Virchows Archiv A Pathol Anat (1989) 416:11–17

Virchows Archiv A
Pathological Anatomy and Histopathology

Quantitative study of centrolobular hepatic fibrosis in alcoholic disease before cirrhosis

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Summary. In an attempt to determine the importance of perivenular fibrosis (PVF) in alcoholic liver disease, we studied 71 liver biopsies using histological grading and a morphometric method. The histological grading used 7 variables which allowed us to classify the patients into 7 groups: controls, patients without alcoholic hepatitis but with steatosis, steato-fibrosis, portal fibrosis and patients with mild, moderate, or severe alcoholic hepatitis. The quantitative analysis examined 3 parameters: (1) The inner diameter of the terminal hepatic veins (THV). (2) The thickness of the THV rims, related to perivenular fibrosis (PVF). (3) Centrolobular fibrosis (CLF) which represented the association of perivenular and perisinusoidal centrolobular fibrosis. No changes in the inner diameter of the terminal hepatic veins was observed for the different groups except in the case of severe alcoholic hepatitis. This fact indicated the absence of veno-occlusive lesions in early stages of mild and moderate alcoholic disease. In severe alcoholic hepatitis, THV were destroyed by centrolobular scars and most of them were indistinguishable and unmeasurable. Of the 26 cases with steatosis (with or without portal fibrosis) only two cases with steatofibrosis showed perivenular fibrosis. In contrast, a significant increase in PVF and in CLF appeared in patients with alcoholic hepatitis. CLF is easier to quantify and more significative than PVF. Thus, it seems to us that CLF is a better indicator of the intensity of sclerosis and of the risk of developing cirrhosis than PVF alone.

Key words: Alcohol – Liver – Fibrosis – Morphometry

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Introduction

Several studies have been carried out on terminal hepatic vein (THV) lesions in alcoholic liver disease: Van Waes and Lieber (1977); Nakano et al. (1982); Worner and Lieber (1985) have pointed out that the thickening of the wall of the THV, so-called "perivenular fibrosis" (PVF) is a warning sign which indicates a propensity for the rapid development of cirrhosis, already observable at the fatty liver stage, without the features of alcoholic hepatitis. Nasrallah et al. (1980) feel that "lobular and subsinusoidal fibrosis" is a better marker of the risk of cirrhosis than the isolated thickening of the THV, but almost all of their specimens had alcoholic hepatitis. Goodman and Ishak (1982) stress that veno-occlusive lesions and phlebo-sclerosis are the most important lesions in the genesis of portal hypertension in alcoholic liver.

In an attempt to better define and assess the significance of these different features using histological and quantitative techniques, we studied 71 liver biopsies collected from the Pathology Laboratory of Bicêtre's hospital.

Material and methods

Sixty-three liver biopsy specimens from alcoholic patients, hospitalized for medical complications of alcoholism and eight control patients were evaluated. For drinkers, the average daily consumption was over 50 g/day. In control patients, biopsies were performed to evaluate clinical stage in non Hodgkin lymphomas; the patients had no history of excess alcohol intake and normal liver function tests. These biopsies were performed with Menghini's trocar, fixed with Bouin's fluid and embedded in paraffin-picolyte. 4 μm sections were cut and stained with safran-haematoxylin-eosin, Masson's trichrome and Gordon-Sweet's technique for reticulin fibres. Sirius red using Sweat

et al's method (1961) was applied for morphometric quantitation, using a quantimeter (Cambridge Instruments 720). Patients who showed liver cirrhosis or early stage of cirrhosis and those with suspected drug toxicity or virus infection were excluded.

The histological analysis of the biopsies was performed by two observers (M.F., S.C.) according to a protocol with the following histological features, each of them graded on a scale from 0 to 3: necrosis, Mallory bodies, polymorphonuclear cells, steatosis, portal fibrosis and thickness of the THV wall (PVF). Alcoholic hepatitis (AH) was classified into mild, moderate and severe grades according to Orrego's classification (1979). Portal fibrosis was graded as follows: 0, normal portal tract; 1, portal tract enlarged by increased connective tissue; 2, portal tract enlarged with irregular borders; 3, irregular portal tract enlarged with fibrous septa.

