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Morphometric demonstration of portal vein stenosis and hepatic arterial medial hypertrophy in patients with biliary atresia.

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Abstract (word limit 200 / now 198 words)

Purpose: Portal hypertension in patients with biliary atresia (BA) is generally thought to result from portal vein (PV) narrowing secondary to hepatic fibrosis. To test the hypothesis, we morphometrically analyzed the PVs and hepatic arteries (HAs).

Methods: Morphometrical analyses of 25 BA and 26 non-BA liver biopsy specimens from patients treated from 2000 to 2014. The total specimen area, the fibrotic portal area, vessel diameter and medial thickness of the HAs were measured.

Results: The PV diameter in BA patients was significantly smaller than that in non-BA patients. In BA, the numbers of normal-sized PVs and capillaries were decreased and increased, respectively. The PV diameter was not significantly correlated with the degree of fibrosis. We newly found that medial hypertrophy and the HA diameter increased with the number of endothelial cells in BA. The PV diameter was not significantly correlated with the medial thickness and was positively correlated with the HA diameter in BA.

Conclusions: The narrowing of the PV is unlikely to occur secondarily to liver fibrosis. The medial hypertrophy of the HA is not correlated with the decrease in the PV blood flow. These findings seem to be unique to the primary vascular lesions of BA.

Key words: Biliary atresia, Portal vein, Hepatic artery, Morphometrical analysis

Introduction

Biliary atresia (BA) is a disease with a poor prognosis that causes refractory obstructive jaundice due to obliteration of the extrahepatic and intrahepatic bile duct during early infancy. Although Kasai portoenterostomy (KPE) has been established as a standard surgical procedure, approximately half of BA patients undergo liver transplantation by 20 years of age because of hepatic failure, repeated cholangitis, progressive liver fibrosis or portal hypertension—even in jaundice-free survivors. The pathogenesis of BA has not been clarified. Various etiopathogenetic hypotheses have been proposed, including viral infection [1] and graft-versus-host disease (GvHD) associated with maternal microchimerism (MMc) [2].

In patients with BA, varying degrees of liver fibrosis have been observed at the time of KPE. The fibrosis often progresses, even if the jaundice disappears after KPE. Portal hypertension, a major cause of liver transplantation, has been intuitively thought to be a result of the fibrosing process. However, only a few reports have described the narrowing of the portal veins in liver biopsy specimens from patients with BA [3,4].

The aim of the present study was to clarify the characteristics of the vascular lesions of the portal veins and hepatic arteries in patients with BA by morphometrical analyses using liver biopsy specimens sampled at KPE and compare them to those of patients without BA.

We herein describe the detailed analyses of the narrowing of portal veins and medial hypertrophy of the hepatic arteries in BA and discuss their possible significance.

1. Materials and methods

1.1. Patients

A total of 25 patients with BA (male, n = 13; female, n = 12) who underwent KPE at our institution from 2000 to 2014 were registered in the present study. The median age at KPE in the BA patients was 62 days (range: 44 - 143 days). Twenty-six patients (male, n = 11; female, n = 15) with various other diseases who underwent liver biopsy during the same period were used as a control group. The median age at the liver biopsy in the non-BA patients was 2 years (range 12 days - 13 years). The underlying diseases of these non-BA patients included choledochal cyst (n = 18), neonatal hepatitis (n = 3) and extensive aganglionosis, neuroblastoma, portosystemic shunt, gastroesophageal reflux and Alagille syndrome (each n = 1).

1.2. Pathological preparations

The liver biopsy specimens were fixed in formalin and embedded in paraffin (FFPE). Combined Verhoeff and Masson trichrome was performed on 4-μm-thick sections to evaluate the degree of fibrosis and tunica media of the artery. As we coincidentally recognized an increase in the lymphatic vessels in the portal areas of the BA livers, we excluded the lymphatic vessels from our morphometric analysis of the vascular structures by double chromogenic immunostaining for CD34 + podoplanin (D2-40) as follows: the antigen retrieval was performed using antigen retrieval buffer at pH 9 (DAKO, Glostrup Denmark) for 30 minutes at 95°C. Mouse mononclonal anti-podoplanin antibody (clone D2-40; DAKO) was applied at 1:200 for 40 minutes at room temperature, followed by the secondary antibody

(anti-mouse Simple stain conjugated by Alkaline phosphatase) (Nichirei, Tokyo, Japan) for 30 min. Fast Red (Nichirei) was used as a chromogen. The specimens were treated with pH 9 buffer for 10 minutes at 95°C. Mouse monoclonal anti-CD34 class II antibody (clone QBEnd-10) was applied at 1:1000 overnight at 4°C. Anti-mouse Simple stain (Horseradish peroxidase; Nichirei) was applied for 30 minutes. DAB (Nichirei) was used as a chromogen. Hematoxylin was used for counterstaining of nuclei.

1.3. The parameters included in the analysis

Fig. 1

Fig. 1a

Fig. 1b

Figure 1 shows a schematic illustration of the measured parameters. Figure 1a shows the portal vein. “D” indicates the shortest portal vein diameter. Figure 1b shows the hepatic artery. “T” indicates the thickness of the hepatic artery media. “d” indicates the shortest diameter of the lumen of the hepatic artery.

The parameters measured in this study were as follows: total area of the specimen, Ishak score [5], total area of the fibrotic portal area that we individually measured for this study, luminal area of the portal vein, diameter of the portal vein (shortest diameter of the histological specimen measured as its true diameter “D”) in Figure 1a, the length of the luminal circumference of the hepatic artery, area and length of the outer circumference of the hepatic artery, diameter of the lumen of the hepatic artery (shortest diameter of the histological specimen measured as its true diameter “d”) in Figure 1b, and the number of endothelial cells in the hepatic artery. The thickness of media of the hepatic artery was directly measured as “T”.

Fig. 1a

Fig. 1b

The measurement was performed using the cellSens Standard 1.16® software program (Olympus Corporation, Tokyo, Japan).

1.4. Statistical analyses

The measurements of the portal veins and hepatic arteries in the BA and non-BA groups were compared using an unpaired t-test, and the correlations between the luminal area of the portal veins and the degree of fibrosis, and between the diameter of the portal veins and the parameters of the hepatic arteries were assessed using Pearson's correlation test. The statistical analyses were performed using the R software program.

