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Immunohistochemical assessment of lymphatic vessels in human livers with chronic hepatitis C – relation to histological variables

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ABSTRACT – Background – Viral hepatitis C is a significant public health challenge. The disease may remain clinically silent in both acute and chronic forms, and chronic infections may progress to advanced disease such as cirrhosis and hepatocellular carcinoma, requiring costly treatment, compromising the patient's quality of life and even leading to death. For this reason, it is one of the most frequent indications for liver transplantation. Although treatment with direct-acting antivirals represents remarkable progress, many patients are still infected and even those who cleared the viral infection must be followed due to their previous hepatic lesions, especially regarding the disturbances of lobular architecture and the sanguineal and lymphatic vessels. **Objective –** To assess immunohistochemical aspects of lymphatic sprouts and mature lymphatic vascularity with histological variables of liver injury attributable to hepatitis C virus (HCV) and fatty disease. **Methods –** The present study included 72 liver biopsies of cases with chronic hepatitis C. Morphologic changes reflecting "staging" and "activity" were analyzed. Immunohistochemical reactions were performed with monoclonal antibody D2-40 anti-podoplanin. Major histological variables were also semiquantified so as to enable the search for possible associations among histological and Immunohistochemical criteria, as well as with genotypes 1 and 3 of HCV. **Results –** Histological findings showed that the different degrees of strutural changes were well represented in this casuistic. Intralobular/parenchymal necro-inflammatory activity was predominantly mild to moderate. Most cases did not show major evidences of fatty disease, which was found significantly higher in cases infected with HCV genotype 3. The amount of portal lymphatic sprouts increased along with the progression of structural changes, maximal at cirrhosis. Portal lymphatic sprouts as well as portal mature lymphatic vessels also showed an increase parallel to the increase in the degree of portal/septal inflammatory infiltrate. In the present study, no significant association was found between the proportion of portal lymphatic sprouts or portal mature lymphatic vessels and the degree of periportal/periseptal activity. No significant relations were detected between lymphatic sprouts/mature vessels and periportal or parenchymal inflammatory activity, nor with infections due to HCV genotype 1 or 3. **Conclusion –** Visualization and semiquantitation of sprouts and mature lymphatic vessels were clearly yielded by Immunohistochemical staining with monoclonal antibody D2-40. The amount of lymphatics was increased along fibrogenic process, significantly related to progression of liver disease and maximal at cirrhosis. No significant relations were detected with necro-inflammatory activity at interface or in the parenchyma.

Keywords – Hepatitis C; chronic; immunohistochemistry; lymphatic vessels; lymphangiogenesis; liver; pathology.

INTRODUCTION

Although the introduction of treatment with direct-acting antivirals (DAA) represents a remarkable progress, chronic hepatitis C remains an important challenge in Public Health, both worldwide and more especifically in Brazil[1-10].

Pathological presentation of chronic hepatitis C includes various degrees of inflammation, necrosis and liver injury, resulting in different prognosis[4,11].

Despite poorly studied in chronic hepatitis C, the lymphatic system is essential to maintain tissue homeostasis by collecting excess fluid from the tissues and returning it to the bloodstream, providing a favorable environment for immune cells to find and respond to antigens in the peripheral lymphoid tissues. The lymphatics also play an important role in lipid absorption and transportation[12-15].

In chronic liver diseases, increased lymphatic flow is an important mechanism by which fluid can circumvent the increased sinusoidal/post-sinusoidal resistance in animals[16]. The sinusoidal hydrostatic pressure increases due to the increase in sinusoidal blood resistance and consequently, the filtered plasma components that form lymph increase[13].

Impaired lymphatic drainage was reported by Ribera et al.[17] in cirrhotic rats with ascites suggest that regulation of NO in lymphatic endothelial cells of cirrhotic rats causes long-term lymphatic remodeling, which is characterized by a loss of surrounding smooth muscle cells.

An increase in vasodilation combined with intrahepatic vascular resistance due to fibrosis results in portal hypertension[18]. Oikawa et al.[19] report that the area of portal lymphatic vessels increases in idiopathic portal hypertension (IPH) and speculate

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that the formation of new lymphatic vessels in cirrhosis occurs as a mode of adaptation to increased lymph flow. This compensatory lymphangiogenic response may help reduce the elevated portal pressure seen in both idiopathic portal hypertension and portal hypertension that develops in liver cirrhosis.