Thus, specimens were subdivided in seven groups: normal liver: pure steatosis; steatofibrosis (steatosis with portal fibrosis); portal fibrosis without steatosis; mild AH; moderate AH; severe AH. Steatosis was noted as follows: 0, less than 5% of the hepatocytes with fatty vacuoles; 1, less than 25%; 2, between 25%–50%; 3, over 50%. PVF was scored 0 when the thickness of the THV rim was normal (Fig. 1); 1 with a thin irregular rim of fibrosis (Fig. 2); 2 with a moderate regular or irregular rim (Fig. 3) and 3 when a thick surrounding fibrosis was present (Fig. 4). PVF was graded by the two observers. Perisinusoidal centrolobular fibrosis (PSF) (Fig. 4) was noted as present or absent.

Morphometric quantitation was applied to the terminal hepatic vein (THV) (so-called centrolobular or central vein). Two parameters were studied (Fig. 5):

(a) An assessment of the inner diameter of the THV was calculated by projecting the largest circle which could be contained inside the lumen of the vessel (magnification $\times 80$).

(b) The thickness of the wall (PVF) was measured using the average of three measurements carried out on the minimal, maximal and medium thickness of the THV by projecting the largest circle which could be contained inside the wall of the vessel (magnification $\times 80$).

Secondly, centrolobular fibrosis (CLF) was measured by placing the centrolobular zone in a circle of constant diameter with automated quantification of the area of fibrosis contained inside the circle, using thresholding (Fig. 6) (magnification $\times 32$). The data were expressed as a ratio of the CLF to the total surface measured (CLF density). This process, measuring PVF + PSF, seemed necessary to avoid the difficulty of evaluating the exact thickness of the THV wall when the veins were surrounded by perisinusoidal centrolobular fibrosis (Fig. 7) or totally collapsed (Fig. 8). These centrolobular scars were distinguished from fibrous portal tracts by the absence of any arterial vessel and biliary ducts. Tangential THV cuts were excluded and an average of 8 THV zones were evaluated on each biopsy. The relationship between PVF and CLF and the age of the patients was also studied.

For each biopsy and group, the results were expressed as mean ± standard error of the mean (SEM). Comparisons between each group of patients were evaluated with the parametric Fisher-Snedecor's and the non-parametric *U* Mann-Withney tests. The distribution of the data was studied in each group, using thresholds for the two parameters (PVF and PCF): these thresholds were set at the mean value of the control group plus one standard deviation.

Results

Results of the semi-quantitative histological analysis are shown in Table 1.

In the 7 cases with pure fatty liver, two cases with mild or moderate steatosis showed a thickening of the THV rim (scored 1 and 2). In the four patients with severe steatosis (scored 3) three of them had a normal rim and the last case presented an irregular thin rim (scored 1).

Among the 19 steato-fibrosis cases, only one had no PFV; Ten of the patients had severe steatosis (scored 3), with PVF scored 1 in 7 cases and a uniform thickening of the THV in 3 others (two scored 2, one scored 3).

For the five patients with portal fibrosis, four had PVF (one scored 1 and three scored 2).

No correlation between the fat content and the perivenular fibrosis was observed.

All the patients with alcoholic hepatitis except one had PVF. This PVF was more important than the PVF found in the fatty and fibrous liver biopsies. There was no relation between the intensity of PVF and the degree of alcoholic hepatitis on light microscopy.

There was no perisinusoidal centrolobular fibrosis (PSF) in any of the cases of pure steatosis. In contrast 12 of the 19 patients with steato-fibrosis (SF) had PSF. The presence of this perisinusoidal fibrosis was not related to the degree of steatosis or portal fibrosis, but PSF was associated with the increase of the degree of PVF in the twelve cases of steatofibrosis and in the five cases of portal fibrosis.

Perisinusoidal centrolobular fibrosis was quasi-constant in the cases of AH (30/32 cases).

