemia reperfusion injury in rats compared with untreated controls [34]. Moreover, in rats, chemotherapy-induced alopecia was reduced by DHLHZn administration [36]. In addition, we reported that DHLHZn administration attenuated the hepatic ischemia–reperfusion injury, and the

1,1-diphenyl-2picrylhydrazyl radical scavenging activity of DHLHZn was significantly higher than that of ALA [18]. However, the molecular morphologic mechanisms underlying the effects of DHLHZn are still unclear and require further investigation.

In conclusion, the hepatic fibrosis induced by $CCl_4$ was significantly attenuated by the administration of DHLHZn. In addition, the mRNA expression of TGF-β1 and collagen α1 (I) was significantly decreased by DHLHZn in LI90 cells cultured with AMA. Thus, DHLHZn may be an effective agent for attenuating the hepatic fibrosis induced by oxidative stress.

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**Conflict of interest** Y. Kawano and co-authors have no conflicts of interest.

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Original article

# Effect of laparoscopic splenectomy on portal haemodynamics in patients with liver cirrhosis and portal hypertension

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**Background:** The effect of splenomegaly in patients with liver cirrhosis and portal hypertension is not fully understood. This study was designed to determine the effect of laparoscopic splenectomy on portal haemodynamics in these patients.

**Methods:** Patients with liver cirrhosis and portal hypertension who underwent laparoscopic splenectomy in Kyushu University Hospital from January 2006 to March 2009 were evaluated retrospectively. Correlations between splenic size and portal haemodynamics, and changes in portal haemodynamics and in levels of the vasoactive agents endothelin (ET) 1 and nitric oxide metabolites (NOx) before and 7–10 days after laparoscopic splenectomy were analysed.

**Results:** Portal venous (PV) blood flow, PV cross-sectional area and PV congestion index correlated significantly with splenic size ($P < 0{\cdot}050$). All three were significantly reduced following splenectomy in 59 patients. The hepatic venous pressure gradient, measured in 18 patients, decreased by 25 per cent after splenectomy ($P < 0{\cdot}001$). Portal vascular resistance was also reduced, by 21 per cent ($P = 0{\cdot}009$). The peripheral blood concentration of ET-1 decreased from 2·95 to 2·11 pg/ml ($P < 0{\cdot}001$), and that of NOx tended to decrease (from 29·2 to 25·0 pg/ml; $P = 0{\cdot}068$). In hepatic venous blood, the level of ET-1 decreased from 2·37 to 1·83 pg/ml ($P = 0{\cdot}006$), whereas NOx concentration tended to increase (from 24·5 to 30·9 pg/ml; $P = 0{\cdot}067$).

**Conclusion:** In patients with liver cirrhosis and portal hypertension, splenectomy reduced portal venous pressure. A decrease in splanchnic blood flow, by eliminating splenic blood flow, and reduction in intrahepatic vascular resistance, by normalizing hepatic concentrations of ET-1 and NOx, may both have contributed.

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## Introduction

By the end of the 19th century splenectomy was already regarded as a therapeutic procedure for patients with Banti's disease, defined as the triad of splenomegaly, leucopenia and liver cirrhosis[1]. Splenectomy was reported to have beneficial effects in patients with Banti's syndrome and portal hypertension, including improvements in bleeding tendency, hypersplenism and gastrointestinal bleeding. During the 1940s and 1950s, however, many studies reported that splenectomy failed to stop oesophagogastric variceal haemorrhage in patients with portal hypertension, suggesting that splenectomy may not be curative in these patients[2]. Splenectomy has therefore been combined with other procedures, such as devascularization of the upper stomach and oesophageal transection, in order to control variceal haemorrhaging. Splenectomy alone is not proposed to treat portal hypertension, because of its serious risks, including surgical bleeding, thrombosis in the portal venous system and sepsis[3,4].

Technical advances in surgery, and the recognition of risk factors and effective treatments for portal venous (PV) thrombosis, have resulted in safer and less invasive forms of splenectomy, including the laparoscopic approach[5–7]. Owing to the development of treatments for chronic liver diseases, such as interferon (IFN) therapy for chronic hepatitis C virus (HCV) infection, laparoscopic splenectomy has again attracted attention as a treatment

for patients with liver cirrhosis and portal hypertension[4]. Currently, splenectomy is performed to improve thrombocytopenia in patients with chronic HCV infection, before treatment with pegylated IFN plus ribavirin, and in patients with cirrhosis undergoing treatment for hepatocellular carcinoma (HCC)[4,8]. Concurrent splenectomy has also shown beneficial effects in living-donor liver transplantation (LDLT), controlling excessive portal flow in small-for-size liver grafts and alleviating persistent thrombocytopenia[9].

Portal hypertension in patients with liver cirrhosis is characterized primarily by increased intrahepatic vascular resistance and increased splanchnic blood flow[10,11]. In this setting, splenomegaly is due not only to dilatation of the splenic sinus resulting from portal congestion induced by increased intrahepatic vascular resistance, but also to splenic tissue hyperplasia and fibrosis[3]. As splenic blood flow is indisputably increased, splenomegaly may have a role in the pathogenesis of portal hypertension by increasing splanchnic blood flow. However, the relationship between an enlarged spleen and PV pressure is unclear[3], as the splenectomy-associated reduction in PV pressure is not always substantial and splenectomy is usually performed in conjunction with other procedures such as oesophageal transection. As few reports have assessed haemodynamic changes after splenectomy alone, it is uncertain whether splenomegaly is involved in the pathogenesis of portal hypertension.

Recent studies have reported that, despite reducing PV blood flow by eliminating splenic blood flow, splenectomy often improves liver function[4,8]. This may imply that splenomegaly contributes actively to the pathophysiology of liver cirrhosis. This hypothesis is similar to that of Banti, that an enlarged spleen may secrete substances that injure the liver[1,3]. The present study analysed the effects of splenomegaly on the pathogenesis of liver cirrhosis with portal hypertension by investigating postsplenectomy changes in portal haemodynamics and vasoactive agents related to hepatic microcirculation, such as endothelin (ET) 1 and nitric oxide metabolites (NOx).

## Methods

All consecutive patients with liver cirrhosis and portal hypertension who underwent laparoscopic splenectomy at the Department of Surgery and Science, Kyushu University Hospital, from January 2006 to March 2009 were evaluated retrospectively. All patients had a detailed demographic, clinical and biochemical assessment. They all underwent Doppler ultrasonography and contrast-enhanced CT to evaluate portal haemodynamics and to screen for splanchnic vein thrombosis, before and 7–10 days after surgery. Upper gastrointestinal endoscopy was performed in all patients to assess the severity of oesophagogastric varices before and within 1 month of operation. Risky oesophageal varices were defined as moderate or huge in size with red colour signs, according to the criteria of the Japan Society for Portal Hypertension[12]. No patient enrolled in this study had thrombi in the portal venous system, and none had been administered portal pressure-reducing agents such as beta-blockers. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

### Operative procedures for laparoscopic splenectomy

Operative procedures for laparoscopic splenectomy were performed as described previously[5]. Each patient had preoperative CT to evaluate splenic volume and to determine the location and extent of collateral vessels. Patients whose spleen had a volume of 1000 ml or more, as assessed by CT volumetry, those with large perisplenic collateral vessels and/or patients with a Child–Pugh score of 9 or above underwent hand-assisted laparoscopic surgery (HALS); the remaining patients were operated on totally laparoscopically. Patients were placed in the supine position with the left flank elevated at a 60° angle. Splenic attachments were divided using electrocautery, ultrasound dissection and/or the LigaSure™ vessel sealing system (Covidien, Boulder, Colorado, USA). Once the upper pole of the spleen had been dissected from the diaphragm, the splenic hilar pedicle was transected with an endoscopic linear vascular stapler. The resected spleen was placed into a plastic bag, morcellated and extracted. Splenic size was based on the weight of the morcellated spleen.