1.5. Ethical Approval

This study was approved by the Research Ethics Committee of Kagoshima University Hospital (registration number: 170347) and was performed in accordance with the Ethical Guidelines for Clinical Research from the Japanese Ministry of Health, Labor and Welfare. The committee decided that informed consent from each patient was not required in the present study.

2. Results

2.1. The portal veins

Fig. 2

Figure 2 shows representative histopathological findings in the BA and non-BA specimens. Figures 2a, 2b and 2c show the portal area of a BA patient. Figures 2d, 2e and 2f show the portal area of a non-BA patient (choledochal cyst). Figures 2a and 2d show hematoxylin and eosin staining. The portal vein diameter in BA patients was smaller than that in non-BA patients, and the thickness of the tunica media of the hepatic artery in BA patients was larger than that of non-BA patients. Figures 2b and 2e show images of combined Verhoeff and Masson trichrome staining. The portal area in BA patients showed fibrotic changes, and the vessel findings were the same as those described above. Figures 2c and 2f show images of double-chromogenic immunostaining of CD34 + D2-40. We defined the portal vein as the vascular structure without a prominent tunica media with endothelial cells that were positive for CD34 (stained in brown) and negative for D2-40. The vascular structures with prominent tunica media were defined as arteries. We defined the lymphatic duct as the vascular structure with endothelial cells that were positive for D2-40 (stained in red) and negative for CD34.

Fig. 2abc

Fig. 2def

Fig. 2ad

Fig. 2be

Fig. 2cf

In BA, the portal area contains a considerable number of lymphatic vessels. The portal vein and artery findings were the same as those described above. Figure 3 shows a high-power view of combined Verhoeff and Masson trichrome staining of the hepatic artery. The thickness of the media of the hepatic artery in BA (Figure 3a) was greater than that of the hepatic artery in non-BA (Figure 3b), and the proliferation of the epithelial cells in BA (Figure 3a) was more prominent than that in non-BA (Figure 3b).

Fig. 3

Fig. 3a

Fig. 3b

Figure 4a shows the average portal vein diameter in each patient. The average diameter in the BA specimens was significantly smaller than that in the non-BA specimens (6.05 ± 2.26 μm in BA and 22.21 ± 8.88 μm in non-BA, $p < 0.001$). The diameter of each portal vein branch was $10^{0.67 \pm 0.27}$ μm in BA and $10^{1.22 \pm 0.35}$ μm in non-BA ($p < 0.001$). As shown in Figure 4b, the histograms for the portal vein diameter showed a quasi-lognormal distribution in both BA and non-BA patients. The modes of the portal vein diameter were 5 and 20 μm in BA and non-BA patients, respectively. This indicates that BA patients show a marked decrease in the number of normally-sized portal veins and a marked increase in the number of capillaries. We analyzed the distribution of the density of blood vessels. The number of portal veins per unit area of the whole liver specimen in the BA patients was larger than that in the non-BA patients (Figure 4c) (3.98 ± 2.00 vs. 1.85 ± 0.79/mm^2, $p < 0.0001$), but that per unit area in the fibrotic portal area showed no significant difference (Figure 4d). The measurement of the luminal area in a single specimen had no significance and was only valuable in comparisons between two groups. Figure 4e shows the ratio of the sum of the luminal area of the portal veins divided by the fibrotic portal area in each patient. The luminal area in BA patients was significantly smaller than that in non-BA patients (0.38 % ± 0.91 % in BA and 5.84 % ± 5.43 % in non-BA, $p < 0.001$)

Fig. 4a

Fig. 4b

Fig. 4c

Fig. 4d

Fig. 4e

We also evaluated the degree of fibrosis using the Ishak score. The grades of the 25 patients with BA, were as follows: grade 3, n=10; grade 4, n=12; and grade 5, n=3. The average portal vein diameter in the individual patients did not differ among grades 3, 4, and 5 (Figure 4f). Figure 4g shows a scatter diagram with the ratio of the fibrotic area divided by the total area of the specimen plotted on the X axis and the average portal vein diameter plotted on the Y axis in each BA patient. There was no significant correlation between the

Fig. 4f

Fig. 4g

average portal vein diameter and the ratio of the fibrotic area divided by the total specimen area (correlation coefficient = − 0.22, 95 % confidence interval: − 0.564 - + 0.195, $p = 0.298$). The results suggest that the decrease in the diameter of the portal veins in BA is not associated with the progression of fibrosis in the portal areas. Similarly, no significant correlation was noted between the age at KPE and the luminal area of the portal veins ($p = 0.881$).

2.2. Hepatic arteries

Table 1 compares the number of hepatic arteries per unit specimen area, diameter of the lumen, and medial thickness between BA and non-BA patients. There were no significant differences in the number of hepatic arteries per unit specimen area between the BA and non-BA liver specimens (1.44 ± 0.57/mm^2 in BA and 2.50 ± 4.63 /mm^2 in non-BA, $p = 0.35$). The diameter of the lumen in BA specimens was significantly larger than that in non-BA specimens (16.49 ± 3.62 μm in BA and 14.13 ± 4.44 μm in non-BA, $p = 0.045$). The medial thickness in BA specimens was significantly larger than that in non-BA specimens (15.63 ± 3.77 μm in BA and 9.48 ± 3.57 μm in non-BA, $p < 0.0001$).

Table 1

Figure 5a shows the number of endothelial cells per hepatic artery in each specimen. The number of cells in BA liver specimens was significantly greater than that in non-BA specimens (14.4 ± 5.7 in BA and 7.3 ± 4.3 in non-BA, $p = 0.0056$). Figure 5b shows the ratio of the thickness of the media divided by the diameter of the lumen (which represents the medial thickness of the hepatic artery per unit diameter of the lumen) in the BA specimens was significantly larger than that in non-BA specimens (1.21 ± 0.46 in BA and 0.87 ± 0.27 in non-BA, $p = 0.0024$). This indicates that medial hypertrophy and endothelial proliferation are

Fig. 5a

Fig. 5b

histopathologically significant, and do not occur secondarily to an increase in the diameter of the arterial lumen.