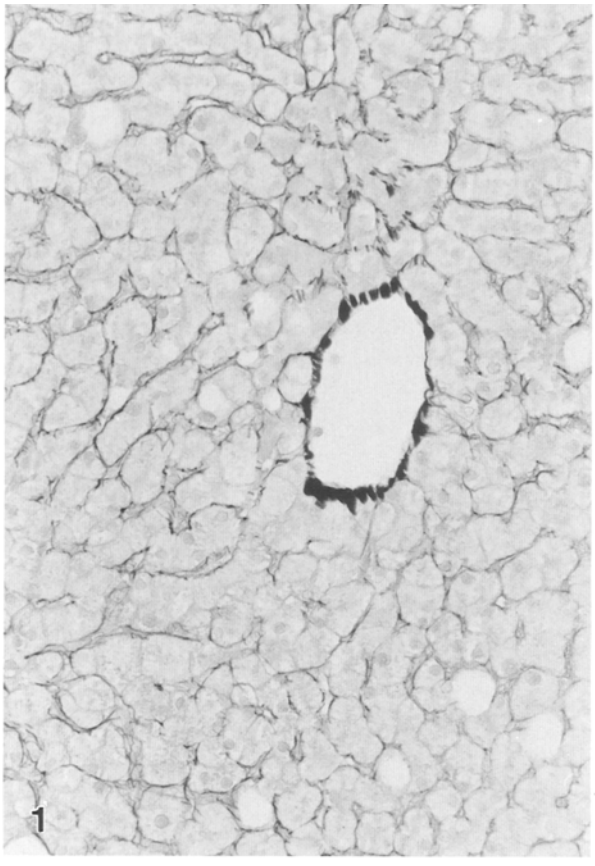
The details of the quantitative studies are seen in Table 2. In that performed on the inner diameter of the THV (Fig. 9) we found an average of 17.7 μm ± 3.8 μm for the control group; there were no significant differences between the controls and all the other groups of patients: patients without

Fig. 1. Normal THV (score 0). Sirius red stain (× 562)

Fig. 2. Thin irregular thickening of the THV rim (PVF score 1). Sirius red stain (× 562)

Fig. 3. Moderate regular or irregular thickening of the THV rim (PVF score 2). Sirius red stain (× 562)