### Doppler ultrasonography

After an overnight fast, patients underwent Doppler ultrasonography in the supine position using a duplex ultrasound device (ProSound SSD-5500SV; Aloka, Tokyo, Japan) with a 3·75-MHz probe provided with pulsed Doppler and a colour-flow mapping device. During the examination, patients were asked to hold their breath. All Doppler examinations were performed by two experienced examiners. PV blood flow (ml/min) was calculated from the PV velocity (cm/s) and the PV cross-sectional area ($cm^2$). The PV cross-sectional area was calculated using the formula: PV cross-sectional area $= \pi (R^2/4)$ (where R is

the diameter of the portal vein). The PV congestion index (cm·s) was calculated using the formula: PV congestion index = PV cross-sectional area/PV velocity[13]. Doppler shift signals were obtained from the centre of the portal vein. The angle between the ultrasonic beam and the longitudinal axis of the vessel never exceeded 60°. All measurements were repeated three times and averaged. The coefficients of variation of all parameters were less than 5 per cent.

## Measurement of hepatic venous pressure gradient

The hepatic venous pressure gradient (HVPG) was measured on the same days as the Doppler ultrasound examinations, before and after surgery, in a subset of patients. The patients were transferred to the angiography room after an overnight fast and placed in the supine position. Hepatic venous pressure ($cmH_2O$) was measured in triplicate with a high-sensitivity transducer (TC-704; Nihon Kohden, Tokyo, Japan) following catheterization of the main right hepatic vein under fluoroscopic guidance using a 6.5-Fr balloon catheter (B-RTO type II catheter; Create Medic, Tokyo, Japan)[14].

The zero reference point for each measurement was set at the mid-axillary line of the patient. The HVPG was calculated as the difference between the wedged hepatic venous pressure and the free hepatic venous pressure. The HVPG was used to assess the PV pressure. Intrahepatic portal vascular resistance was calculated using the formula: intrahepatic portal vascular resistance = HVPG/PV blood flow ($cmH_2O$ per ml per min)[15].

## Measurement of endothelin 1 and nitric oxide metabolites

ET-1 and NOx levels were measured in peripheral blood and in hepatic venous blood of some patients, before and after surgery. Plasma ET-1 concentration was determined by means of radioimmunoassay with a rabbit anti-ET-1 serum (Peninsula Laboratories, Belmont, California, USA)[16]. Plasma nitric oxide metabolites (nitrite and nitrate as NOx) were measured using high-pressure liquid chromatography[10,16]. Nitrate ($NO_3^-$) in each sample was reduced by the cadmium column to nitrite ($NO_2^-$), and the concentration of an azo dye compound formed from nitrite by the Griess reaction was measured spectrophotometrically at 540 nm.

## Statistical analysis

All results are reported as mean(s.d.) or median (range). Linear regression analyses were used to assess the correlations between splenic size and each parameter. Data before and after surgery were compared using Student's *t* tests for paired data or the Wilcoxon signed-rank test, as appropriate. $P < 0{\cdot}050$ was considered statistically significant. All calculations were performed using the software package StatView® version 5.0 for Windows® (SAS Institute, Cary, North Carolina, USA).

**Table 1** Patient characteristics

| | No. of patients ($n = 59$) |
|---|---|
| Age (years)* | 57·5 (9·2) |
| Sex ratio (M : F) | 30 : 29 |
| Aetiology of cirrhosis | |
| HBV | 6 (10) |
| HCV | 48 (81) |
| Alcoholism | 3 (5) |
| Other | 2 (3) |
| Child–Pugh class | |
| A | 20 (34) |
| B | 30 (51) |
| C | 9 (15) |
| Child–Pugh score* | 7·4 (1·6) |
| Ascites | |
| Yes | 24 (41) |
| No | 35 (59) |
| Encephalopathy | |
| Yes | 0 (0) |
| No | 59 (100) |
| Platelet count ($\times 10^3/\mu l$)* | 50 (15) |
| Leucocytes (per μl)* | 2898 (948) |
| Oesophageal varices | |
| Yes | 26 (44) |
| No | 33 (56) |
| Splenic weight (g)* | 556 (318) |
| Indication | |
| Bleeding tendency† | 14 (24) |
| Difficulty in induction or continuation of IFN therapy | 33 (56) |
| Severe portal hypertension‡ | 12 (20) |

Values in parentheses are percentages unless indicated otherwise; *values are mean(s.d.). †Platelet count below $30 \times 10^3/\mu l$; ‡severe portal hypertensive gastropathy, endoscopic treatment-resistant oesophageal varices or refractory ascites. HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon.

## Results

A total of 97 patients with liver cirrhosis and portal hypertension underwent laparoscopic splenectomy in the study interval. Of these, 38 were excluded: five with persistent hypersplenism after LDLT, 15 who had concomitant treatment for HCC (radiofrequency ablation or hepatic resection), 11 who underwent concomitant occlusion of huge splenorenal shunts, and seven with postoperative PV thrombosis. Thus, 59 patients with liver cirrhosis and portal hypertension were included in the study (*Table 1*).

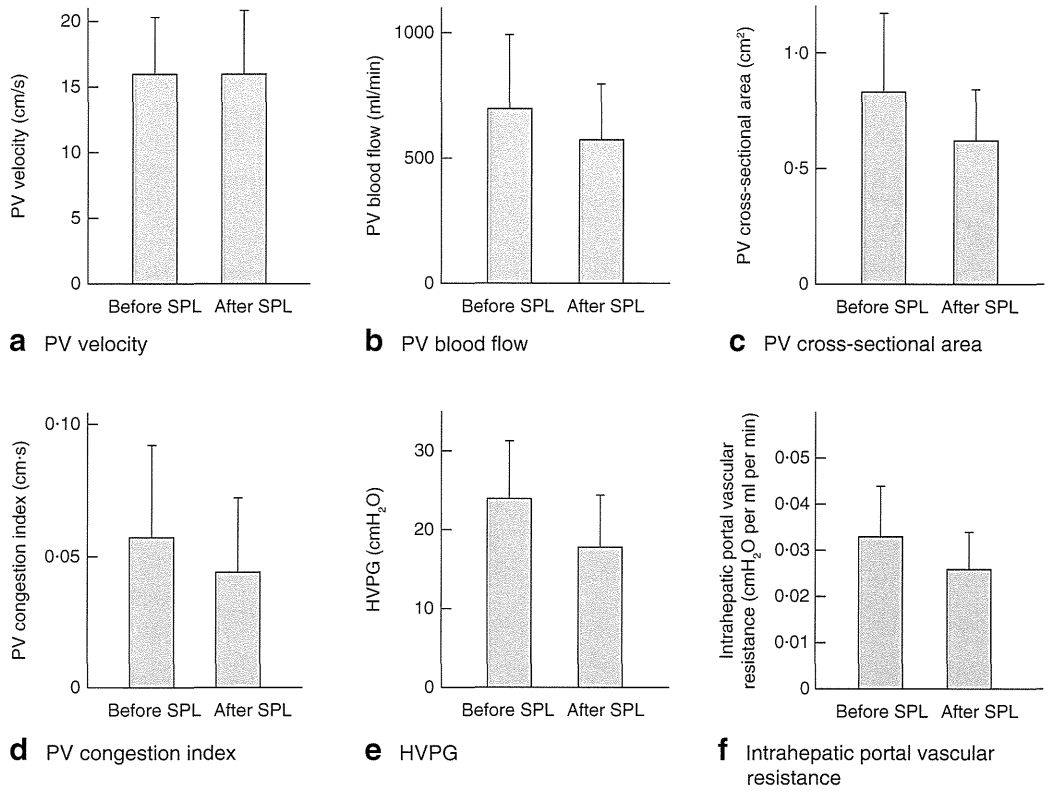



**Fig. 1** Comparison of **a** portal venous (PV) velocity, **b** PV blood flow, **c** PV cross-sectional area, **d** PV congestion index, **e** hepatic venous pressure gradient (HVPG) and **f** intrahepatic portal vascular resistance in patients before and after splenectomy (SPL). Values are mean(s.d.). **a** $P = 0{\cdot}928$, **b,c,e** $P < 0{\cdot}001$, **d** $P = 0{\cdot}035$, **f** $P = 0{\cdot}009$ (before *versus* after SPL, Student's *t* test)