In many occasions, the lymphatic capillaries are difficult to identify, sometimes because they are collapsed, sometimes because they are dilated, have a delicate wall, with an endothelial coating very similar to that of small blood vessels, supported by delicate conjunctive tissue[20-22].

Both in the study of inflammatory diseases and of neoplasms, discrimination between blood vessels and lymphatic vessels has been performed via immunohistochemical markers for lymphatic vessels, but the most sensitive and specific marker for the endothelium of lymphatic vessels is podoplanin, identified using the D2-40 monoclonal antibody[20,21,23].

The present study aims to assess how lymphatic sprouts and lymphatic vessels relate to histological aspects of liver lesions in several stages of chronic hepatitis C, to hepatitis C virus (HCV) genotype and to evidence of fatty liver disease.

METHODS

The study was approved by the Ethics Committee for Analysis of Research Projects of *Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo* (HC-FMUSP) on 11/24/2016 under online registration number 15847.

This was a retrospective study of liver samples obtained by needle biopsy archived at the Division of Anatomic Pathology (DAP) of HC-FMUSP, ensuring confidentiality of personal data, and no interference in diagnostic or in therapeutic procedures.

As depicted at FIGURE 1, from a universe of 190 HCV-infected patients, cases with the following criteria were selected: diagnosis of HCV infection previously established by serological and virological methods and sufficient amount of residual tissue sample in the paraffin block for histopathological and immunohistochemical studies, defining the minimum tissue length as 1 centimeter and/or the identification of at least 10 portal tracts in the sample. All cases with HCV co-infection with Hepatitis B virus (HBV) or with human immunodeficiency virus (HIV) or with evidences of autoimmune hepatitis were excluded. According to these criteria, the casuistic of the present study includes 72 patients assisted at the Gastroenterology and Infectious Diseases Outpatient Services of HC-FMUSP in the period from 2000 to 2015. Twenty-nine (40%) patients were male and 43 (60%) were female, with a mean age of 48.7 years old, ranging from 22 to 78 years old.

Information about the Genotype of HCV was available in all 72 cases.

Liver samples were collected by 16-gauge needle biopsy and fixed in 4% buffered formalin saline solution at pH=7.4.

The slides were stained with hematoxylin-eosin and picrosirius red and submitted for collaborative histopathological analysis by two experienced liver pathologists (APF and VAFA).

Morphologic changes related to "staging" and "activity" were analyzed, meaning structural alterations and necro-inflammatory findings, respectively.

The histological alterations were semi-quantitated acccording to the proposal by Gayotto et al.[24] in the classification of the Brazilian Society of Pathology, which grades from 0–4 changes related