Fig. 4. Severe thickening of the THV rim associated with a perisinusoidal fibrosis (PVF score 3). Sirius red stain (× 562)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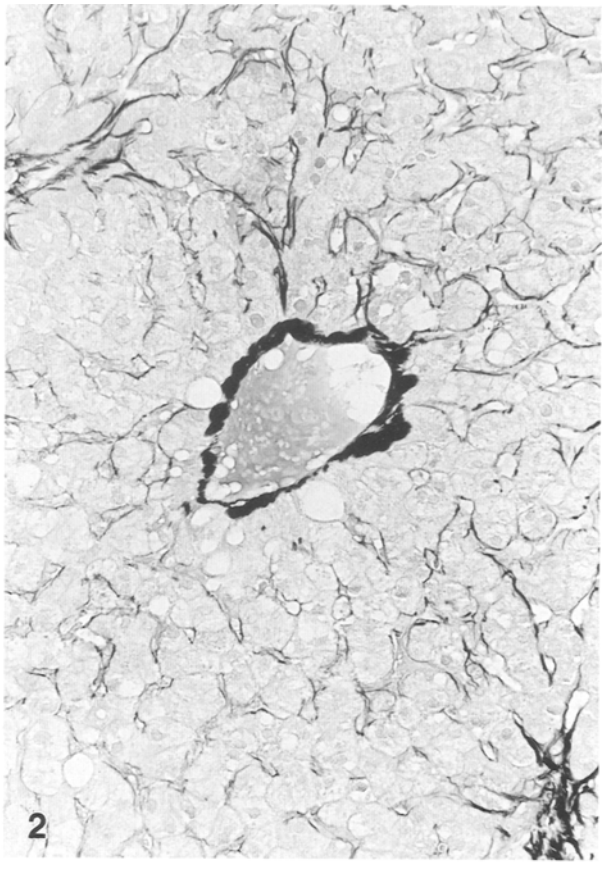
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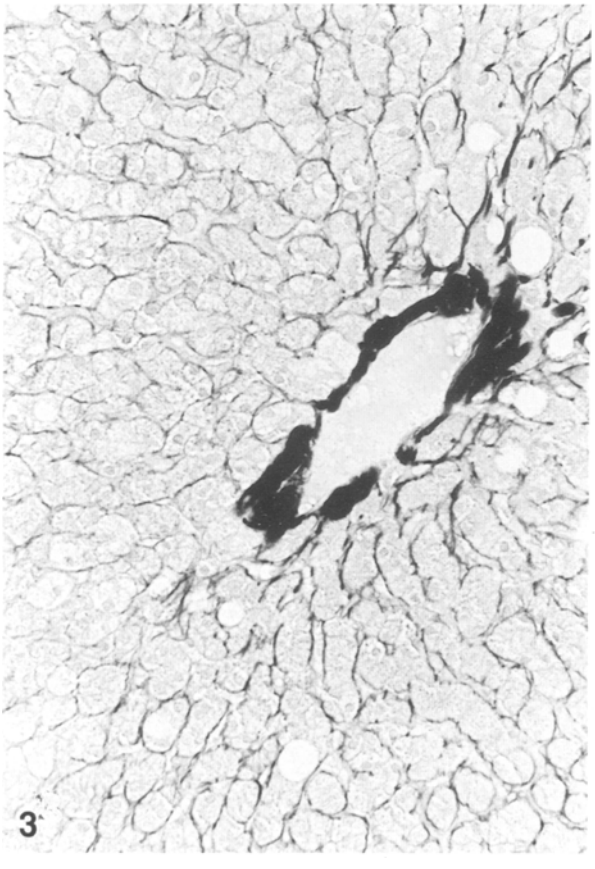
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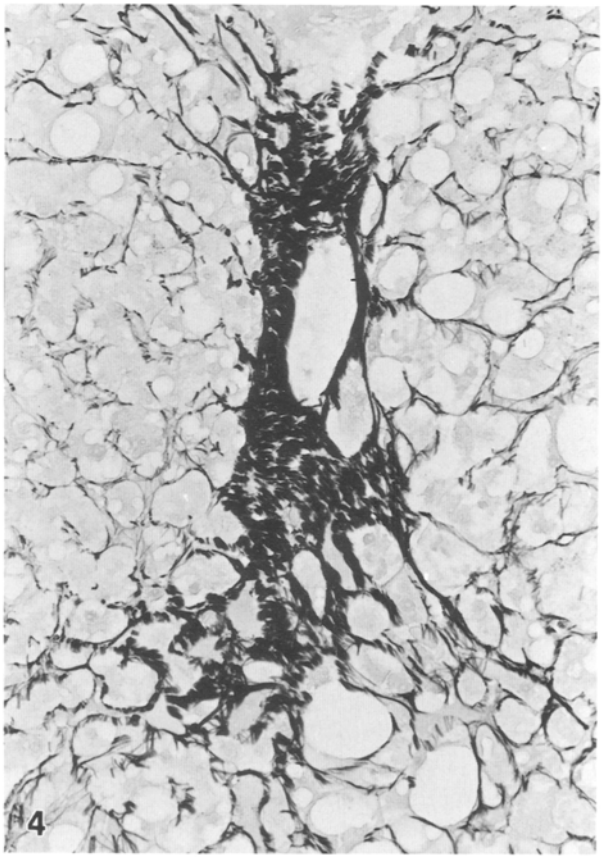
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Table 1. Semi-quantitative analysis: results

Histological features		Groups						Total	
		Steatosis $n=7$	Steatofibrosis $n=19$	Portal fibrosis $n=5$	Mild AH $n=13$	Moderate AH $n=10$	Severe AH $n=9$	Non hepatitis $n=31$	Hepatitis $n=32$
Portal fibrosis									
Score:	0	7	/	/	1	/	/	7	1
	1	/	5	1	1	3	2	6	6
	2	/	10	4	8	5	3	14	16
	3	/	4	/	3	2	4	4	9
Steatosis									
Score:	0	/	/	5	3	/	/	5	3
	1	2	2	/	3	3	2	11	8
	2	1	1	/	7	5	3	2	15
	3	4	9	/	/	2	4	13	6
PVF									
Score:	0	3	1	1	1	/	/	5	1
	1	2	10	1	/	3	3	13	6
	2	1	7	3	6	3	1	11	10
	3	1	1	/	6	4	5	2	15
PSF									
Absent:		7	7	3	1	1	/	17	2
Present:		/	12	2	12	9	9	14	30

A.H.: alcoholic hepatitis; PVF: perivenular fibrosis; PSF: perisinusoidal centrolobular fibrosis. Histological features are graded on a scale from 0 to 3

