Association between liver steatosis and angiogenesis in chronic hepatitis C

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Background: The relationship between steatosis and angiogenesis in chronic hepatitis C (CHC) is unclear.
Aim and methods: The aim was to explain whether liver steatosis presence and its extent are associated with the number of new-formed blood vessels in lobules and portal tracts in CHC. 72 CHC patients infected with viral genotype 1b, 35 of whom had steatosis were evaluated. Monoclonal antibody anti-CD34 was used to identify new-formed blood vessels.
Results: Patients with steatosis had a significantly more advanced stage of fibrosis ($p = 0.002$) and higher inflammatory activity grade ($p = 0.062$). CD34 expression in portal tracts (CD34pt), lobules and fibrous septa (CD34lfs) and total (CD34) were significantly higher in patients with steatosis ($p = 0.034$; $p = 0.021$; $p = 0.023$, respectively). CD34, CD34pt and CD34lfs differed significantly between patients with various steatosis grade ($p = 0.006$; $p = 0.009$; $p = 0.013$, respectively). CD34 and CD34pt differed significantly between each steatosis grade whereas CD34lfs between grade 1 and 3. Fibrosis stage and inflammatory grade were positively associated with steatosis extent ($p = 0.015$; $p = 0.003$, respectively).
Conclusions: Our observations suggest that extensive steatosis of liver parenchyma in CHC patients is associated with formation of new blood vessels in lobules and portal tracts. Understanding the relationship between steatosis, fibrosis and angiogenesis is therefore of great importance for the introduction of new therapeutic approaches and in the evaluation of CHC progression.

Key words: steatosis, angiogenesis, liver, chronic hepatitis C, CD34.

Introduction

Hepatic steatosis is one of morphologic features encountered in samples from patients with chronic hepatitis C (CHC) [1]. The reported prevalence of steatosis in CHC patients varies between 40% and 80% [2-9]. Steatosis may be associated with "host-related" factors such as BMI, obesity or insulin resistance and "viral" including HCV genotype 3. It is likely that two types of steatosis can coexist in at least some CHC patients, although, in genotype 3 infection steatosis will be primarily of viral origin and in genotype non-3 – primarily metabolic [10]. Steatosis has been recognized as one of factors capable of influencing liver fibrosis and inflammation [2, 5]. Fibrogenic effect of steatosis is mediated by increased oxidative stress and insulin resistance, induction of apoptosis, activation of hepatic stellate cells (HSCs) and proinflammatory cytokines [10].

Angiogenesis belongs to the factors contributing to the liver damage during CHC. The formation of new blood vessels has been considered to play a pivotal pathogenic role [11, 12]. Structures responsible for proliferation and maturation of new blood vessels in the liver are HSCs, Kupffer cells, regenerating hepatocytes and existing endothelial cells (ECs) [11] which produce vascular endothelial growth factor (VEGF), placental growth factor which potentiates the effects of VEGF, fibroblast growth factors (aFGF and bFGF), hepatocyte growth factor (HGF) and transforming growth factors (TGF-α and TGF-β) [11].

CD34 is a cell surface, sialomucin-like glycoprotein which is expressed on hematopoietic progenitor cells, normal vascular endothelium and fibroblasts [13]. CD34 is an endothelial antigen which has been used to highlight the density of vessels as a direct marker of the degree of neoangiogenesis in tumours [14].

The relationship between angiogenesis and steatosis in CHC is unclear. As far as we are concerned, our study for the first time compared angiogenesis in the liver of CHC patients with and without steatosis. It evaluated the relationship between hepatic steatosis extent and the number of new-formed blood vessels in lobules and portal tracts in the liver of CHC patients. Moreover, the study assessed the localization of new blood vessels in steatotic areas of liver parenchyma.

Material and methods

Patients and liver biopsy

The study encompassed 72 patients with CHC, infected with hepatitis C virus genotype 1b, who had undergone liver biopsy before treatment with pegylated interferon α and ribavirin. The diagnosis of CHC was confirmed by the presence of serum HCV-RNA assayed with the reverse transcription polymerase chain reaction (RT-PCR) method (Amplicor Roche/Promega v.2 Diagnostic Test, New Jersey, USA). Virus genotype was assessed by a reverse-hybridization line probe assay (LiPA Versant Test, Milwaukee, USA). All the patients had abnormal serum aminotransferase activity for at least 6 months.