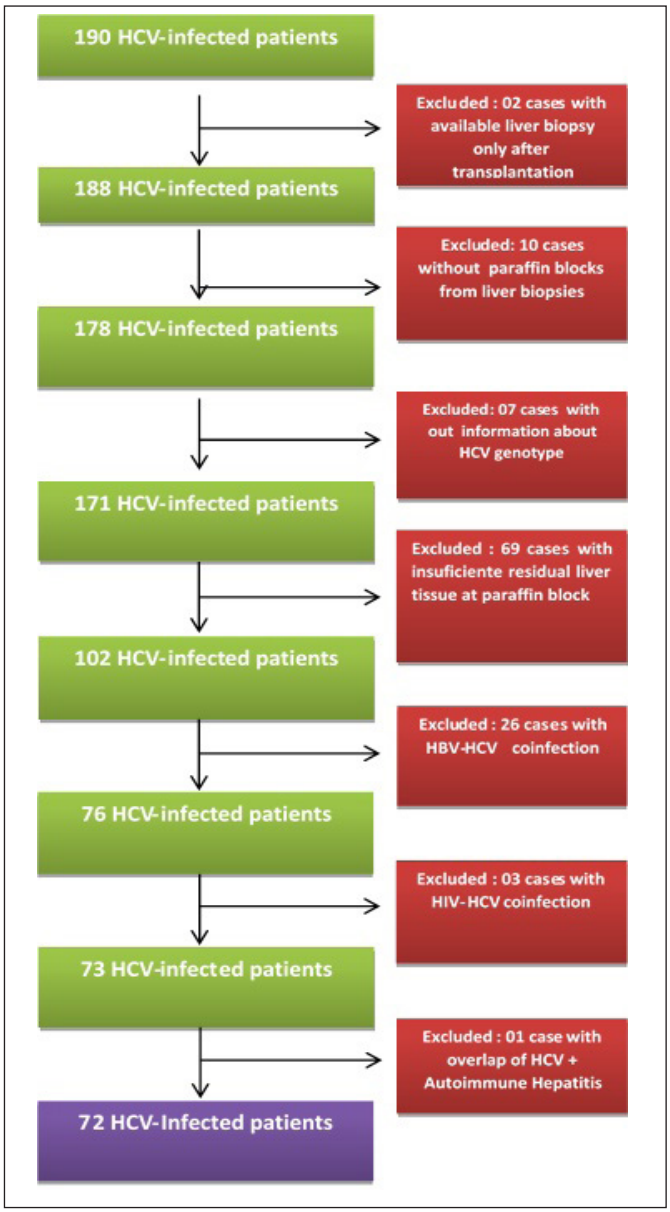

FIGURE 1. Flowchart of hepatitis C infected patients included in the present study.

to lobular architecture, portal inflammation, periportal interface inflammatory activity and parenchymatous necro-inflammation.

As hepatitis C may present features associated with fatty liver disease and also due to the possible comorbidity with metabolic syndrome, we also searched for histological criteria related to fatty liver disease as proposed by Kleiner et al.[25]: steatosis, hepatocytic ballooning, Mallory-Denk bodies and intralobular inflammation were annotated, as well as classification of perivenular and pericellular fibrosis defined as 0= absent, 1= present, slight or 2= marked (or severe).

Immunohistochemical reactions were performed manually with antigen retrieval (Citrate buffer, ph6.0, Spring, 100x) in a steamer for 35 minutes, followed by blocking of endogenous tissue peroxidase with hydrogen peroxide (H2O2) 20 volumes and

methyl alcohol in volume/volume proportion in three incubations for 10 minutes each. After washings in water followed by 0.01M phosphate buffered saline (PBS) pH 7.4, the sections were incubated with non-specific binding blocker (Cas Block, Life Technologies) in an incubator at 37°C for 10 minutes. The primary anti-podoplanin antibody (mouse monoclonal, clone D2-40, DAKO M3619, dilution 1:100) was incubated at 37°C for 30 minutes and then further incubated at 4°C "overnight" for 16 hours. Signal amplification was performed by polymer-based immunoperoxidase method (Novolink Max Polymer DS, Leica Biosystems/Novocastra) at 37°C for 30 minutes, developed with diaminobenzidine (100 mg/100 mL) for 5 minutes and counterstained with Harris hematoxylin.

The detected lymphatic vessels were quantified in the following components: portal lymphatic sprout (PLS), portal lymphatic vessel (PLV), lymphatic sprout at interface, lymphatic vessel at interface, lymphatic sprout in parenchyma and lymphatic vessel in parenchyma. Quantification was performed on digital images acquired by scanning the immunostained slides. Morphometric analysis of the images was performed using Image Pro Plus© 4.1 software.

In each case, five fields estimated as presenting the highest concentration ("hot spots") of sprouts or lymphatic vessels in the region of the portal spaces were analyzed using the 20x objective. Only the sprouts and vessels with well-defined markings were considered (FIGURE 2).

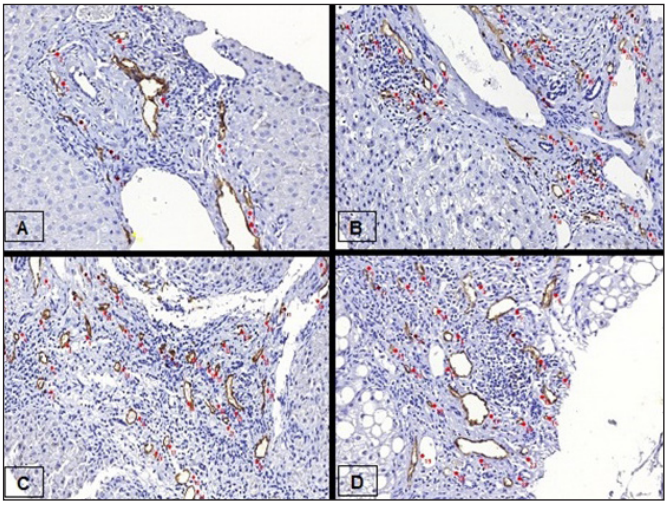

FIGURE 2. Immunohistochemical aspects of lymphatic vessels in chronic hepatitis C.