## Surgical outcomes of laparoscopic splenectomy in patients with cirrhosis and portal hypertension

For safety reasons, HALS was performed in 23 (39 per cent) of the 59 patients. The remaining 36 patients had totally laparoscopic splenectomy; none required conversion to open splenectomy and there were no deaths related to laparoscopic splenectomy. The mean duration of surgery was 247(69) min and median blood loss was 143 (10–900) g. No patient had postoperative pancreatic fistula or post-operative bleeding requiring emergency haemostasis.

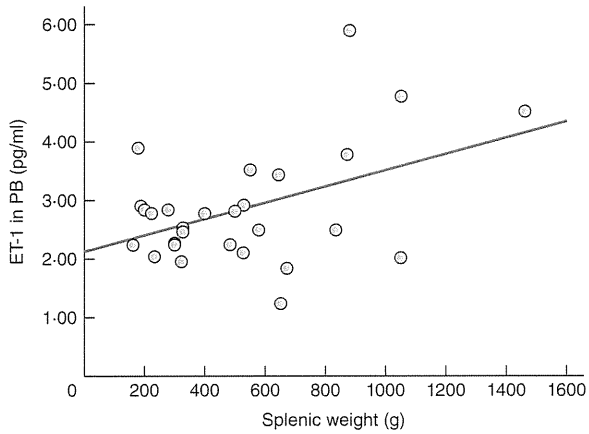
## Relationship between splenic size and portal haemodynamics

As the haemodynamic characteristics of the right and left portal veins and the main portal trunk were similar, only the characteristics of the right portal vein are shown (*Fig. S1*, supporting information). PV blood flow, cross-sectional area and congestion index were significantly correlated with splenic size, whereas PV velocity and HVPG were not.

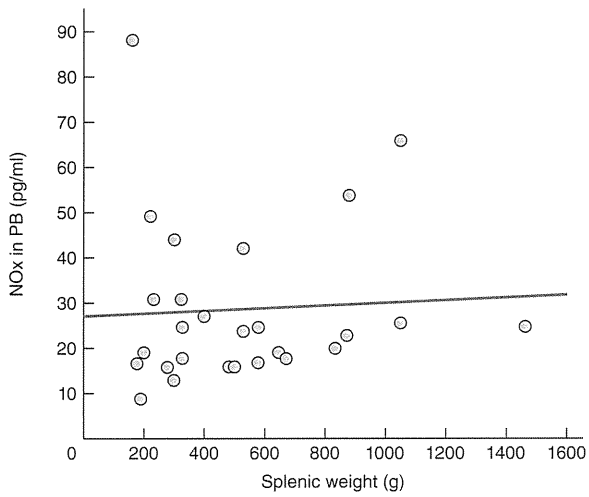
## Changes in portal haemodynamics after splenectomy

Haemodynamic changes in the right portal vein are shown in *Fig. 1*. Although PV velocity did not change significantly after splenectomy, PV blood flow, cross-sectional area and congestion index decreased significantly.

In the subgroup of 18 patients assessed for HVPG, the gradient decreased by a mean of 25(14) per cent after splenectomy ($P < 0{\cdot}001$) (*Fig. 1e*). In this subgroup, mean PV blood flow decreased from 716(245) to 623(206) ml/min ($P < 0{\cdot}001$) (mean reduction 12(18) per cent). Intrahepatic portal vascular resistance also decreased after splenectomy ($P = 0{\cdot}009$), with a mean reduction of 21(21) per cent (*Fig. 1f*). These patients were divided into those with mild (splenic weight less than 500 g) and those with severe (splenic weight 500 g or more) splenomegaly, and changes in portal haemodynamics were compared in these two groups. In the six patients with mild splenomegaly (mean splenic weight 229(52) (range 170–300) g), mean

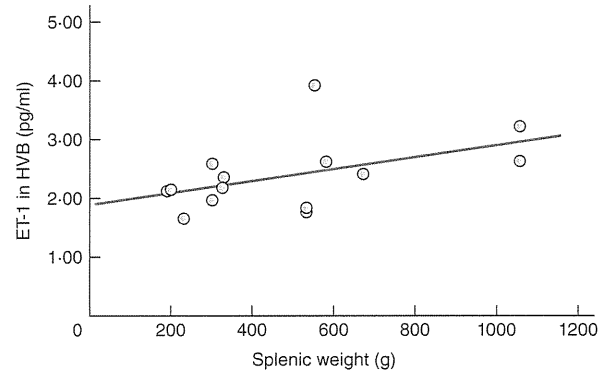



**a** ET-1 in PB and splenic weight

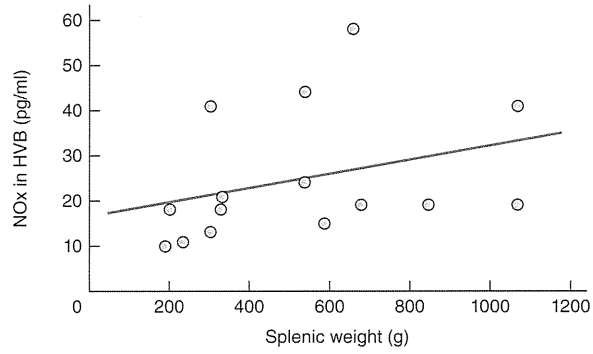



**b** NOx in PB and splenic weight

**Fig. 2** Correlation between splenic size and concentration of **a** endothelin (ET) 1 and **b** nitric oxide metabolites (NOx) in peripheral blood (PB). **a** $R = 0{\cdot}446$, $P = 0{\cdot}017$; **b** $R = 0{\cdot}049$, $P = 0{\cdot}807$ (linear regression analysis)




**a** ET-1 in HVB and splenic weight




**b** NOx in HVB and splenic weight

**Fig. 3** Correlation between splenic size and concentration of **a** endothelin (ET) 1 and **b** nitric oxide metabolites (NOx) in hepatic venous blood (HVB). **a** $R = 0{\cdot}483$, $P = 0{\cdot}080$; **b** $R = 0{\cdot}327$, $P = 0{\cdot}234$ (linear regression analysis)

PV blood flow decreased from 655(178) to 589(174) ml/min ($P = 0{\cdot}046$), intrahepatic portal vascular resistance decreased from 0·038(0·010) to 0·026(0·007) $cmH_2O$ per ml per min ($P = 0{\cdot}068$) and HVPG decreased from 23·7(9·0) to 19·6(8·0) $cmH_2O$ ($P = 0{\cdot}048$). In the 12 patients with severe splenomegaly (mean splenic weight 745(305) (range 500–1450) g), mean PV blood flow decreased from 777(290) to 657(235) ml/min ($P = 0{\cdot}007$), intrahepatic portal vascular resistance decreased from 0·031(0·011) to 0·024(0·008) $cmH_2O$ per ml per min ($P = 0{\cdot}048$) and HVPG decreased from 24·4(7·7) to 17·0(5·6) $cmH_2O$ ($P < 0{\cdot}001$). Although there was no difference in the reduction of intrahepatic portal vascular resistance between the two groups, the decreases in PV blood flow and HVPG were significantly greater in patients with severe splenomegaly ($P = 0{\cdot}045$ and $P = 0{\cdot}034$ respectively).