The patients with infection with other HCV genotypes, obesity (BMI over 30 kg/m^2), drug or alcohol (more than 20 mg/day) abuse and autoimmune, neoplastic, thyroid and psychiatric diseases, hepatitis B or HIV or cytomegalovirus coinfection, diabetes mellitus or impaired glucose tolerance, renal and heart failure, metabolic syndrome were excluded from the study.

The study was approved by the Ethical Committee of the Medical University of Silesia in Katowice and conformed to the ethical guidelines of the Declaration of Helsinki. Informed consent was obtained for the whole study series.

Table I. Grading for steatosis according to Brunt's scale

Grading for steatosis (1-3)
Grade 1: < 33% of hepatocytes affected
Grade 2: 33-66% of hepatocytes affected
Grade 3: > 66% of hepatocytes affected

Histopathological evaluation

Liver biopsy was performed with Hepafix kit (B. Braun, Melsungen AG, Germany) before antiviral therapy, as part of the diagnostic routine. Biopsy samples included at least 6 portal tracts. Liver biopsy specimens were fixed in 5% buffered formalin and embedded in paraffin. Histopathological preparations were made using haematoxylin-eosin, and with methods according to Gomory and Azan (fibrotic stage evaluation). Five-micrometer thick tissue sections were cut. For further immunohistochemical examinations each paraffin block was used to make at least two preparations including 4 biopsy sections. The preparations were evaluated with a microscope (Labophot, Nikon, Japan) which provided magnifications ranging from 40× to 400×.

Histopathological examination was carried out retrospectively by two experienced pathologists using a double-headed microscope. The steatosis grade was assessed according to Brunt's (Table I) whereas the necro-inflammatory activity grade and fibrosis stage -according to Scheuer's scale [15, 16].

Immunohistochemistry for CD34

Formalin-fixed paraffin-embedded sections were deparaffinized, dehydrated and demasked in a microwave oven for 20 minutes in 0,01 M sodium citrate buffer (pH 6.0). Monoclonal anti-CD34 (N-1632, DAKO, Glostrup, Denmark) was used as the primary antibody. The antigen-antibody reaction was visualized with LSAB 2 System-HRP (K0675, DAKO, Glostrup, Denmark) using 3,3-diaminobenzidine (DAB) as a chromogen. The sections were counterstained with Mayer's haematoxylin. Human placenta tissue was used for positive control of the performed determination CD34 expression.

The reaction of antigen CD34 in new created blood vessels was evaluated in two separate analyses of 6 portal spaces and 6 areas of lobules or fibrous septa in lobules. Areas of fibrous septa were defined as a zone of lobules closely adjacent to fibrous septum visualized in a high power field (400×).

Subsequently, the median value of vessel counts in a portal tract and fibrous septum was calculated

Table II. The results of histopathological examination in CHC patients. The comparison of the fibrosis stage and inflammatory activity grade between CHC patients with and without steatosis

PARAMETER	CHC PATIENTS	NO STEATOSIS	STEATOSIS		STEATOSIS GRADE 1	STEATOSIS GRADE 2	STEATOSIS GRADE 3
Fibrosis							
Stage 0	7	5	2		2	0	0
Stage 1	14	12	2		2	0	0
Stage 2	16	6	10	p = 0.002	3	7	0
Stage 3	24	13	11		2	4	5
Stage 4	11	1	10		1	4	5
Inflammatory activity							
Grade 1	8	7	1		1	0	0
Grade 2	30	17	13		7	6	0
Grade 3	25	10	15	p = 0.062	2	7	6
Grade 4	9	3	6		0	2	4
	72	37	35		10	15	10

(CD34 index). The number of vessels was estimated with a "hot-spot" technique according to Vermeulen criteria [17]. In statistical analysis, the amount of blood vessels was expressed by CD34 index in portal tracts (CD34pt), CD34 index in lobules and fibrous septa (CD34lfs) and by summarized index in portal tracts and lobules (CD34).

Statistical analysis

In describing the study results, medians and interquartile ranges (for continuous variables: age, CD34pt, CD34lfs, CD34) and frequency distributions (for categorical variables: gender, fibrosis stage, inflammatory and steatosis grade) were used. Kruskal-Wallis analysis and Mann-Whitney U test were used for comparison of continuous variables between groups. The Spearman's correlation coefficient was calculated to investigate the correlation between variables. Statistical significance was set at $p < 0.05$. Analysis was carried out with Statistica 7.0 software (StatSoft, Tulsa, OK, USA).