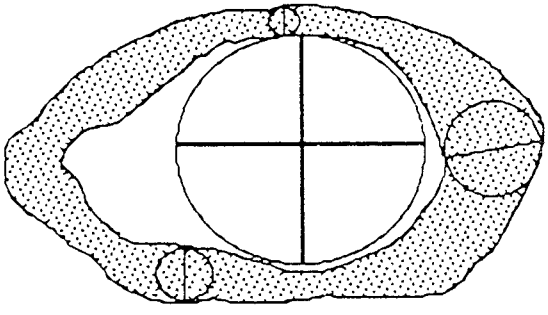

Fig. 5. Measurements of the inner diameter and thickness of THV corresponding to perivenular fibrosis (PVF) (average of 3 measurements, $\times 80$)

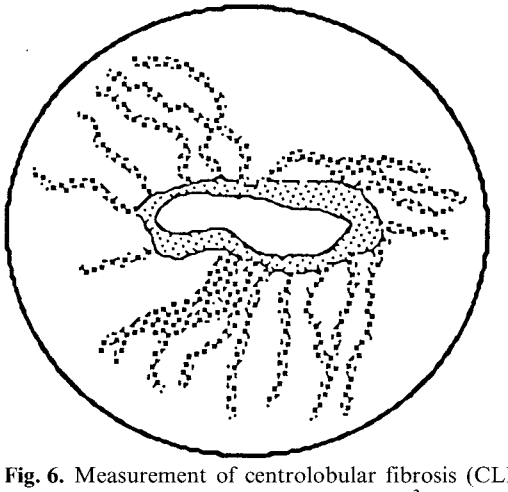

Fig. 6. Measurement of centrolobular fibrosis (CLF) in given surface ($\times 32$); area of the circle = 392 μm^2

AH had a THV's mean diameter of 19.7 $\mu m \pm$ 3.4 μm and those with AH had a diameter of 19.9 $\mu m \pm 4.1$ μm. On each biopsy, the inner diameter showed important variations depending on the THV assessed; but these variations were identical in each group with a SEM of 4.2 μm.

The thickness of the THV rim (PVF) (Fig. 10) in the normal liver biopsies was 2.02 $\mu m \pm 0.35$ μm using the Sirius red stain. The threshold of the PVF was set at 3 μm. The statistical analysis showed no differences between the controls and patients with portal fibrosis, steatosis, steatofibrosis and no differences between these groups and the mild AH group. Nor were there significant differences between the three AH groups, but there were significant differences between non-hepatitis and hepatitis groups on the one hand ($p < 10^{-2}$), and between non-hepatitis and severe AH or moderate AH groups on the other.