A) Presence of several lymphatic vessels and few lymphatic sprouts in a portal tract with minor structural alteration IHC, x200. B) expanded portal tract (F2) with moderate periportal necro-inflammation presenting many lymphatic vessels. IHC, x200. C) Fibrovascular septum (F3) with moderate periportal necroinflammation depicting many sprouts and mature lymphatic vessels IHC, x200. D) wide fibrovascular septum (F4) showing many sprout and mature lymphatic vessels, moderate inflammatory infiltrate and mild periportal activity and minimal parenchymal activity. Presence of grade 3 steatosis and steatohepatitis. IHC, x200.

Since the section from one of the cases did not show portal tracts (case with fibrous expansion of portal tracts – stage 1), the expression of podoplanin in the lymphatic endothelium was assessed in 71 cases.

The distribution of values obtained by counting sprouts and lymphatic vessels in both the portal tracts and the interface in each of the 71 cases were presented as medians of the mean positivities, thus grading the cases as "low grade" and "high grade".

In order to assess associations between the immunohistochemical variables related to the pattern of lymphatic vessels and the histological criteria of chronic hepatitis C and the histological criteria of fatty liver disease the chi-square test adopting the significance index as $P<0.05$.

RESULTS

Histological criteria for chronic hepatitis C

Among the 72 HCV serologically positive patients, 7 (10%) cases showed architectural stage 0 (no significant injury in lobular architecture), 19 (26%) cases stage 1 (fibrous expansion of portal tracts), 15 (21%) cases stage 2 (portal expansion with portal-port septa), 16 (22%) cases stage 3 (porto-portal septa and porto-central septa and incomplete nodular formation), whereas 15 (21%) cases were already at stage 4 (cirrhosis, fully identified or predominance of nodular areas over remaining lobules).

Regarding portal/septal inflammatory infiltrate, we found no cases with rare portal lymphocytes (grade 0), whereas 12 (17%) cases had mild lymphocytic infiltrate (grade 1), 31 (43%) cases depicted moderate portal lymphocytes (grade 2), 25 (35%) cases with major portal lymphocytic infiltrate (grade 3). Only 4 (5%) cases presented an exhuberant portal lymphocytic infiltrate (grade 4).

As to the histological variable peri-portal/peri-septal activity, or interface activity, we found 8 (11%) cases with no interface activity (grade 0), 8 (11%) cases presenting only "spill over" of lymphocytes without true hepatocytic lesion (grade 1). True interface lesion with so-called "piece-meal necrosis" (grade 2) was found in 23 (32%) cases, 31 (43%) cases presented moderate interface hepatitis (grade 3), whereas only 2 (3%) cases showed extensive interface hepatitis (grade 4).

As expected for a cohort of chronic hepatitis C cases, intralobular/parenchymal necro-inflammatory activity was mild to moderate in most cases: The hepatocytes were almost normal and lobular infiltrate was not evident in 3 (4%) cases, 28 (39%) cases presented mild lymphohistiocytic infiltrate, and rare hepatocytic apoptosis or focal necrosis (grade 1), 29 (40%) cases with several figures of focal hepatocytic necrosis surrounded by lymphocytes and histiocytes (grade 2). Higher lobular necro-inflammation was detected in 11 (16%) cases presenting grade 3 lesions, whereas only 1 (1%) case showed extensive/multiple confluent necrosis (grade 4).

Histological criteria for fatty liver disease

Twenty-five (35%) cases presented grade 0 steatosis (absence or up to 5% of hepatocytes with macrovesicular steatosis, 27 (38%) cases showed up to 30% of hepatocytes with steatosis (grade 1), whereas 14 (19%) cases had steatosis in 40–60% of hepatocytes (grade 2), and 6 (8%) cases with more than 70% of hepatocytes with steatosis (grade 3).

As for the presence or absence of steatohepatitis, 54 (75%) cases did not present diagnostic criteria sufficient for the diagnosis of steatohepatitis (grade 0) while 18 (25%) cases were diagnosed as steatohepatitis (grade 1).

Ballooning was not present in 35 (49%) cases (grade 0), was mild in 20 (28%) cases (grade 1), and was moderate to severe in 17 (24%) cases (grade 2).

Genotype of hepatitis C virus

Genotype 1 was detected in 50 (69%) cases. In two cases, genotype 1 was reported without other specification (3%), 24 cases geno-

type 1a (33%), 23 cases genotype 1b (32%), and one case genotype 1a/1b (1%). The remaining 22 cases were positive for genotype 3: 18 cases genotype 3a (25%) and four cases with genotype 3 without other specification (6%).