Figure 6a shows a scatter diagram with the portal vein diameter of each patient plotted on the X axis and the diameter of the hepatic artery lumen plotted on the Y axis. A positive correlation was observed in the BA group (correlation coefficient = 0.398, 95% confidence interval: 0.00297-0.685, $p = 0.049$), while no significant correlation was observed in the non-BA group ($p = 0.33$). Figure 6b shows a scatter diagram with the portal vein diameter in each patient plotted on the X axis and the hepatic artery medial thickness plotted on the Y axis. There was no significant correlation in either the BA group ($p = 0.95$) or the non-BA group ($p = 0.92$). Figure 6c shows a scatter diagram with the diameter of the hepatic artery lumen plotted on the X axis and the thickness of media of the hepatic arteries on the Y axis in each BA patient. No significant correlation was observed in the BA group ($p = 0.95$), while a positive correlation was observed in the non-BA group (correlation coefficient = 0.676, 95% confidence interval: 0.383-0.845, $p = 0.0002$).

Fig. 6a

Fig. 6b

Fig. 6c

3. Discussion

The present morphometrical study clarified two major vascular changes in BA: 1) the narrowing of portal veins with increased numbers of capillaries, which was independent of the progression of fibrosis in the portal area, and 2) endothelial proliferation and medial hypertrophy, which were independent of portal vein narrowing.

A significant number of patients with BA show progressive hepatic fibrosis, even after the resolution of jaundice by KPE. Ohuchi et al. previously reported that the number of portal veins was reduced in the BA liver, and hypothesized that this was caused by compression due to the proliferation of fibrous tissue [6]. Under this hypothesis, the degree of fibrosis would be inversely correlated with the luminal area of the portal vein. However, the present study showed no significant correlation between the degree of fibrosis and the diameter of the portal veins. Kang and Davenport also failed to find a relationship between the degree of fibrosis and the development of esophageal varices [7]. Kasai et al. reported that the portal pressure was not correlated with the morphometrically defined interstitial volume % in biliary atresia [8]. They reported that the length of the portal vein per volume of liver in BA patients was significantly shorter than that in non-BA patients. In the present study, we performed double chromogenic immunostaining of CD34 + podoplanin (D2-40) to evaluate the capillary-level portal veins more accurately than conventional methods by excluding lymphatic vessels. These results suggest that the narrowing of the portal veins may occur due to factors other than the fibrosing process. The proliferation of capillaries in our study could represent the presence of a collateral pathway due to the obstruction of the branches of the normal sized portal vein. As the fibrotic area increases, the number of capillary level branches of the portal vein increases.

The presence of medial hypertrophy with increased endothelial cells in the hepatic arteries was another major finding of our study. To the best of our knowledge, the present study is the first to report on the morphology of the hepatic artery in BA. The significant increase in the area of the arterial lumen observed in the present study is suspected to reflect the increase in hepatic arterial blood flow. However, in the BA group, the correlation coefficient between the diameters of the portal veins and the diameters of the hepatic arteries was positive. The narrower the portal veins, the smaller the diameter of the arteries. Furthermore, there was no significant correlation between the diameters of the portal veins and the medial thickness of the hepatic arteries in BA group. These results suggest that hepatic arterial blood flow did not increase to compensate for a reduction in portal blood flow in BA. Medial hypertrophy is a common finding in patients with hypertension [9]. However, none of the BA patients in this series showed systemic hypertension at their first visit.

Endothelial proliferation is often observed in chronic rejection after liver transplantation. Medial hypertrophy of the arteries is also a common finding of delayed-type hypersensitivity in the chronic rejection of a liver graft [10]. The disappearance of the peripheral bile duct and ductular proliferation are common findings among these diseases. Vascular endothelial cells are the targets of immune-mediated insults in liver graft rejection and hepatic GvHD. These observations are compatible with the hypothesis that BA is caused by GvHD which is associated with maternal microchimerism [2,11,12]. Further analyses are required.

The limitation of this study is that the ages of the patients with BA were not precisely matched with those of the patients in the non-BA control group. We need to conduct a

multicenter study to overcome the limitation of patient volume and to perform a more comprehensive analysis.

Conclusions

The narrowing of the portal veins with an increase in the number of capillaries and medial hypertrophy with an increase in the number of endothelial cells in the hepatic arteries were confirmed. These are considered to be essential vascular lesions of BA and may not occur secondarily to liver fibrosis.

Compliance with ethical standards

The authors declare no conflicts of interest in association with the present study.

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Figures and Table Legends

Figure 1. Illustration of the parameters included in the morphometric analyses of the portal veins and arteries

a: Portal vein

Dotted area: The lumen of the portal vein

D: The diameter of the portal vein

b: Hepatic artery

Inner circle: The lumen of the artery.

The peripheral length of the lumen is represented by the circumference of the inner circle.

Outer circle: The outer circumference of the artery.

Dotted area: The media of the hepatic artery.

T: The medial thickness of the hepatic artery.

D: The diameter of the lumen of the hepatic artery.

○: The endothelial cells.

●: The smooth muscle cells of the media of the hepatic artery.

Figure 2. Representative pathological findings of vasculopathy in BA and non-BA (choledochal cyst).

A, b, c: Portal area of a BA case.

D, e, f: Portal area of a non-BA case.

A, d: Hematoxylin and eosin staining.

B, e: Combined Verhoeff and Masson trichrome staining.

C, f: Double chromogenic immunostaining for CD34 + D2-40.

The vascular endothelium is stained brown, and the lymphatic endothelium is red.

Magnification, × 200

Dashed ellipse: Portal vein, Circle: Artery.

Figure 3. Representative pathological findings of the hepatic artery in BA and non-BA (choledochal cyst).

Combined Verhoeff and Masson trichrome staining**.**

A: Hepatic artery of a BA case.

B: Hepatic artery of a non-BA case.

Magnification, ×400

Arrowhead: The media of the hepatic artery

Figure 4. Morphometric analyses of the portal veins and portal area

A The average portal vein diameters in BA and non-BA specimens (expressed as the mean ± SD).

b A histogram of the diameter of each portal vein in BA and non-BA specimens (logarithmic conversion). A total of 3395 portal veins were measured in 25 BA patients, while 1509 were measured in 26 non-BA patients.

c Comparison of the number of portal veins per unit area of the whole liver specimen [/ mm^2]. Expressed as the mean ± SD.

d Comparison of the number of portal veins per unit of fibrotic portal area [/ mm^2] (expressed as the mean ± SD).

e A box plot comparing the ratios of the sum of the luminal area of the portal veins divided by the fibrotic portal area.

f Comparison of the diameter of the portal veins above the Ishak score in BA patients. The values are expressed as the mean ± SD.

g A scatter diagram plotting the proportion of the fibrotic area divided by the total area of the specimen in each BA patient on the X axis and the average portal vein diameter on the Y axis.