The correlation coefficient between the inner

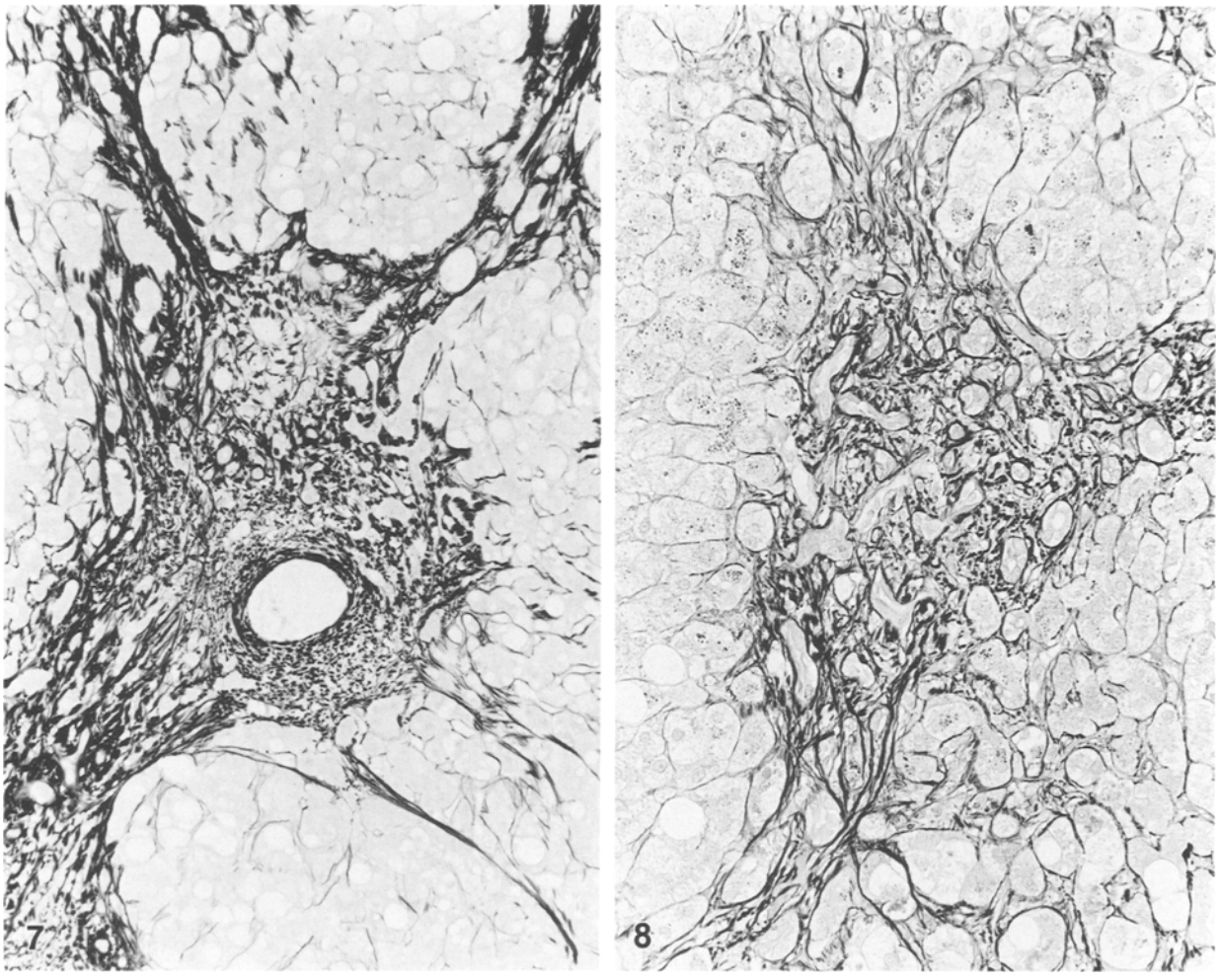

Fig. 7. Important perisinusoidal centrolobular fibrosis associated with a laminated wall of the THV. Sirius red stain (× 360)

Fig. 8. Centrolobular scars with indistinguishable THV. Sirius red stain (× 360)

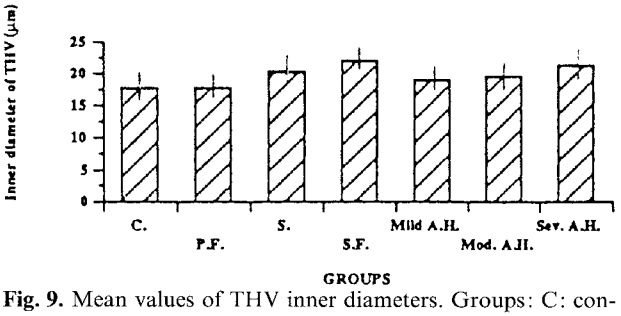

Fig. 9. Mean values of THV inner diameters. Groups: C: control; P.F.: portal fibrosis; S: steatosis; SF: steatofibrosis (Mod: moderate, Sev: severe); AH: alcoholic hepatitis. Bars indicate the mean value with SEM in brackets. No significant difference between controls and all the other groups

diameter and the thickness of the THV rim was low and not significant for any of the groups (Table 2).

Analysis of CLF density (PVF + PSF) (Fig. 11) showed no statistical differences between the controls and the portal fibrosis, steatosis, and steatofibrosis groups; but there was a significant difference between the non AH and AH groups (considered individually or together) ($p < 10^{-5}$). There was a significant increase in the CLF between the mild or moderate and severe AH groups (Table 3).

The mean age of subjects with perivenular or centrolobular fibrosis (47.6 and 45.7 years respectively) and those without fibrosis (46.3 and 46.8 years) were comparable.