## Relationship between splenic size and levels of endothelin 1 and nitric oxide metabolites

The level of ET-1 in peripheral blood was correlated with splenic size ($R = 0{\cdot}446$, $P = 0{\cdot}017$) (*Fig. 2a*) and was reduced after splenectomy (from 2·95(0·90) to 2·11(0·47) pg/ml; $P < 0{\cdot}001$). The concentration of NOx in peripheral blood did not correlate with splenic size (*Fig. 2b*), but tended to decrease following splenectomy (from 29·2(18·6) to 25·0(10·4) pg/ml; $P = 0{\cdot}068$). The level of ET-1 in hepatic venous blood showed a slight correlation with splenic size ($R = 0{\cdot}483$, $P = 0{\cdot}080$) (*Fig. 3a*) and decreased after splenectomy (from 2·37(0·59) to 1·83(0·37) pg/ml; $P = 0{\cdot}006$). NOx concentration in hepatic venous blood did not correlate with splenic size (*Fig. 3b*), but tended to increase

**Table 2** Changes in haematological data and liver function after splenectomy

| | Child–Pugh class A ($n = 20$) | | | Child–Pugh class B or C ($n = 39$) | | |
|---|---|---|---|---|---|---|
| | Before SPL | 1 month after SPL | $P$† | Before SPL | 1 month after SPL | $P$† |
| Leucocytes (per μl) | 3250(1093) | 5505(1277) | < 0·001 | 2661(810) | 5280(1323) | < 0·001 |
| Platelet count (× $10^3$/μl) | 55(17) | 189(88) | < 0·001 | 46(15) | 174(81) | < 0·001 |
| Total bilirubin (mg/dl) | 1·0(0·4) | 0·8(0·3) | 0·001 | 1·7(0·6) | 1·1(0·5) | < 0·001 |
| Albumin (g/dl) | 3·7(0·4) | 3·8(0·5) | 0·022 | 3·0(0·4) | 3·5(0·5) | < 0·001 |
| Prothrombin time (%) | 86(10) | 87(20) | 0·828 | 66(9) | 77(11) | < 0·001 |
| Ascites* | | | 1·000‡ | | | < 0·001‡ |
| Yes | 0 (0) | 0 (0) | | 24 (62) | 7 (18) | |
| No | 20 (100) | 20 (100) | | 15 (38) | 32 (82) | |
| Child–Pugh score | 5·3(0·5) | 5·3(0·5) | 0·329 | 8·3(1·3) | 6·6(1·1) | < 0·001 |

Values are mean(s.d.) unless indicated otherwise; *values in parentheses are percentages. SPL, splenectomy. †Student's t test, except ‡Wilcoxon signed-rank test.

**Table 3** Changes in portal haemodynamics

| | Child–Pugh class A ($n = 5$) | | | Child–Pugh class B or C ($n = 13$) | | |
|---|---|---|---|---|---|---|
| | Before SPL | After SPL | $P$* | Before SPL | After SPL | $P$* |
| PV blood flow (ml/min) | 709(344) | 572(206) | 0·010 | 693(262) | 577(233) | 0·005 |
| Intrahepatic portal vascular resistance ($cmH_2O$ per ml per min) | 0·025(0·016) | 0·026(0·019) | 0·586 | 0·036(0·009) | 0·025(0·005) | 0·004 |
| HVPG ($cmH_2O$) | 14·2(4·7) | 11·5(5·6) | 0·028 | 27·4(4·7) | 20·1(4·5) | < 0·001 |
| ET-1 level (pg/ml) | | | | | | |
| In peripheral blood | 2·70(0·59) | 1·98(0·48) | 0·033 | 3·08(1·06) | 2·17(0·46) | 0·007 |
| In hepatic venous blood | 2·14(0·27) | 1·87(0·33) | 0·020 | 2·51(0·68) | 1·82(0·40) | 0·019 |
| NOx level (pg/ml) | | | | | | |
| In peripheral blood | 31·0(24·3) | 27·1(13·5) | 0·499 | 30·0(15·6) | 22·2(7·7) | 0·013 |
| In hepatic venous blood | 17·3(7·0) | 15·0(2·0) | 0·667 | 25·9(15·9) | 36·1(23·4) | 0·023 |
| Weight of spleen (g) | 438(241) | – | | 596(365) | – | |

Values are mean(s.d.). SPL, splenectomy; PV, portal venous; HVPG, hepatic venous pressure gradient; ET-1, endothelin 1; NOx, nitric oxide metabolites. *Student's *t* test.

following splenectomy (from 24·5(14·4) to 30·9(20·7) pg/ml; $P = 0·067$).

## Changes in haematological data, liver function and oesophageal varices after splenectomy

Leucocyte and platelet counts were significantly higher 1 month after splenectomy compared with preoperative values (*Table 2*). Improvements in total bilirubin and albumin concentrations were observed in patients with Child–Pugh A and those with Child–Pugh B/C, and improvements in prothrombin time occurred in patients with Child–Pugh B/C 1 month after splenectomy. The probability of ascites requiring diuretics was lower 1 month after than before splenectomy. None of the 59 patients had encephalopathy before or after splenectomy; thus, none showed worsening of liver function, and patients with Child–Pugh B/C had improvements in the Child–Pugh score. Even in the nine patients with Child–Pugh C (score 10), mean total bilirubin concentration decreased from 2·2(0·1) to 1·2(0·6) ($P = 0·009$), albumin increased from 2·6(0·2) to 3·0(0·5) ($P = 0·032$), prothrombin time increased from 61(7) to 71(11) ($P = 0·002$), and the Child–Pugh score decreased from 10(0) to 7·9(1·1) ($P = 0·002$).

Risky oesophageal varices, observed in 26 (44 per cent) of the 59 patients before splenectomy, were found in only 12 patients (20 per cent) after splenectomy ($P < 0·001$). All 12 patients underwent successful endoscopic variceal ligation.

## Changes in portal haemodynamics categorized by liver function

HVPG was significantly reduced after splenectomy both in patients with Child–Pugh A and in those Child–Pugh B/C (*Table 3*). There was a greater percentage reduction in the latter group, owing to the significant decrease in intrahepatic portal vascular resistance, despite the similar reduction in PV blood flow in the two groups. The ET-1 concentration in peripheral blood and hepatic venous blood was significantly lower after splenectomy in both groups, whereas the level of NOx was significantly reduced in peripheral blood and significantly increased in hepatic venous blood after splenectomy only in the Child–Pugh B/C group.

## Discussion

Orthotopic liver transplantation is the best therapeutic option for liver cirrhosis and portal hypertension, but many patients are currently awaiting this operation because of the lack of liver donors. This problem is especially serious in Japan, because LDLT is the main form of liver transplantation. Recent advances in laparoscopic surgery and the treatment of PV thrombosis have made splenectomy more safe and less invasive, even in patients with liver cirrhosis and portal hypertension[5–7]. In Japan, splenectomy is of increasing importance for patients with liver cirrhosis and portal hypertension, and as a bridging therapy to LDLT[4]. The present study was therefore designed to assess the role of splenomegaly in the pathogenesis of liver cirrhosis and portal hypertension, and the impact of laparoscopic splenectomy on portal haemodynamics.