Figure 5. Comparison of the hepatic arteries of BA and non-BA patients.

a Number of endothelial cells per luminal length [/ mm].

b Medial thickness divided by the diameter of the lumen.

Figure 6. A correlation analysis of the diameter of the portal vein, diameter of the hepatic artery lumen and medial thickness of the hepatic arteries in BA and non-BA patients

a A scatter diagram with the diameter of the portal vein in each patient plotted on the X axis and the diameter of the lumen of the hepatic artery plotted on the Y axis.

b A scatter diagram with the diameter of the portal vein in each patient plotted on the X axis and medial thickness of the hepatic artery plotted on the Y axis.

c A scatter diagram with the diameter of the lumen of the hepatic artery in each BA patient plotted on the X axis and the medial thickness of the hepatic artery plotted on the Y axis.

●: BA group

○: non-BA group

continuous line: fitted curve of BA group

dotted line: fitted curve of non-BA group

Table 1. **Comparison of the number of hepatic arteries per unit specimen area, diameter of the lumen, and medial thickness between BA and non-BA patients.**

Figure 1

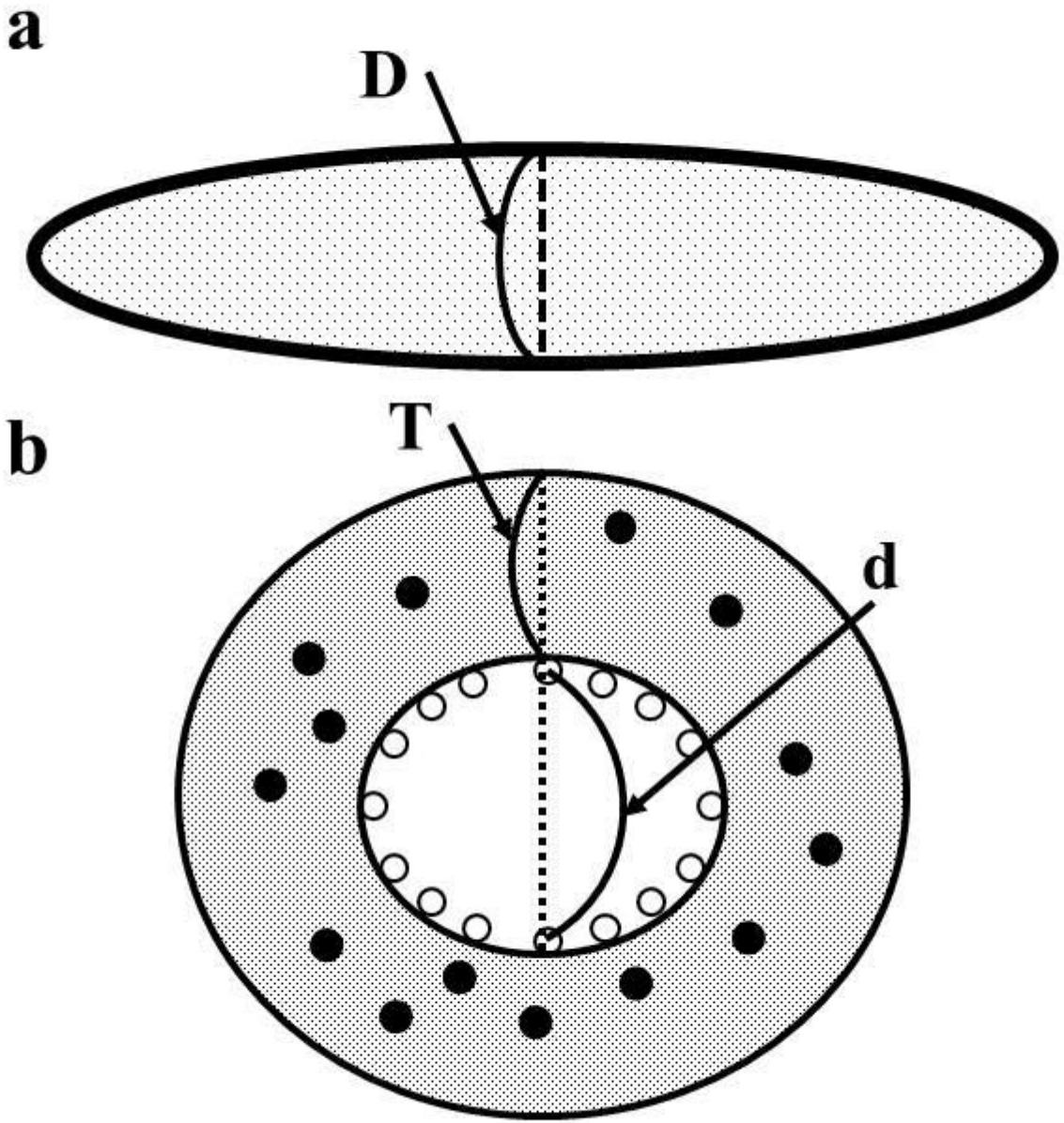


Figure 2

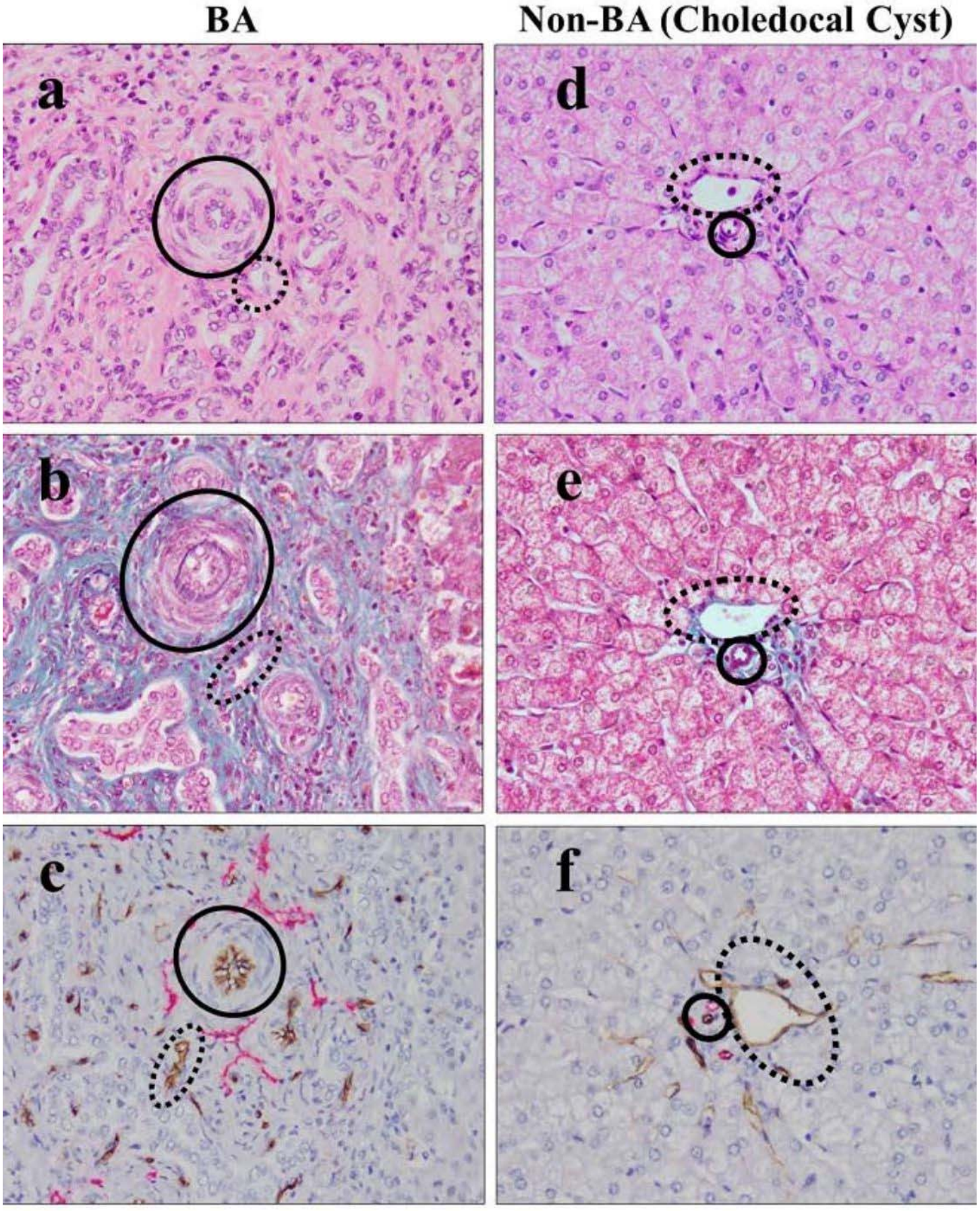


Figure 3

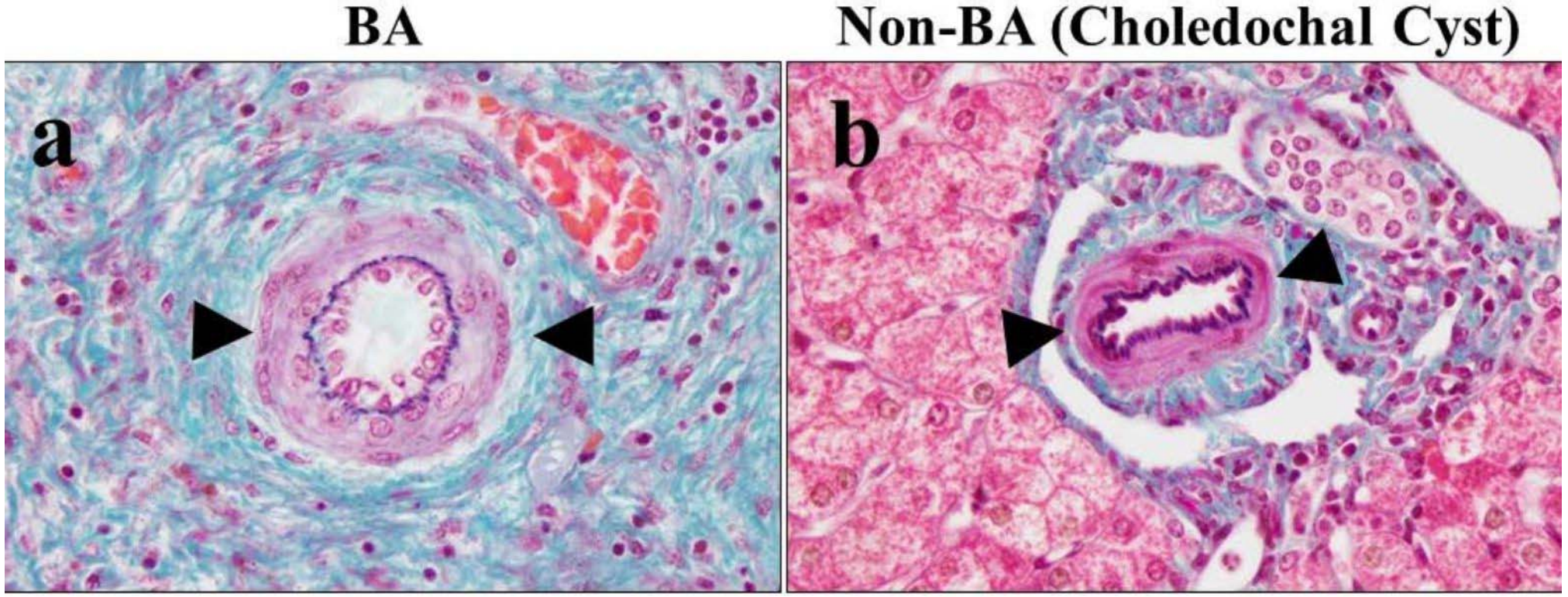


Figure 4

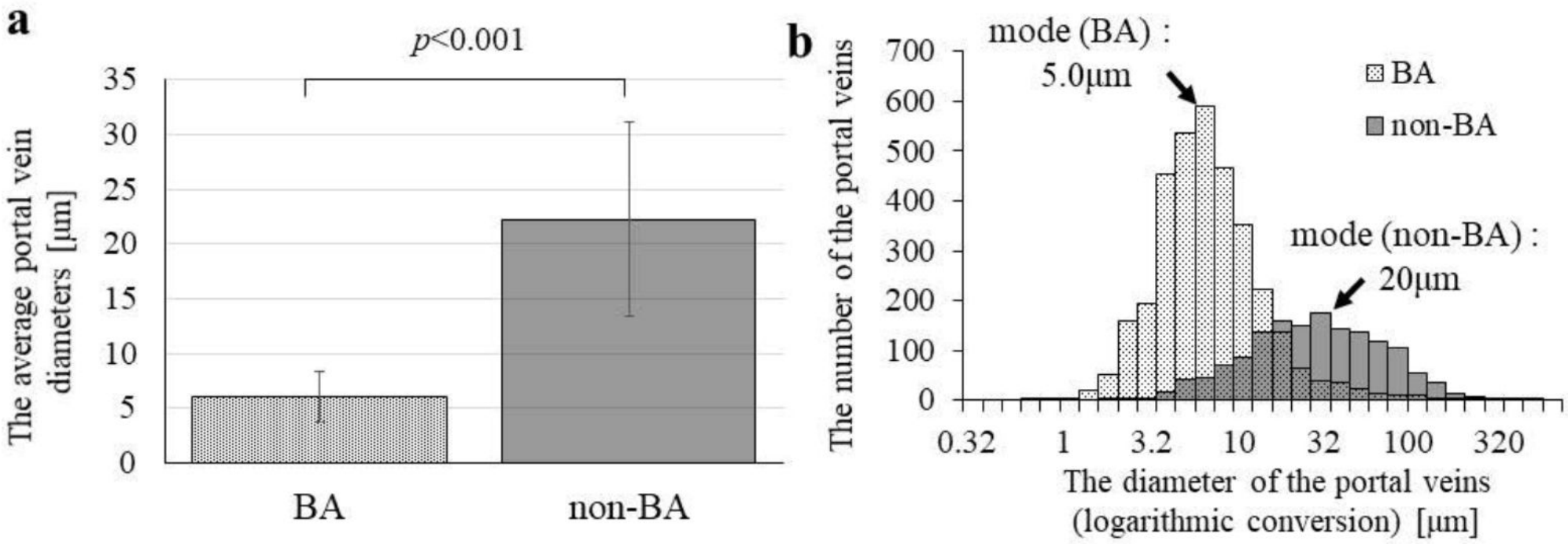

a
p<0.001
The average portal vein diameters [μm]
35
30
25
20
15
10
5
0
BA
non-BA
b
The number of the portal veins
700
600
500
400
300
200
100
0
mode (BA) :
5.0μm
mode (non-BA) :
20μm
BA
non-BA
0.32
1
3.2
10
32
100
320
The diameter of the portal veins
(logarithmic conversion) [μm]