Results

Characteristics of the patients studied

Biopsy specimens of the liver from 72 patients (36 males and 36 females) suffering from CHC were evaluated. Median (interquartile range) age was 49.0 (33.5-55.5) years and BMI 23.4 (18.3-27.1) kg/m^2. Males and females were comparable according to age ($p = 0.48$) and BMI ($p = 0.55$). There was no difference with respect to sex and BMI between patients with steatosis and with no steatosis. Patients with steatosis were older than patients without steatosis [52 (44.0-58.0) vs. 52 (27.0-51.0) years, $p = 0.002$].

Histological evaluation

The results of histopathological examination in CHC patients are presented in Table II.

Steatosis was observed in 35 patients. The comparison of histopathological features in CHC patients with and without steatosis is shown in Table II. Patients with steatosis had a significantly more advanced stage of fibrosis ($p = 0.002$). The inflammatory activity grade was higher in steatotic patients but the difference was on the threshold of statistical significance ($p = 0.062$). The comparison of patients with and without steatosis regarding fibrosis and inflammatory activity is shown in Table II.

Immunohistochemistry

All analyzed patients with steatosis had a significantly higher total CD34 expression as well as expression in portal tracts (CD34pt) and fibrous septa and lobules (CD34lfs) ($p = 0.023$; $p = 0.034$; $p = 0.021$, respectively). The value of CD34 expression index is shown in Table III. CD34, CD34pt and CD34lfs differed significantly between patients with various grade of steatosis ($p = 0.006$; $p = 0.009$; $p = 0.013$, respectively) (Table IV, Figures 1-2). Post-hoc analysis revealed statistically significant difference of CD34 between each grade of steatosis. There was no difference of CD34pt between patients with steatosis grade 1 and grade 2. CD34lfs expression differed significantly in post-hoc analysis only between patients with grade 1 and grade 3 of steato-

Table III. The comparison of CD34 expression in CHC patients with and without steatosis

	No steatosis	Steatosis	p
CD34	13.8 (6.6-26.7)	25.4 (14.2-34.0)	0.023
CD34pt	11.4 (6.6-20.3)	17.4 (11.0-23.0)	0.034
CD34lfs	3.7 (0-8.5)	8.0 (3.0-10.8)	0.021

CD34pt – CD34 expression index in portal tracts, CD34lfs – CD34 expression index in lobules and fibrous septa

Table IV. The comparison of CD34 expression in CHC patients with a different grade of steatosis

	Steatosis			
	Grade 1	Grade 2	Grade 3	p
CD34	12.6 (5.8-25.4)	24.2 (15.4-32.2)	34.5 (28.1-36.0)	0.006
CD34pt	9.4 (5.8-17.4)	16.2 (11.2-21.0)	22.5 (17.8-25.0)	0.009
CD34lfs	1.6 (0-8.0)	7.0 (3.7-9.8)	10.9 (3.0-12.0)	0.013

CD34pt – CD34 expression index in portal tracts, CD34lfs – CD34 expression index in lobules and fibrous septa

sis. When compared in post-hoc analysis patients with and without steatosis CD34 and CD34pt expression was higher in the liver of patients with steatosis grade 2 and 3 whereas CD34lfs only in the case of grade 3 (Table V).

There were single, sparse new formed blood vessels within lobule area in the case of grade 1 of steatosis (Fig. 1). When steatosis was more extent - grade 2 and 3 – evident CD34 expression was observed in steatotic areas of lobules and was localized mainly in periportal zone (Fig. 2). CD34 expression in steatotic areas of lobules is also presented and described in Figures 3-7.

Fibrosis stage and inflammatory activity grade were positively associated with grade of steatosis ($p = 0.015$; $p = 0.003$, respectively).

In some biopsies CD34 expression was arranged linearly, similar to that of septal structures, and accompanied by bile duct proliferation. Moreover, there was no inflammatory infiltration near these alterations (Fig. 8).

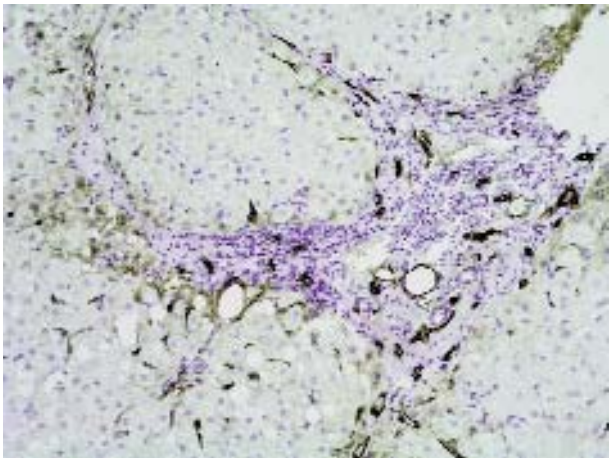

Fig. 1. Steatosis grade 1 and periportal fibrosis – abundant vessels in portal tracts and in periportal area of lobules (CD34 immunostaining, magnification 200×)