Immunohistochemical detection of podoplanin

The distribution of values obtained by counting sprouts and lymphatic vessels in both the portal tracts and the interface in each of the 71 cases were presented as medians of the mean positivities, thus grading the cases as "low grade" and "high grade".

The median of the average number of portal lymphatic sprouts (PLS) was 2: 40 cases were classified as low grade and the other 31 as high grade.

The median of the average number of mature portal lymphatic vessels was 7: 39 cases were classified as low grade and 32 cases as high grade.

The immunohistochemical study of D2-40 did not identify lymphatic sprouts at the interface. Regarding mature lymphatic vessels, the median was also 0 vessels / 5 fields. Only two cases presented median = 1 vessel / field at the interface. The data shows that lymphangiogenesis was very low at the interface, not allowing comparison with histopathological variables indicating stage and inflammatory activity in the various compartments of the liver lobules.

The search for sprouts or lymphatic vessels in the parenchyma resulted negative in all 71 cases, which prevents its comparative analysis with the other immunohistochemical variables and histological criteria.

With the exception of one case, the TABLE 1 show the distribution between the patterns of portal lymphatic sprouts and portal mature lymphatic vessels was almost identical, demonstrating the direct relationship between the two types of findings.

TABLE 1. Comparison between the values of portal lymphatic sprouts and portal lymphatic vessels.

	Portal lymphatic sprouts			*P*
	PLS low grade	PLS high grade	Total	
Portal lymphatic vessels				
PLV low grade	39	0	39	
PLV high grade	1	31	32	*P*<0.01
Total	40	31	71	

PLV: portal lymphatic vessels; PLS: portal lymphatic sprouts.

TABLES 2 and 3 show the distribution of cases with lower or higher proportion of portal lymphatic sprouts and portal mature lymphatic vessels according to histological variables indicating staging and inflammatory activity in the various compartments of the liver lobules.

The proportion of cases with more portal lymphatic sprouts showed an increase along with the progression of structural alterations. Such an increase was shown to be significant in the grouped analysis, and it was also apparent that the increase occurred at each degree of increasing structural injury, suggesting early and sustained activation of portal lymphangiogenesis.

The proportion of cases with more portal lymphatic vessels was significantly higher in the group of cases with major structural alterations, with an apparent discontinuity of this increase among the cases with grade 3 structural alterations.

TABLE 2. Distribution of portal lymphatic sprouts and portal mature lymphatic vessels marked by expression of podoplanin in lymphatic endothelium according to structural alteration and portal/septal inflammatory infiltrate.

Structural alteration	F0	F1	F2	F3	F4	*P*
Portal lymphatic sprouts						
Low grade	6	12	9	7	6	0.03
High grade	1	6	6	9	9	
Portal lymphatic vessels						
Low grade	7	13	7	9	3	0.01
High grade	0	5	8	7	12	
Portal/septal inflammatory infiltrate	**0**	**1**	**2**	**3**	**4**	***P***
Portal lymphatic sprouts						
Low grade	0	10	20	9	1	<0.01
High grade	0	1	11	16	3	
Portal lymphatic vessels						
Low grade	0	9	20	9	1	0.01
High grade	0	2	11	16	3	

TABLE 3. Distribution of portal lymphatic sprouts and portal mature lymphatic vessels marked by expression of podoplanin in lymphatic endothelium according to periportal/perisseptal activity and parenchymal activity.

	0	1	2	3	4	*P*
Periportal/perisseptal activity						
Portal lymphatic sprouts						
Low grade	8	6	10	16	0	0.21
High grade	0	1	13	15	2	
Portal lymphatic vessels						
Low grade	8	4	12	14	1	0.13
High grade	0	3	11	17	1	
Parenchymal activity						
Portal lymphatic sprouts						
Low grade	3	21	12	3	1	<0.01
High grade	0	6	17	8	0	
Portal lymphatic vessels						
Low grade	3	19	14	3	1	<0.01
High grade	0	8	15	8	1	

The proportion of cases with more portal lymphatic sprouts as well as with more portal mature lymphatic vessels showed an increase parallel to the increase in grade of portal/septal inflammatory infiltrate. This association was shown to be significant in the grouped analysis, and it was also apparent that the increase occurred at each degree of increase in portal inflammatory infiltrate, suggesting a direct relationship between inflammatory activity and portal lymphangiogenesis.