During laparoscopic splenectomy, the splenic artery and vein were transected simultaneously using an endoscopic linear vascular stapler without intraoperative ligation of the splenic artery. It was assumed that the weight of the resected spleen was representative of splenic volume. Patients in this study had liver cirrhosis of any stage (Child–Pugh score 5–10), except for end-stage disease, along with portal hypertension. All patients underwent laparoscopic splenectomy safely using a standardized technique[5], with no serious complications. Therefore, this population of patients with liver cirrhosis and portal hypertension was considered suitable for investigating the role of splenomegaly and the portal haemodynamic effects of splenectomy as a single operation.

Splenic size was associated with increases in PV blood flow and congestion index, but not HVPG, consistent with previous findings[3]. Portal hypertension in liver cirrhosis is characterized by increased intrahepatic vascular resistance and increased splanchnic blood flow, which may explain the lack of correlation between splenic size and HVPG. Splenectomy was followed by reductions in PV blood flow, congestion index and HVPG, suggesting that splenomegaly contributes actively to the pathogenesis of portal hypertension by increasing splanchnic flow (active congestion).

In liver cirrhosis, ET-1 concentration is increased in both the splanchnic and the systemic circulation[17–19]. ET-1 in liver cirrhosis is thought to derive from hepatic stellate cells and the splanchnic bed, including the spleen. The ET-1 concentration in peripheral blood correlated, and the level in hepatic venous blood tended to correlate, with splenic size, with ET-1 levels in both peripheral and hepatic venous blood decreasing significantly after splenectomy. These findings suggest that the spleen may be a major source of ET-1 in patients with liver cirrhosis, and that spleen-derived ET-1 could reach the hepatic and systemic circulation via the splanchnic circulation.

Splenectomy reduced HVPG by 25 per cent, more than that expected from the reduction in PV blood flow. Intrahepatic portal vascular resistance was also reduced, by 21 per cent following splenectomy. Intrahepatic portal vascular resistance is regulated by the contractility of hepatic stellate cells, which is regulated by the balance between vasoactive agents such as ET-1 and vasorelaxing agents such as nitric oxide (NO). ET-1 has dual vasoactive effects, mediating vasoconstriction by binding to endothelin A ($ETR_A$) and endothelin B ($ETR_B$) receptors on hepatic stellate cells and vasodilatation by binding to $ETR_B$ on sinusoidal endothelial cells, resulting in the production of NO[11,17]. In liver cirrhosis, ET-1 production is increased in the liver and splanchnic circulation, upregulating ETR–rho-kinase signalling and resulting in the contraction of hepatic stellate cells[17,18]. In sinusoidal endothelial cells of liver cirrhosis, NO production is reduced by impaired signalling of $ETR_B$-mediated NO production[10,11,20]. Decreased sinusoidal circulation reduces shear stress on sinusoidal endothelial cells, leading to a further decrease in NO production[11]. Thus, in cirrhotic liver, increased levels of ET-1 and decreased levels of NO contribute to the contraction of hepatic stellate cells and increased intrahepatic vascular resistance. In the present study, splenectomy significantly reduced the level of ET-1 and increased the level of NOx in hepatic venous blood. Splenectomy, by eliminating spleen-derived ET-1, may therefore lead to relaxation of hepatic stellate cells and a reduction of intrahepatic portal vascular resistance. Subsequent improvements in the sinusoidal circulation may increase the shear stress on sinusoidal endothelial cells, restoring NO production.

The splanchnic and systemic hyperdynamic circulation in liver cirrhosis and portal hypertension is characterized by increased NO production in endothelial cells and decreased response to vasoconstrictive agents such as ET-1. Despite the increase in ET-1 concentration, splanchnic and systemic vascular tone is reduced, possibly owing to increased $ETR_B$-mediated NO production in endothelial cells and the decreased responses to ET-1 resulting from impaired ETR–rho-kinase signalling in vascular smooth muscle cells[11,17,21,22]. The resulting increases in splanchnic and systemic blood flow result in additional NO overproduction, worsening the hyperdynamic circulation. As a result, raised levels of ET-1 and NO are associated with splanchnic and systemic hyperdynamic circulation. Splenectomy reduced the levels of both ET-1 and NOx in peripheral blood, the former significantly. Splenectomy may decrease systemic ET-1 concentration

by eliminating spleen-derived ET-1, subsequently reducing $ETR_B$-mediated NO production in endothelial cells. Although increasing levels of NO in hepatic venous blood and decreasing levels of NO in peripheral blood after splenectomy are seemingly contradictory findings, these results suggest that splenectomy may improve not only intrahepatic portal vascular resistance, but also splanchnic and systemic hyperdynamic circulation.

Long-term clinical studies have consistently reported that reducing HVPG below 12 mmHg, or by at least 20 per cent, markedly lowers the risk of variceal bleeding[14]. In the present study, splenectomy reduced HVPG by 25 per cent, surpassing the therapeutic goals for oesophageal varices. Risky oesophageal varices were eradicated by laparoscopic splenectomy alone in most patients. However, several papers from the 1940s and 1950s reported that splenectomy failed to control variceal bleeding[2]. In contrast, splenectomy with oesophageal transection was able to control variceal haemorrhage[3,4], because the procedure has two effects in treating varices: reducing PV pressure and obliterating varices by devascularization of perioesophagogastric collateral vessels. Following splenectomy, reduced PV pressure can lead to a transient diminution in oesophageal varices, but these varices may cause problems without further treatment. Endoscopic treatments for oesophagogastric varices have been shown to be effective, safe and easy to perform[23]. Therefore, laparoscopic splenectomy in combination with endoscopic treatment should have the same effects as oesophageal transection, with the former being less invasive and safer. Currently, in the present authors' institution, laparoscopic splenectomy is not considered first-line therapy for risky oesophageal varices, but is regarded as an alternative or adjunctive procedure.

Interestingly, liver function improved after splenectomy, with more pronounced improvements in patients with Child–Pugh B/C (score 7–10). The improvements in intrahepatic portal vascular resistance, together with the normalization of ET-1 and NOx concentrations may explain, at least in part, the mechanisms underlying the improvements in liver function. Expression of $ETR_A$ and $ETR_B$ was enhanced on hepatic stellate cells of patients with liver cirrhosis, with the levels of expression of these receptors correlating with the degree of portal hypertension[24]. Therefore, the same decrease in ET-1 concentration may result in a greater reduction of intrahepatic portal vascular resistance and a greater increase in resulting NO production in patients with Child–Pugh B/C than in those with Child–Pugh A. In the peripheral circulation, ET-1 in advanced liver cirrhosis is associated with reduced vascular tone, owing to increased $ETR_B$-mediated NO production in endothelial cells, whereas ET-1 in early liver cirrhosis still contributes to the maintenance of vascular tone[25]. Therefore, the same reduction of ET-1 by splenectomy may result in a greater decrease in $ETR_B$-mediated NO production in endothelial cells in patients with Child–Pugh B/C than in those with Child–Pugh A.

The portal decompressing effect was significantly greater in severe (splenic weight 500 g or more) than in mild splenomegaly. This effect was also greater in patients with Child–Pugh B/C than in those with Child–Pugh A. Splenectomy may therefore have fewer benefits, except for prolonged haematological effects, in patients with Child–Pugh A and mild splenomegaly, where the aim is often induction of IFN therapy, than in those with Child–Pugh B/C or severe splenomegaly. If patients with Child–Pugh A require short-term improvements in thrombocytopenia during IFN therapy, partial splenic artery embolization or the use of eltrombopag, a new oral platelet growth factor, may be a better choice than laparoscopic splenectomy[8,26]. In patients with a Child–Pugh score of 7–10 or severe splenomegaly, many of whom have severe portal hypertension, laparoscopic splenectomy may be optimal, although long-term follow-up is still necessary. Laparoscopic splenectomy may provide clinically meaningful outcomes in patients who otherwise would be marginal candidates for liver transplantation. Moreover, it may be useful as a bridging therapy to liver transplantation.