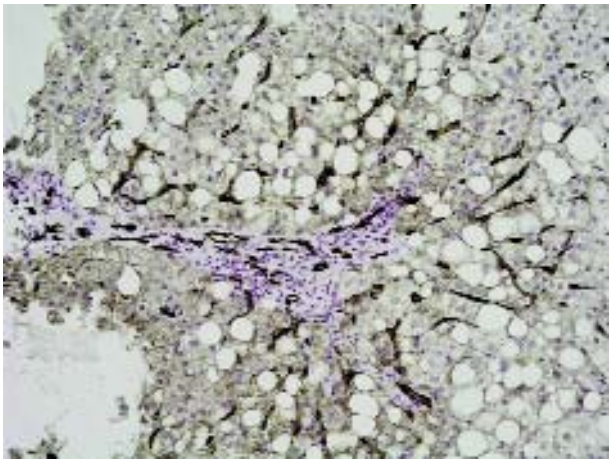

Fig. 2. Steatosis grade 3 and periportal fibrosis - evident microvessels in the portal tract and periportal area of lobules within steatotic focuses (CD34 immunostaining, magnification 200×)

Discussion

The pathophysiological significance of chronic viral hepatitis-associated angiogenesis is currently unclear. The new vessels are an integral part of tissue remodelling that accompanies chronic inflammation and provide a portal of entry for the continuing recruitment of inflammatory cells [11, 18]. It has also been proposed to exert a beneficial role by contributing to tissue repair and regeneration after liver damage [11, 12]. Angiogenesis in chronic liver diseases (CLDs) can be interpreted according to two main pathways. First, neo-angiogenesis is stimulated in hepatic tissue by progressive tissue hypoxia evoked by inflammation and fibrosis. Second, the

Table V. Post-hoc analysis of difference of CD34, CD34pt and CD34lfs expression between patients with various steatosis grade and without steatosis

	No steatosis	Grade 1	Grade 2	Grade 3
CD34				
No steatosis		0.649	**0.046**	**0.002**
Grade 1	0.649		**0.049**	**0.007**
Grade 2	**0.046**	**0.049**		**0.027**
Grade 3	**0.002**	**0.007**	**0.027**	
CD34pt				
No steatosis		0.585	**0.049**	**0.003**
Grade 1	0.585		0.067	**0.007**
Grade 2	**0.049**	0.067		**0.046**
Grade 3	**0.003**	**0.007**	**0.046**	
CD34lfs				
No steatosis		0.845	0.051	**0.003**
Grade 1	0.845		0.067	**0.009**
Grade 2	0.051	0.067		0.052
Grade 3	**0.003**	**0.009**	0.052	

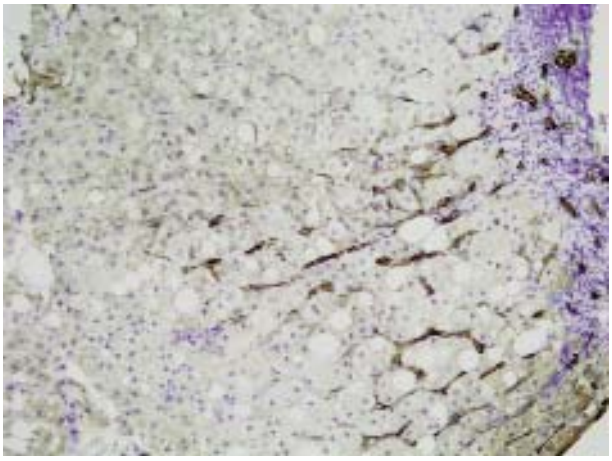

Fig. 3. CD34 expression in new formed blood vessels within steatotic areas in the periportal lobular zone and portal tract (CD34 immunostaining, magnification 200×)