In the present study, no significant association was found between the proportion of portal lymphatic sprouts or portal mature lymphatic vessels and the degree of periportal/perisseptal activity. However, a remarkable increase in cases presenting more portal

lymphatic sprouts and portal mature lymphatic vessels was detected starting with grade 2 necro-inflammatory activity.

Acknowledging that only one case presented with grade 4 inflammatory activity in the parenchyma, the proportion of cases presenting more portal lymphatic sprouts as well as mature portal lymphatic vessels was significantly related to the increase in parenchymal necroinflammatory activity. It is also evident that the increase with each degree of parenchymal activity. In particular, the transition from grade 1 to 2 parenchymal activity, which is highly significant on histopathology, was accompanied by greater activation of portal lymphangiogenesis.

TABLE 4 show the distribution of cases with lower or higher proportion of portal lymphatic sprouts and portal mature lymphatic vessels according to the grade of steatosis and to histological criteria of steatohepatitis, respectively.

TABLE 4. Distribution of portal lymphatic sprouts and portal mature lymphatic vessels marked by expression of podoplanin in lymphatic endothelium according to steatosis and presence of steatohepatitis diagnostic criteria.

Steatosis	0	1	2	3	*P*
Portal lymphatic sprouts					
Low grade	15	13	8	4	0.69
High grade	10	13	6	2	
Portal lymphatic vessels					
Low grade	19	11	7	2	0.29
High grade	6	15	7	4	
Steatohepatitis	**Absence**		**Presence**		***P***
Portal lymphatic sprouts					
Low grade	32		8		0.37
High grade	22		9		
Portal lymphatic vessels					
Low grade	32		7		0.19
High grade	22		10		

The proportion of cases with more portal lymphatic sprouts as well as with more portal mature lymphatic vessels was not significantly related to either steatosis or to the presence of histological criteria of steatohepatitis.

TABLE 5 depicts the distribution of lymphatic structures according to HCV genotypes. Despite the proportion of cases with a higher quantity of portal lymphatic sprouts and portal mature lymphatic vessels being apparently higher in patients infected with HCV genotype 1, this difference was not statistically significant.

TABLE 5. Distribution of portal lymphatic sprouts and portal mature lymphatic vessels marked by expression of podoplanin in lymphatic endothelium according to HCV genotype.

HCV genotype	1	3	*P*
Portal lymphatic sprouts			
Low grade	25	15	0.17
High grade	24	7	
Portal lymphatic vessels			
Low grade	28	11	0.57
High grade	21	11	

HCV: hepatitis C virus.

DISCUSSION

Studies addressing the morphological variations of the lymphatic system in liver biopsy samples throughout the span of major liver lesions in chronic hepatitis are scarce. For this, we aimed to assess the presence of lymphatic vessels and sprouts in chronic hepatitis C marked by the semiquantitative immunohistochemical assessment of podoplanin with the monoclonal antibody D2-40, expecting that the present data may inspire future studies in which further analysis and morpho-molecular correlations may be possible.

Although the experimental data presented in the introduction raise some intriguing questions about lymphangiogenesis in chronic liver disease, Saxena et al.[26], cautioned about the large differences in liver microvasculature between animal and human models. Furthermore, none of the mentioned studies employed quantitative or semiquantitative morphological approaches to study lymphatic shoots and mature lymphatic vessels.

In the present study, the assessment of lymphatic vessels through immunohistochemical labelling with podoplanin yielded the definition of sprout as solid aggregates of lymphatic endotelial cells, versus mature lymphatic vessels which are thin structures with a lumen without red blood cells. The attempt to identify each of these components in different compartments of hepatic lobules was only partially successful, since lymphatic sprouts and mature vessels were identified and quantitated only at portal tracts in early stages of chronic hepatitis C and at fibrous septa in advanced stages, including cirrhosis. Our hypotheses of finding lymphatic structures at the periportal interface and in the hepatic lobule was not substantiated with the present immunohistochemical approach. While acknowledging these important drawbacks, the present study demonstrated several interesting associations.

The almost identical distribution of lymphatic sprouts and mature lymphatic vessels clearly demonstrate by immunohistochemistry and light microscopy that these structures are directly related. It reinforces the concept that a morphological sequence of lymphangiogenesis is reflected in the portal spaces and fibrous septa, starting with the activation of podoplanin-positive lymphatic endothelial cells that give origin to lymphatic sprouts. This relationship is maintained from early stages of chronic hepatitis to advanced stages of HCV-associated cirrhosis. Also, in future studies, the presence of either of these structures may be chosen as a marker of the hepatic lymphatic system.