Figure 4

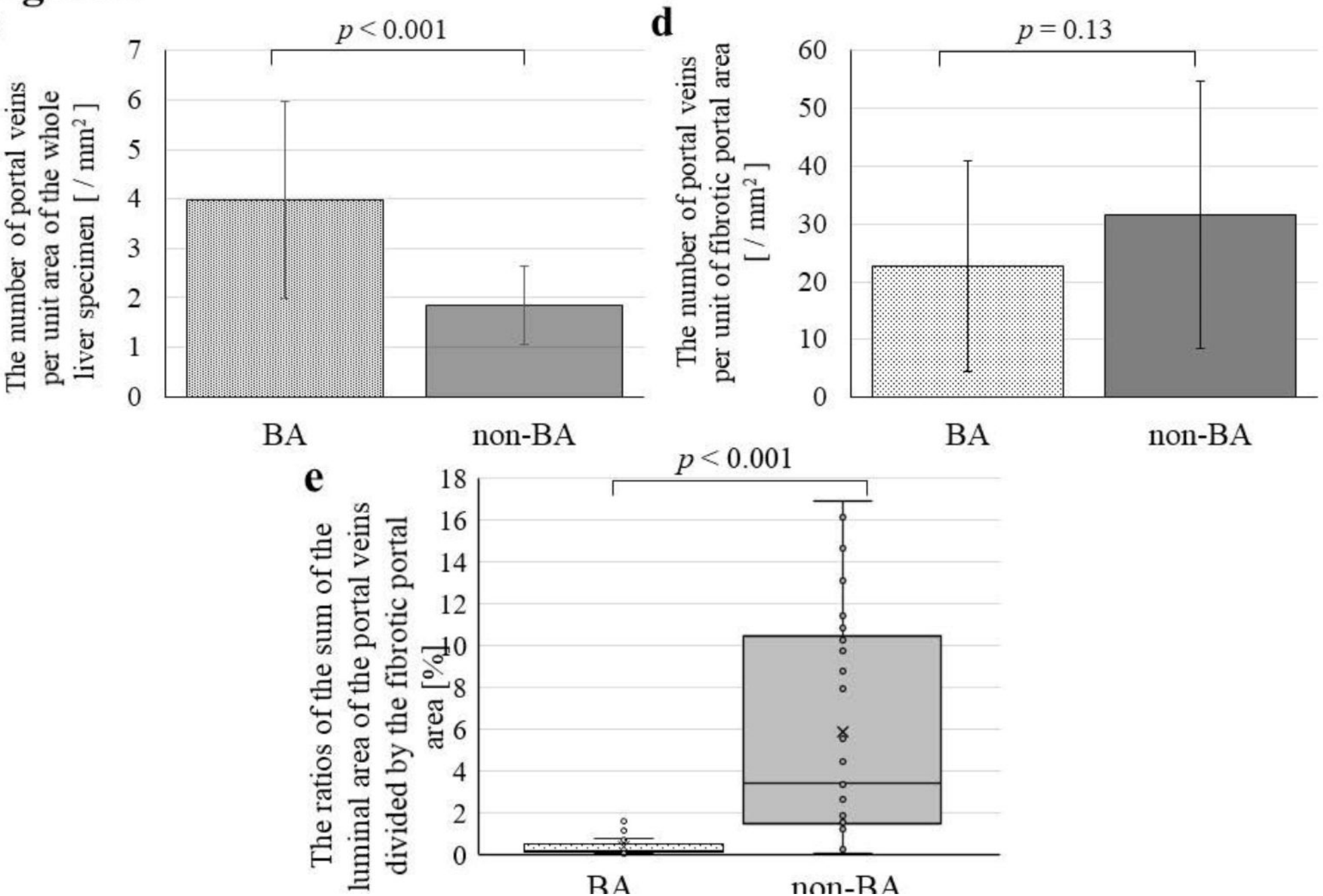

c
p < 0.001
The number of portal veins per unit area of the whole liver specimen [/ mm²]
0
1
2
3
4
5
6
7
BA
non-BA
d
p = 0.13
The number of portal veins per unit of fibrotic portal area [/ mm²]
0
10
20
30
40
50
60
BA
non-BA
e
p < 0.001
The ratios of the sum of the luminal area of the portal veins divided by the fibrotic portal area [%]
0
2
4
6
8
10
12
14
16
18
BA
non-BA

Figure 4

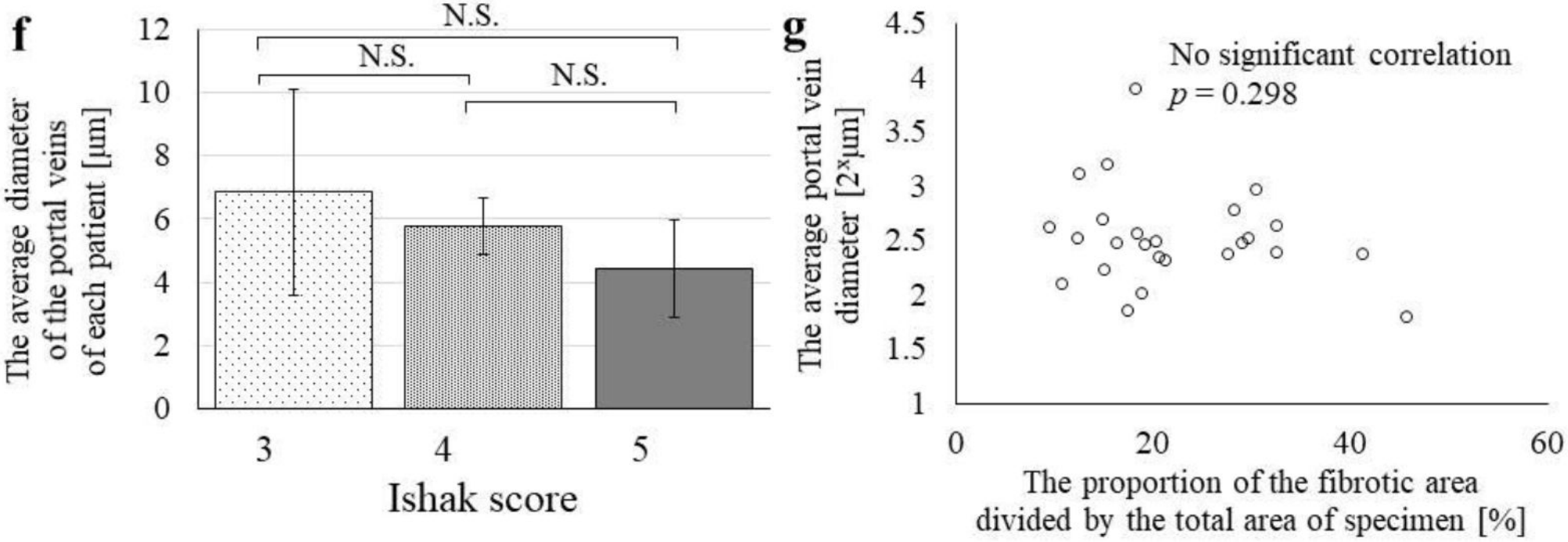

f
The average diameter of the portal veins of each patient [μm]
12
10
8
6
4
2
0
N.S.
N.S.
N.S.
3
4
5
Ishak score
g
The average portal vein diameter [2^x μm]
4.5
4
3.5
3
2.5
2
1.5
1
No significant correlation
p = 0.298
0
20
40
60
The proportion of the fibrotic area divided by the total area of specimen [%]

Table 1 Comparison of the number of hepatic arteries per unit specimen area, diameter of the lumen, and medial thickness between BA and non-BA patients.

Group	Number of hepatic arteries per unit specimen area [μm^2]		Diameter of the lumen per one artery [μm]		Medial thickness per one artery [μm]	
BA (n = 25)	1.44 ± 0.57	$p = 0.35$	16.49 ± 3.62	$p = 0.045$	15.63 ± 3.77	$p < 0.0001$
Non-BA (n = 26)	2.50 ±4.63		14.13 ± 4.44		9.48 ± 3.57	