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**Supporting information**

Additional supporting information may be found in the online version of this article:

**Fig. S1** Correlation between splenic size and portal venous (PV) velocity, PV blood flow, PV cross-sectional area, PV congestion index and hepatic venous pressure gradient (HVPG) (TIFF file)

# Optimizing Risk Stratification in Portal Vein Thrombosis after Splenectomy and its Primary Prophylaxis with Antithrombin III Concentrates and Danaparoid Sodium in Liver Cirrhosis with Portal Hypertension

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**BACKGROUND:** Decreased antithrombin III (ATIII) activity and large splenic vein diameter (SVD) are risk factors for portal vein thrombosis (PVT) after splenectomy in liver cirrhosis with portal hypertension. Antithrombin III concentrates can prevent PVT. This study was designed to stratify risks for PVT after splenectomy in cirrhotic patients and to develop prophylactic protocols for PVT.

**STUDY DESIGN:** In 53 patients (testing cohort), the cutoff level of preoperative ATIII activity ($\leq$60%) was evaluated for administration of ATIII concentrates. Antithrombin III activity and SVD were re-evaluated as criteria for prophylaxis of PVT. In 57 patients (validation cohort), the risk stratification of PVT and prophylactic protocols were validated.

**RESULTS:** In the testing cohort, 10 (19%) of 53 patients had PVT. Risk level of PVT was stratified and prophylactic protocols were developed. Patients at low risk (ATIII activity $\geq$70% and SVD $<$10 mm) were not treated; those at high risk (ATIII activity $<$70% or SVD $\geq$10 mm) received ATIII concentrates (1,500 U/day) for 3 days; and those at highest risk (SVD $\geq$15 mm) received ATIII concentrates for 3 days, followed by danaparoid sodium (2,500 U/day) for 14 days and warfarin. In the validation cohort, 0 of 14 low-risk and 2 of 32 high-risk patients had PVT. Although 8 of 11 patients at highest risk had temporary PVT, it disappeared within 3 months postoperatively. Finally, only 2 (3.5%) of 57 patients had PVT.

**CONCLUSIONS:** Risk stratification of PVT after splenectomy and prophylaxis with ATIII concentrates and danaparoid sodium dramatically reduced the incidence of PVT. (J Am Coll Surg 2014; 219:865–874. © 2014 by the American College of Surgeons)

Recent technical advances in laparoscopic surgery have enabled safer and less-invasive laparoscopic splenectomy, even in patients with liver cirrhosis and portal hypertension.[1] The evolution of treatments for chronic liver diseases, such as interferon (IFN) therapy for hepatitis C virus (HCV), has shed light on laparoscopic splenectomy for liver cirrhosis and hypersplenism.[2] Currently, splenectomy is performed to improve thrombocytopenia before the start of IFN therapy for HCV infection[3] and during treatment for hepatocellular carcinoma.[4] Splenectomy is also performed concurrently with living donor liver transplantation to reduce graft congestion and after living donor liver transplantation to alleviate persistent portal hypertension and thrombocytopenia.[5,6] Although portal vein thrombosis (PVT) is not a rare complication of splenectomy and can be fatal in patients with liver cirrhosis, it remains to be resolved. It is important to determine risk factors for PVT and to prevent PVT after splenectomy in cirrhotic patients.

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**Abbreviations and Acronyms**

ATIII = antithrombin III
HCV = hepatitis C virus
IFN = interferon
LMWH = low-molecular weight heparin
POD = postoperative day
PVT = portal vein thrombosis
ROC = receiver operating characteristic
SVD = splenic vein diameter
UFH = unfractionated heparin
US = ultrasonography

Bleeding complications, such as esophageal variceal hemorrhage, are frequent in patients with liver cirrhosis. These patients are considered to be in a state of auto-anticoagulation, accompanied by prolonged prothrombin time and INR, as well as thrombocytopenia.[7] Hemostatic systems in patients with liver cirrhosis are delicately balanced between pro- and anticoagulant factors and can be easily tipped to a hypo- or hypercoagulable status, resulting from decreased levels of procoagulant and anticoagulant factors synthesized by hepatocytes and sinusoidal cells.[7-9] Hypercoagulability has an underestimated but crucial role in many aspects of liver cirrhosis. Thrombin generation, as a more balanced marker of coagulation status, is normal, despite the abnormal prothrombin time and INR.[10-12] Reduced production of anticoagulants, such as protein C, protein S, and antithrombin III (ATIII), coupled with the increased production of the procoagulants factor VIII and von Willebrand factor, put cirrhotic patients at increased risk for hypercoagulopathy.[8,13,14]

Portal vein thrombosis is a frequent complication in patients with liver cirrhosis, with a prevalence of about 8% to 25%.[7-9] Portal vein thrombosis results from several local and systemic factors, including decreased portal venous flow, hypercoagulable status, and genetic thrombophilic factors, such as factor V Leiden and prothrombin mutations.[15-17] Cirrhotic patients after splenectomy show decreased levels of ATIII activity, which are associated with hypercoagulable status, and reduced portal venous flow, resulting from the elimination of increased splenic blood flow. This has been found to amplify the incidence of PVT considerably, as much as 24% to 36%.[18,19] Although antithrombotic prophylaxis is recommended for patients at high risk for thrombotic complications, including splenectomy in patients with liver cirrhosis, safe and effective prophylactic methods that do not increase the risk of bleeding have not been identified in cirrhotic patients.

The results of our previous study suggested that prophylactic administration of ATIII concentrates, correcting one of the risk factors for PVT, can prevent PVT without increasing the risks of postoperative hemorrhage after splenectomy.[18] Antithrombin III concentrates can restore the hemostatic balance from a hypercoagulable status to equilibrium. However, it remains unclear whether administration of ATIII concentrates is necessary in all splenectomized patients, or whether ATIII concentrates alone can prevent PVT in patients at higher risk for this complication. We sought to stratify risk levels of PVT after splenectomy in patients with liver cirrhosis and portal hypertension and to establish a prophylactic protocol for preventing PVT. In the testing cohort, we initially determined the cutoff level of preoperative ATIII activity as an indicator of PVT, as described in our previous study,[18] and administered ATIII concentrates based on this cutoff. We re-evaluated the results of the testing cohort by ATIII activity and splenic vein diameter (SVD), which is related to decreased portal venous flow,[19] to stratify the risk levels of PVT, and we developed a prophylactic protocol centered on ATIII concentrates to prevent PVT after splenectomy. In the validation cohort, we validated the stratified risk level of PVT and the prophylactic protocol for PVT after splenectomy in patients with liver cirrhosis and portal hypertension.

## METHODS

### Testing cohort

Our previous study showed that 9 (36.0%) of 25 cirrhotic patients who received no prophylactic anticoagulation therapy had postoperative PVT develop, and that preoperative low ATIII activity was the most important predictive factor for PVT after splenectomy.[18] Using receiver operating characteristic (ROC) curve analysis to define the cutoff of preoperative ATIII activity to diagnose postoperative PVT in these 25 patients, we found that the area under the ROC curve was 0.852 (Fig. 1). A threshold of ATIII activity $\leq$61% to predict PVT had a sensitivity of 100% and a specificity of 67%; a threshold of ATIII activity $\leq$53% had a sensitivity of 78% and a specificity of 73%; and a threshold of ATIII activity $\leq$48% had a sensitivity of 78% and a specificity of 80%. To reduce the incidence of false negatives as much as possible, the initial criteria for administering ATIII concentrates to prevent PVT was set at ATIII $\leq$60%.