Discussion

When we compare the results of the semi-quantitative and quantitative studies, it appears evident that PVF and CLF are overestimated using light microscopy. In fact 13/31 non-hepatitis alcoholic patients showed PVF with light microscopy (even

Table 2. Quantitative data

Groups	Controls	Portal fibrosis	Steatosis	Steato-fibrosis	Mild AH	Moderate AH	Severe AH
Number of biopsies	8	5	7	19	13	10	9
Average number of THV	6.5	11	13	10	10	10	10
Inner diameter of THV (μm)	17.7 +3.8	17.7 +3.1	21.7 +4.2	21.7 +2.9	18.9 +3.3	19.6 +4.2	21.3 +4.8
Thickness of the rim (μm)	2.02 +0.3	1.9 +0.3	2.13 +0.3	2.07 +0.3	2.6 +0.5	2.97 +0.8	3.7 +1.8
Coefficient of correlation Diameter/Thickness	0.28	0.62	0.08	0.51	0.44	0.38	0.34
Centrolobular fibrosis density	0.027+0.009	0.031+0.006	0.029+0.006	0.036+0.005	0.103+0.026	0.099+0.035	0.175+0.031

Diameter and thickness of the THV rim are expressed in mean ± SEM

Table 3. Repartition of PVF and CLF in the groups. Thresholds (respectively 3 μm and 0.05) are set at the mean value of the control group increased with one standard deviation

Groups	Controls	Portal fibrosis	Steatosis	Steato-fibrosis	Mild AH	Moderate AH	Severe AH	Total
Number of cases	8	5	7	19	13	10	9	63
PVF < 3 μm	8	5	7	17	10	5	5	49
PVF > 3 μm	0	0	0	2	3	5	4	14
CLF < 0,05	8	5	7	16	2	3	0	33
CLF > 0,05	0	0	0	3	11	7	9	30

PVF is noted in 0% of pure steatosis, 10% of SF and 37% of AH. CLF is noted in 0% of pure steatosis, 15% of SF and 84.5% of AH

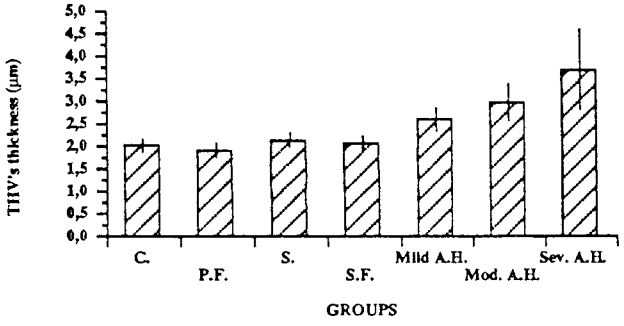

Fig. 10. Thickness of THV rim (PVF). Significant increase of PVF between non-AH and AH groups

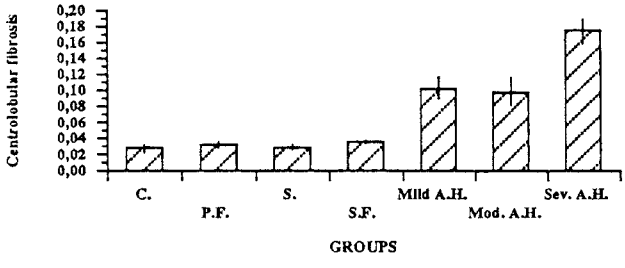

Fig. 11. Centrolobular fibrosis (CLF) Significant increase of CLF between non-AH and AH groups

excluding PVF scored 1) compared to 2 with the morphometric study, and 14/31 biopsies showed peri-sinusoidal centrolobular fibrosis with light microscopy compared to 3 cases with morphometry. This stresses the difficulty of appreciating fibrosis using only light microscopy and probably explains the presence of "PVF on normal liver" biopsy specimens in the work of Nasrallah et al. (1980) which was not a quantitative study.

We did not observe perivenular fibrosis in our patients with pure steatosis, in contrast to Lieber and his co-workers (1977, 1982, 1985). We quantified the thickness of the wall of normal THV, using Sirius red dye, as in the work of Nakano et al. (1982) using Masson's trichrome stain, at a value of 2.02 μm ± 0.35 μm. PVF noted by them was > 4 μm; for us the threshold of the PVF was at 3 μm. This difference may be explained by the use of the Sirius red stain instead of the trichrome stain: we carried out some measurements using the trichrome stain on normal veins and obtained the same value as with Sirius red. We also did some assessments on cases with PVF using Masson's trichrome stain and obtained values greater than 4 μm, similar to Lieber and his co-workers' and greater than that found with Sirius red. Picro-sirius red is known to stain collagens intensely and also to stain reticulin fibres and basement membranes (Puchtler et al. 1961). It is a more specific dye for collagen than Masson's trichrome.