BA: Biliary Atresia

Figure 5

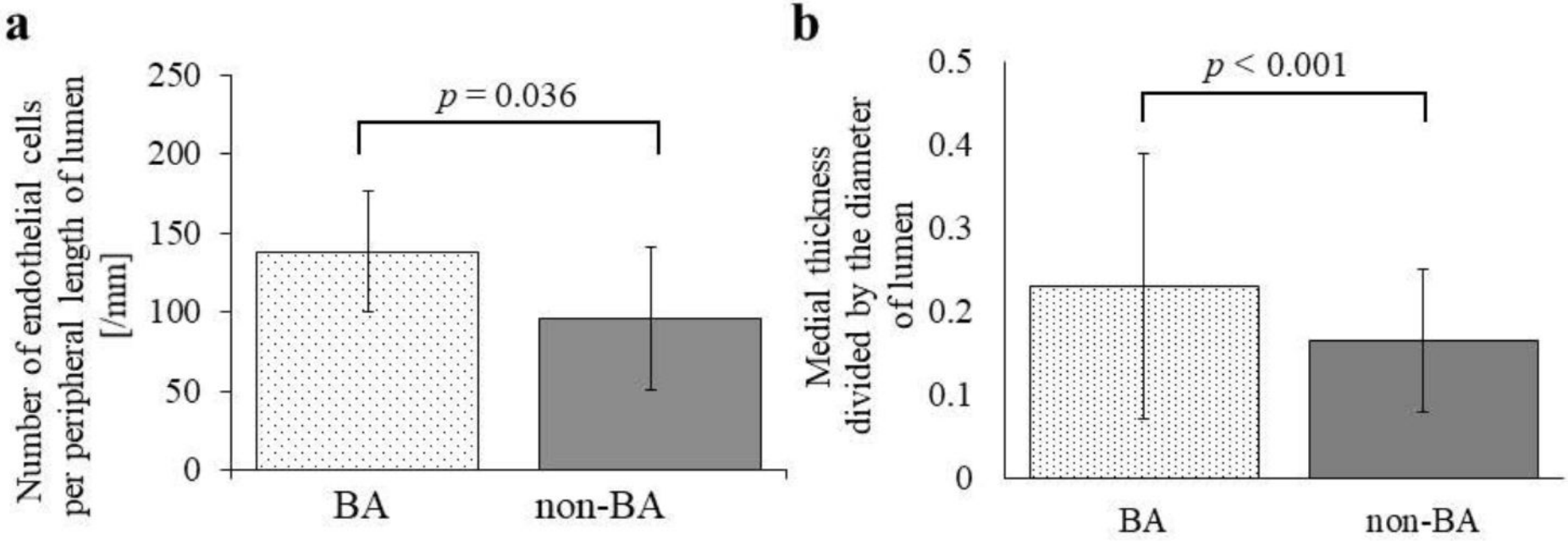
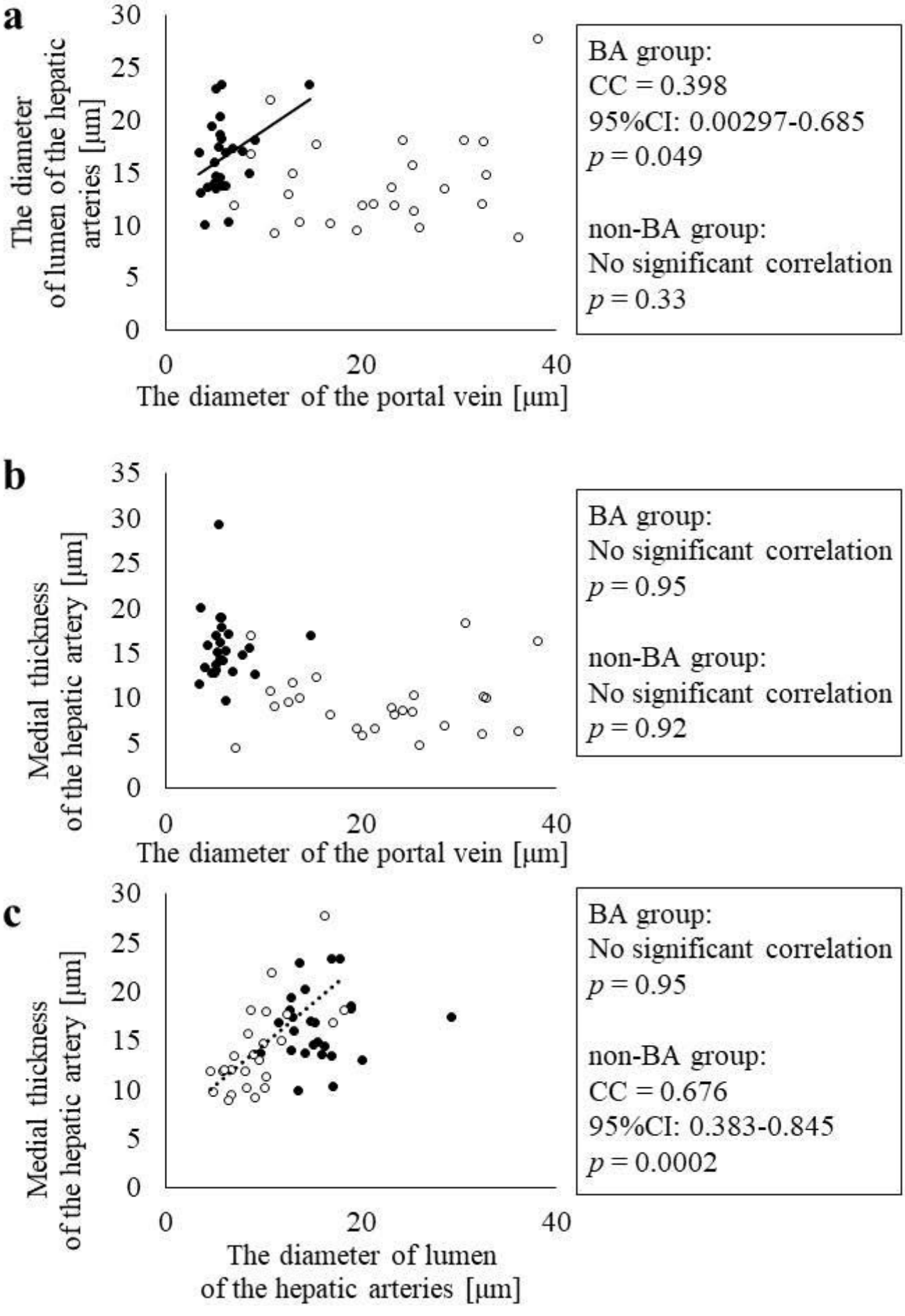

a
Number of endothelial cells per peripheral length of lumen [/mm]
250
200
150
100
50
0
p = 0.036
BA
non-BA
b
Medial thickness divided by the diameter of lumen
0.5
0.4
0.3
0.2
0.1
0
p < 0.001
BA
non-BA

Figure 6


a
The diameter of lumen of the hepatic arteries [μm]
30
25
20
15
10
5
0
0
20
40
The diameter of the portal vein [μm]
BA group:
CC = 0.398
95%CI: 0.00297-0.685
p = 0.049
non-BA group:
No significant correlation
p = 0.33
b
Medial thickness of the hepatic artery [μm]
35
30
25
20
15
10
5
0
0
20
40
The diameter of the portal vein [μm]
BA group:
No significant correlation
p = 0.95
non-BA group:
No significant correlation
p = 0.92
c
Medial thickness of the hepatic artery [μm]
30
25
20
15
10
5
0
0
20
40
The diameter of lumen of the hepatic arteries [μm]
BA group:
No significant correlation
p = 0.95
non-BA group:
CC = 0.676
95%CI: 0.383-0.845
p = 0.0002