Fifty-three patients (26 male and 27 female; mean age 60.6 $\pm$ 8.6 years; range 39 to 77 years) with liver cirrhosis and portal hypertension who underwent laparoscopic splenectomy in the Department of Surgery, Fukuoka City Hospital from April 2008 to March 2011 were prospectively enrolled (Table 1). Of these patients, 2 had hepatitis B virus–related cirrhosis, 45 had HCV-related cirrhosis, 1

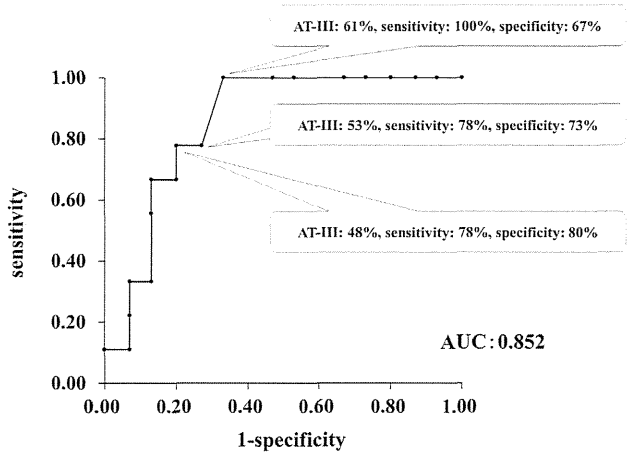


**Figure 1.** Receiver operating characteristics curves for cutoff levels of preoperative antithrombin III (AT-III) activity to diagnose portal vein thrombosis (PVT) in 25 cirrhotic patients who underwent splenectomy with no prophylactic treatment of PVT. AUC, area under the receiver operating characteristics curves.

had alcoholic cirrhosis, 2 had primary biliary cirrhosis, and 3 had cirrhosis of unknown origin. Child-Pugh class was class A in 22 patients, class B in 23, and class C in 8 patients. Indications for splenectomy included bleeding tendency due to thrombocytopenia (platelet counts $<30 \times 10^3/\mu L$; n = 7), difficulties in induction or continuation of pegylated IFN therapy due to thrombocytopenia (n = 19), and severe portal hypertension, such as endoscopic treatment-resistant esophagogastric varices, portal hypertensive gastropathy bleeding, and refractory ascites (n = 27). At enrollment, all the patients underwent Doppler ultrasonography (US), and contrast-enhanced CT. None of the patients had thrombi in the portal vein or its branches, the mesenteric veins, or the splenic veins.

According to the initial criteria, 37 of the 53 cirrhotic patients in the testing cohort had ATIII activity ≤60% and received intravenous infusions of 1,500 units ATIII concentrates (Anthrobin P; CSL Behring) for approximately 1 hour on postoperative days (PODs) 1, 2, and 3. The dosage of ATIII concentrates was based on the disseminated intravascular coagulation guidelines of the Japanese Ministry of Health, Labor and Welfare.[18,20] The other 16 cirrhotic patients had ATIII activity >60% and received no prophylactic therapy. After the operation, all 53 patients were carefully screened for PVT by Doppler US on POD 3 and CT on PODs 7, 30, and 90. Patients who showed evidence of splanchnic vein thrombosis on Doppler US underwent CT to confirm the extent of thrombosis. Portal vein thrombosis was defined as complete or partial occlusion of the portal vein trunk and its

**Table 1.** Characteristics of Cirrhotic Patients Who Underwent Laparoscopic Splenectomy With or Without Prophylactic Treatment With Antithrombin III Concentrates

| Factors | ATIII(+) group (n = 37; ATIII ≤60%) | ATIII(−) group (n = 16; ATIII >60%) | p Value |
|---|---|---|---|
| Sex, male to female ratio | 16:21 | 10:6 | 0.241 |
| Age, y, mean ± SD | 61.9 ± 8.8 | 59.6 ± 8.3 | 0.575 |
| Cause, n | | | |
| HBV | 1 | 1 | 0.278 |
| HCV | 32 | 13 | |
| Alcoholism | 0 | 1 | |
| PBC | 2 | 0 | |
| Unknown | 2 | 1 | |
| Leukocytes/μL, mean ± SD | 2,797 ± 908 | 3,193 ± 1,121 | 0.190 |
| Platelet counts × $10^3$/μL, mean ± SD | 48 ± 19 | 58 ± 18 | 0.075 |
| Prothrombin time, %, mean ± SD | 69 ± 10 | 85 ± 15 | <0.001 |
| ATIII, %, mean ± SD | 54 ± 15 | 83 ± 18 | <0.001 |
| Child-Pugh score, mean ± SD | 7.8 ± 1.6 | 5.6 ± 0.9 | <0.001 |
| Operative procedure, n | | | |
| Purely laparoscopic SPL | 7 | 8 | 0.043 |
| HALS-SPL | 30 | 8 | |
| Operative time, min, mean ± SD | 288 ± 68 | 235 ± 37 | 0.004 |
| Estimated blood loss, g, mean ± SD | 255 ± 231 | 123 ± 119 | 0.020 |
| Resected spleen weight, g, mean ± SD | 641 ± 318 | 473 ± 194 | 0.049 |
| Splenic vein diameter, mm, mean ± SD | 11.8 ± 3.1 | 10.4 ± 2.1 | 0.035 |
| Portal vein thrombosis, n | 3 | 7 | 0.005 |

ATIII, antithrombin III; HALS, hand-assisted laparoscopic surgery; HBV, hepatitis B virus; HCV, hepatitis C virus; PBC, primary biliary cirrhosis; SPL, splenectomy.

branches and the superior mesenteric veins, but not of the splenic vein.[18] Although thrombosis at the splenoportal confluence extending from the splenic vein thrombosis was defined as a PVT and was treated, thrombosis in the splenic vein was not considered a PVT and was not treated.

After the start of this study, we found that SVD correlated significantly with decreased portal venous flow and was related to development of PVT after splenectomy in patients with liver cirrhosis and portal hypertension.[19] Splenic vein diameter was calculated as the mean of the proximal, mid, and distal portions of the splenic vein on CT images. We analyzed the prevalence of PVT after splenectomy not only by ATIII activity but also by SVD. We then stratified the risks of PVT into 3 categories. Low risk was defined as ATIII activity ≥70% and SVD <10 mm; high risk as ATIII activity <70% and/or SVD ≥10 mm; and highest risk as SVD ≥15 mm (Table 2). We developed a prophylactic protocol to prevent PVT, in which patients at low risk received no prophylactic treatment, those at high risk received intravenous ATIII concentrates (1,500 U/day) for 3 days, and those at highest risk received intravenous ATIII concentrates (1,500 U/day) for 3 days, followed by intravenous danaparoid sodium (low molecular weight heparinoid) (2,500 U/day) for 14 days and subsequent warfarin. Before starting warfarin alone, danaparoid sodium and warfarin were combined for 5 days. Warfarin doses were adjusted to achieve an INR of 1.5 to 2.5 (target 2.0), with INR determined every 2 to 3 weeks. Warfarin was continued for 3 months or until the disappearance of PVT was confirmed by CT.

The current study was approved by the Ethics Committees of the participating hospitals and all patients proved written informed consent. The study was also registered in the UMIN Clinical Trials Registry and was assigned ID number UMIN000013134.