The increase of both portal sprouts and mature lymphatic vessels was significantly associated to the progression of liver structural alterations. Interestingly, we found that such increase occurs in each step of disturbance in lobular architecture, not only immediately preceding cirrhosis. This may be useful in future studies aiming at the prevention of pathological lymphangiogenesis, which should be started early in the progression of chronic liver disease.

Our analysis of liver lymphangiogenesis in human samples collected by needle biopsy shows many similarities to those reported in animal experiments by Vollmar et al.[16], to the study of Yamauchi et al.[27] in human liver biopsy samples with chronic hepatitis B and C and Yokomori et al.[23] in surgical samples of human livers with HCV cirrhosis.

Using high-resolution fluorescence microscopy, Vollmar et al.[16] simultaneously evaluated hepatic blood macromolecular exchange from the sinusoidal microvasculature and the hepatic lymphatic system in rats in the early stages after exposure to the hepatotoxic drug CCl4.

Those rats were characterized by a progressive delay in the exchange of hepatic macromolecules into blood, implying the development of diffusion barriers inside the fibrotic and cirrhotic liver.

In parallel, in those animals, a marked increase in both lymphatic vessel density and lymphatic vessel area was observed. Linear regression analysis revealed a significant correlation between impairment of sinusoidal macromolecular exchange and density of the lymphatic network. Thus, the lymphatic network increased in agreement with the fibrotic alterations in rats.

Since there were no reports that the data from Vollmar et al.[16] was applicable to the human liver, Yamauchi et al.[27] studied, by morphometric methods, the alterations that occur in human hepatic lymphatic vessels in chronic viral hepatitis B and C and its progression to cirrhosis using 62 liver samples. Those authors investigated the relationship between the degree of liver fibrosis, the activity of liver inflammation, and changes in lymphatic as well as in blood vessels.

Besides number of vessels, Yamauchi et al.[27] studied the area of each lymphatic vessel, finding that such area was significantly higher in cirrhosis than in lower degrees of fibrosis (mild, moderate, and severe, but not cirrhosis). It is intriguing that no differences were found by Yamauchi et al.[27] regarding both the area of each vessel or the number of vessels between the groups with mild, moderate, and severe fibrosis. However, the number of lymphatic vessels in each section tended to increase in association with staging progression. Their finding of a strong correlation of the number of portal/septal lymphatic vessels with the degree of expansion of the portal space was remarkably similar to what we observed in the present study.

The histochemical and morphometric analyses by Yamauchi et al.[27] produced results in agreement with the results of the study by Vollmar et al.[16], showing that lymphatic vessels in the liver increase in size and number with the progression of fibrosis in chronic hepatitis.

Beyond the relationship between architectural disturbance and lymphatic vessels, our present study also disclosed a correlation between the proportion of portal sprouts and lymphatic vessels and the degree of portal inflammation and parenchymal activity. These findings clearly demonstrate that even in early stages of hepatitis, the necroinflammatory activity elicits lymphangiogenesis, a fact to be considered in future studies. In particular, the transition of periportal activity from grade 1 to 2 in both sprouts and portal lymphatic vessels, and from grade 2 to 3 in portal lymphatic vessels highly valued on histopathology, apparently accompanied by more activation of portal lymphangiogenesis.

Thus, in the item "inflammatory activity", our result differed from that obtained by Yamauchi et al.[27] who concluded that the number and area of lymphatics did not differ significantly with hepatitis activity. On the other hand, the findings of the present study and those of Yamauchi et al.[27] were similar regarding the association of greater formation of lymphatic vessels in cases of hepatitis C in more advanced architectural stages.

The present study also aimed at the search of potential relation of lymphangiogenesis and other important pathological variables. However, in the present study, periportal/perisseptal activity, viral genotype, and variables related to comorbidities such as steatosis and steatohepatitis were not found to be associated with the pattern of lymphatic sprouts or mature vessels.

As previously characterized in the normal liver, the tools applied in the present study did not allow identification of lymphatic vessels in the parenchymal region in any stage of chronic hepatitis C/cirrhosis. Similarly, in a study of 16 surgical samples Yokomori et al.[23] had reported finding few or no lymphatic vessels in the parenchyma in both normal livers and cirrhotic livers with HCV-associated hepatocellular carcinoma.