We also quantified CLF, measuring perivenular fibrosis and fibrosis located in the centrolobular zone outside the THV rim (PSF). PVF was present in 10% of the steatofibrosis group, in 37,5% of AH. CLF was present in 15% of the steatofibrosis group, in 84,3% of AH. PVF is associated with CLF in the majority of our patients (all but 2/14 cases), in agreement with Nakano et al. (1982). However, CLF is detectable quantitatively in many

more cases than PVF (30 and 14 cases respectively). This means that CLF is a more sensitive and earlier detectable feature than PVF alone, this is also shown by the greater significance of the statistical tests for CLF ($p<10^{-5}$) than for PVF ($p<10^{-2}$). These quantitative data differ from those of Nakano et al. (1982) who noted in an ultrastructural study that "myofibroblast proliferation and collagen deposition were, sometimes, present exclusively in the perivenular area, at the early stages". In addition to the fact that PVF is commonly associated with perisinusoidal centrolobular fibrosis, it would seem to be reasonable to quantify them together for physiopathological reasons as well as for practical and reliable assessment.

In our experiment, CLF, like PVF, is absent in patients with pure steatosis and PVF and CLF are associated with alcoholic hepatitis and increased with the degree of AH.

The mean inner diameter of normal THV was 17.7 μm ± 3.8 μm in our study. These data differ from the results of Porto et al. (1989) who reported inner diameters between 35 μm and 75 μm. Two explanations may be proposed: their material consisted of surgical biopsies and the microscopic image was measured with a variable magnification (from ×10 to ×40) in contrast with the use of transparietal biopsies and constant magnification (×80) in our study. The greatest THV were thus not quantifiable, either because they were partially cut by the trocar or because they were partially outside the field of the screen. In agreement with our results, this publication demonstrates the absence of correlations between wall thickness and inner diameter in normal THV, in contrast to Nakano et al. (1982). They propose the ratio of wall surface/internal surface to measure the THV wall rim. This ratio appears to be an independant parameter of the THV caliber in the normal vein. However, it cannot take into account pathological THV wall laminated by surrounding perisinusoidal centrolobular fibrosis or totally collapsed THV.

In our study, we did not observe modification of the THV inner diameter for the different groups except in severe AH where randomly obliterated THV were seen. So we have not demonstrated intimal proliferation, the so-called veno-occlusive (VO) lesions by Goodman and Ishak (1982), in the early stages of alcoholic liver disease. For these authors, using light microscopy, intimal proliferation of connective tissue with partial or complete occlusion of the lumen was observed in 52% of their AH correlated with the severity of the AH. However, Burt and Mac Sween (1986), in a more recent study, rarely noted VO lesions. Goodman and Ishak have also pointed out the physiopathological significance of phlebosclerosis compounded with perivenular fibrosis and perisinusoidal centrolobular fibrosis, which tends to obliterate the THV by external compression (as many authors such Edmonson et al. (1963); Popper et al. (1979); Christoffersen et al. (1981) have found). Goodman and Ishak (1982) noted that phlebosclerosis and VO lesions occur together and are significantly correlated with portal hypertension. In their cases without AH, they showed no vascular lesions with pure steatosis and isolated phlebosclerosis with no VO lesions in 54% of their cases presenting with "steatosis and intralobular fibrosis".

To summarize, we have not shown perivenular fibrosis in patients with pure steatosis but with alcoholic hepatitis. Centrolobular fibrosis, which is the association of perivenular fibrosis and perisinusoidal centrolobular fibrosis, appears to be a better criterion than PVF alone for evaluating the intensity of fibrosis because of its more reliable and quantifiable measurement and its statistical significance. It should be interesting to estimate its value in evaluating the risk of the onset of portal hypertension and cirrhosis in a prospective study. Centrolobular fibrosis was also absent in patients with pure steatosis but increased with the severity of alcoholic hepatitis. This stresses, once more, the importance of alcoholic hepatitis in the genesis of fibrosis in alcoholic liver disease.

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Received April 4, 1989 / Accepted June 26, 1989