### Validation cohort

To validate the risk stratification of PVT and its prophylactic protocol, 57 patients (34 male and 23 female; mean age 56.8 ± 9.7 years; range 27 to 75 years) with liver cirrhosis and portal hypertension who underwent laparoscopic splenectomy in the Department of Surgery, Fukuoka City Hospital and Department of Surgery and Science, Kyushu University from April 2011 to September 2013 were enrolled in the validation cohort (Table 3). Of these patients, 10 had hepatitis B virus–related cirrhosis, 40 had HCV-related cirrhosis, 5 had alcoholic cirrhosis, and 2 had cirrhosis of unknown origin. Child-Pugh class was class A in 26 patients, class B in 27, and class C in 4. Indications for splenectomy included bleeding tendency due to thrombocytopenia (platelet counts $<30 \times 10^3/\mu L$; n = 14), difficulties in induction or continuation of pegylated IFN therapy due to thrombocytopenia (n = 21), and severe portal hypertension, such as endoscopic treatment-resistant esophagogastric varices, portal hypertensive gastropathy bleeding, and refractory ascites (n = 22). After the operation, all of the patients were carefully screened for PVT by Doppler US on POD 3 and CT on PODs 7, 30, and 90.

### Operative procedures for laparoscopic splenectomy

Laparoscopic splenectomy was performed as described previously.[1] Preoperative CT was performed to evaluate the splenic volume and to determine the location and extent of the collateral vessels. Patients with spleens measuring ≥600 mL, as assessed by CT volumetry, those with large perisplenic collateral vessels, and/or those with Child-Pugh score of ≥9 underwent hand-assisted laparoscopic surgery for safety reasons. Patients were placed in a semilateral position with the left flank elevated at a 60-degree angle. A 12-mm laparoscope trocar was inserted through an incision on the left side of the umbilicus, made by a minimal open laparotomy. A $CO_2$-pneumoperitoneum was created using a high-flow electric insufflator, and 3 other trocars were inserted under visual control, 1 each into the epigastric area, the midclavicular line of the subcostal line, and the left flank. The hand port for hand-assisted laparoscopic surgery was inserted through a 7-cm midline incision in the epigastric area. Splenic attachments were divided using electrocautery, an ultrasound dissector, and/or the LigaSure vessel sealing system. Once the upper

**Table 2.** Risk Stratification of Portal Vein Thrombosis after Splenectomy and Prophylactic Treatments for its Prevention in Liver Cirrhosis and Portal Hypertension

| Risk level | Factors | Prophylactic treatments |
|---|---|---|
| Low risk | ATIII activity ≥70% and SVD <10 mm | No treatment |
| High risk | ATIII activity <70% or SVD ≥10 mm | ATIII monotherapy* |
| Highest risk | SVD ≥15 mm or huge hepatofugal collateral vessels ≥10 mm† | ATIII combined therapy followed by danaparoid sodium and warfarin‡ |

*Antithrombin III concentrates are intravenously administered at a dose of 1,500 units once daily from POD 1 to POD 3.
†For patients with huge collateral vessels of ≥10 mm in diameter, careful systematic screening with repeated Doppler ultrasonography and/or CT is necessary.
‡Danaparoid sodium is intravenously administered at a dose of 1,250 units twice daily from POD 4 for 14 days. Subsequently, warfarin is administered adjusted to prothrombin INR of 1.5 to 2.5 (target 2.0) up to elimination of PVT or 3 months after splenectomy. Before starting warfarin, danaparoid sodium and warfarin are combined for 5 days.
ATIII, antithrombin III; PVT, portal vein thrombosis; SVD, splenic vein diameter.

**Table 3.** Characteristics of Cirrhotic Patients Categorized by Risk Level of Portal Vein Thrombosis After Splenectomy

| Factors | Low risk (n = 14) | High risk (n = 32) | Highest risk (n = 11) | p Value |
|---|---|---|---|---|
| Sex, n | | | | |
| Male | 4 | 21 | 9 | 0.012 |
| Female | 10 | 11 | 2 | |
| Age, y, mean ± SD | 59.6 ± 11.7 | 56.6 ± 9.5 | 53.6 ± 6.9 | 0.133 |
| Cause, n | | | | |
| HBV | 2 | 3 | 5 | 0.007 |
| HCV | 12 | 25 | 3 | |
| Alcoholism | 0 | 3 | 2 | |
| Unknown | 0 | 1 | 1 | |
| Leukocytes/μL, mean ± SD | 3,704 ± 1253 | 3,107 ± 1273 | 2,276 ± 818 | 0.023 |
| Platelet counts $\times 10^3$/μL, mean ± SD | 66 ± 12 | 55 ± 23 | 40 ± 12 | 0.001 |
| Prothrombin time, %, mean ± SD | 90 ± 9 | 71 ± 11 | 62 ± 11 | <0.001 |
| ATIII, %, mean ± SD | 84 ± 12 | 58 ± 13 | 62 ± 9 | <0.001 |
| Child-Pugh score, mean ± SD | 5.3 ± 0.6 | 7.0 ± 1.6 | 8.3 ± 1.3 | <0.001 |
| Operative procedure, n | | | | |
| Purely laparoscopic SPL | 12 | 11 | 0 | <0.001 |
| HALS SPL | 2 | 21 | 11 | |
| Operative time, min, mean ± SD | 208 ± 58 | 257 ± 62 | 271 ± 64 | 0.044 |
| Estimated blood loss, g, mean ± SD | 89 ± 100 | 160 ± 194 | 173 ± 224 | 0.272 |
| Resected spleen weight, g, mean ± SD | 274 ± 104 | 590 ± 293 | 1092 ± 403 | <0.001 |
| Splenic vein diameter, mm, mean ± SD | 8.2 ± 0.8 | 10.7 ± 2.7 | 18.3 ± 3.1 | <0.001 |
| PVT, n | | | | |
| Yes | 0 | 2 | 0 (8*) | 0.431 |
| No | 14 | 30 | 11 | |

*Temporal occurrence of PVT.
ATIII, antithrombin III; HALS, hand-assisted laparoscopic surgery; HBV, hepatitis B virus; HCV, hepatitis C virus; PVT, portal vein thrombosis.

pole of the spleen was dissected entirely away from the diaphragm, the splenic hilar pedicles were transected with an endoscopic linear vascular stapler. The resected spleen was placed into a plastic bag, morcellated, and extracted. Operation time was measured from initial incision to skin closure. Splenic weight was based on the weight of the morcellated spleen.

### Anticoagulation therapy for portal vein thrombosis

Anticoagulation therapy was started on the Doppler US and/or CT detection of a complete or partial PVT. Treatment of patients who received no prophylaxis for PVT consisted of intravenous ATIII concentrates (1,500 U/day) for 3 days, followed by intravenous danaparoid sodium (2,500 U/day) for 14 days and subsequent warfarin, as described here. Treatment of patients who received prophylaxis with ATIII concentrates consisted of intravenous danaparoid sodium (2,500 U/day) for 14 days followed by warfarin.

### Statistical analysis

Patient characteristics were compared using Student's *t*-tests for parametric data, Mann-Whitney U tests for nonparametric data, and chi-square tests for categorical data. A p value <0.050 was considered statistically significant. In the testing cohort, the sensitivity and specificity for predicting PVT were calculated for various threshold values of preoperative ATIII activity, and the initial criteria for PVT prophylaxis were determined by ROC analysis. In the validation cohort, the sensitivity and specificity for predicting PVT were calculated for various threshold values of preoperative ATIII activity and SVD. All values were expressed as mean ± SD. All statistical analyses were performed using StatView software version 5.0J (Abacus Concepts).

## RESULTS

### Testing cohort

#### *Prevalence of portal vein thrombosis after splenectomy using the initial criteria for prophylactic treatment with antithrombin III concentrates*

Table 1 shows the characteristics of the cirrhotic patients who underwent laparoscopic splenectomy with or without prophylactic treatment with ATIII concentrates. Of the 53 patients in this cohort, 37 patients with preoperative