Finally, our current study also attempted to identify possible Mall spaces by assessing the presence of any podoplanin-expressing lymphatic endotelial cells at the portal-parenchymal interface, especially because of the recently introduced concepts of hepatic interstitium[28,29]. However, only rare, isolated cells were found at the interface, leading to a median number of zero lymphatic sprouts. The same occurred with mature lymphatic vessels, since only two cases showed more evident lymphatic vessels at the interface. Therefore, no conclusions could be drawn regarding the interstitial space of Mall and lymphatic sprouts/vessels in the current study.

Our study may serve as a basis for future analyses attempting to correlate histological and immunohistochemical findings with molecular data, considering that several growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor (TGF) have been implicated in angiogenesis. We hope our findings may usher in further studies and promote better knowledge about lymphatic endothelial cell proliferation and lymphatic vessel formation.

Authors' contribution

Assato AK: designed and performed the research and the immunohistochemistry reactions, analyzed the data, and wrote the manuscript. Pasinato APBF: helped in obtaining samples and analyzed the histological variables. Cirqueira CS: analyzed the data. Wakamatsu A: supervised all laboratorial activity. Alves VAF: designed and performed the research, analyzed the data, wrote the manuscript, and supervised all the research steps.

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RESUMO – Contexto – A hepatite C é um relevante problema de saúde pública. A doença pode permanecer clinicamente silenciosa tanto na forma aguda como na crônica e as infecções crônicas podem progredir para doenças avançadas, tais como cirrose e carcinoma hepatocelular (CHC), requerendo tratamentos dispendiosos, comprometendo a qualidade de vida do paciente e até mesmo levando à morte. Por esta razão, é uma das indicações mais frequentes para o transplante hepático. Apesar da introdução do tratamento com antivirais de ação directa (AAD) representar um progresso notável, muitos pacientes não receberam o tratamento e continuam infectados, e mesmo aqueles que eliminaram a infecção viral devem ser seguidos devido às lesões hepáticas anteriores, especialmente no que diz respeito às alterações da arquitetura lobular e dos vasos sanguíneos e linfáticos. **Objetivo** – Avaliar os aspectos imuno-histoquímicos dos brotos linfáticos e dos vasos linfáticos "maduros" com variáveis histológicas de lesão hepática atribuíveis ao vírus da hepatite C (VHC) e à doença gordurosa. **Métodos** – O presente estudo incluiu 72 biópsias hepáticas em pacientes com hepatite C crônica. Foram analisadas alterações estruturais relativas a "estadiamento" e "atividade". Reações imuno-histoquímicas foram realizadas com anticorpo D2-40 anti-podoplanina. As principais variáveis histológicas também foram semiquantificadas, de modo a permitir a procura de possíveis associações entre os critérios histológicos e imunohistoquímicos, bem como com os genótipos 1 e 3 do VHC. **Resultados** – Os achados histológicos mostraram que os diferentes graus de alterações estrutural estavam bem representados nesta casuística. A atividade necro-inflamatória lobular/parenquimatosa foi predominantemente leve à moderada. A maioria dos casos não apresentava grandes evidências de doença gordurosa, que foi encontrada significativamente mais elevada nos casos infectados com o genótipo 3 do VHC. A quantidade de brotos linfáticos portais aumentou com a progressão de alterações estruturais, sendo máxima na cirrose. Os brotos linfáticos portais, bem como os vasos linfáticos "maduros" portais também mostraram um aumento paralelo ao aumento do grau de infiltrado inflamatório portal/septal. No presente estudo, não foi encontrada qualquer associação significativa entre a proporção de brotos linfáticos portais ou vasos linfáticos maduros portais e o grau de atividade periportal/periseptal. Não foram detectadas relações significativas entre os brotos linfáticos/vasos maduros e a atividade inflamatória periportal ou atividade inflamatória parenquimatosa, nem com infecções devido ao genótipo 1 ou 3 do VHC. **Conclusão** – A reação imunohistoquímica com anticorpo monoclonal D2-40 possibilitou a visualização e a semiquantitação de brotos e vasos linfáticos "maduros" nas amostras obtidas por biópsia hepática. A quantidade de linfáticos aumentou ao longo do processo fibrogênico, significativamente relacionada com a progressão da doença hepática e máxima na cirrose. Não foram detectadas relações significativas com a atividade necro-inflamatória periportal ou parenquimatosa.

Palavras-chave – Hepatitie C crônica; imuno-histoquímica; vasos linfáticos; linfangiogênese; fígado; patologia.

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