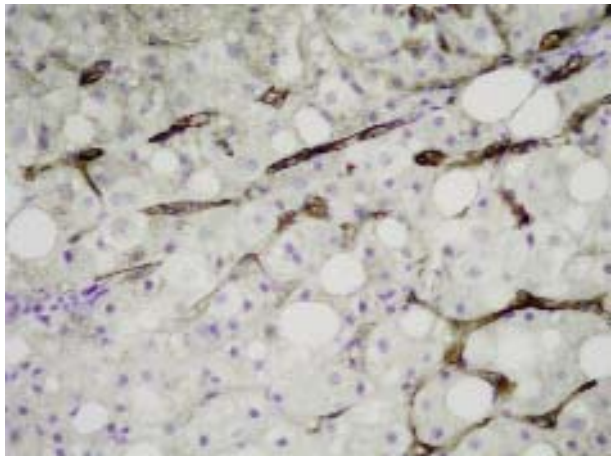

Fig. 4. CD34 expression in longitudinal sections of proliferating blood vessels within lobular areas of macrovesicular steatosis (CD34 immunostaining, magnification 400×)

process of liver chronic wound healing typical of fibrogenic CLDs is characterized by an over-expression of pro-angiogenic factors [19]. Garcia-Monzon *et al.* found expression of CD31 and cadherin 5 on endothelial cells in inflamed portal tract of patients with CHC. It was more evident in more inflamed portal tracts acquiring a characteristic form of capillary tube formation [12]. Also a soluble form of CD31 [soluble platelet endothelial cell adhesion molecule (PECAM)-1] is increased in CHC patients [20]. Amarapurkar *et al.* described angiogenesis in chronic liver diseases, having shown expression of CD34 in 66% of CHC patients, but the group was small. These authors described more frequent CD34 expression in the more advanced fibrosis stage [21]. Mazzanti *et al.* assessed microvessel density in the liver of patients with chronic viral hepatitis C or B and indicated that angiogenesis was particularly linked to HCV infection [22]. The relationship between steatosis and angiogenesis in CHC is unclear.

Our study showed that higher grades of steatosis were associated with a more advanced stage of fibrosis. It is in accordance with the results presented by Adinolfi *et al.* [2] and Hourigan *et al.* [5]. Moreover, Castera *et al.* showed that worsening of steatosis was an independent factor associated with progression of fibrosis in paired liver biopsies [23]. In a study by Leandro *et al*., steatosis has been shown to be associated with an increased liver inflammatory activity [24]. Our investigation showed that the difference of inflammatory activity between steatotic and non-steatotic patients was on the threshold of statistical

Fig. 5. CD34 expression within areas of the lobule with steatosis. The absence of CD34 expression in areas of the lobule without steatosis (CD34 immunostaining, magnification 200×)

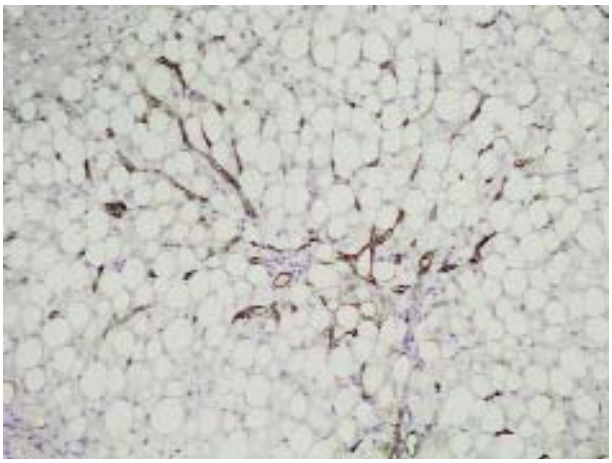

Fig. 6. The vast area of macrovesicular steatosis. CD34 expression present around fatty granuloma – longitudinal sections of new created blood vessels (CD34 immunostaining, magnification 200×)

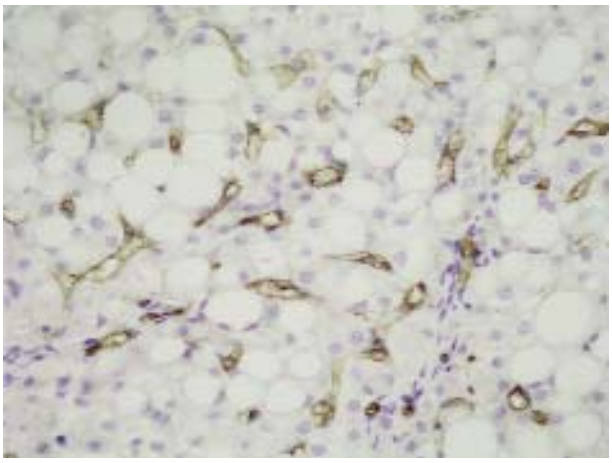

Fig. 7. The cross-section of new created blood vessels with evident CD34 expression within vast area of macrovesicular steatosis (CD34 immunostaining, magnification 400×)

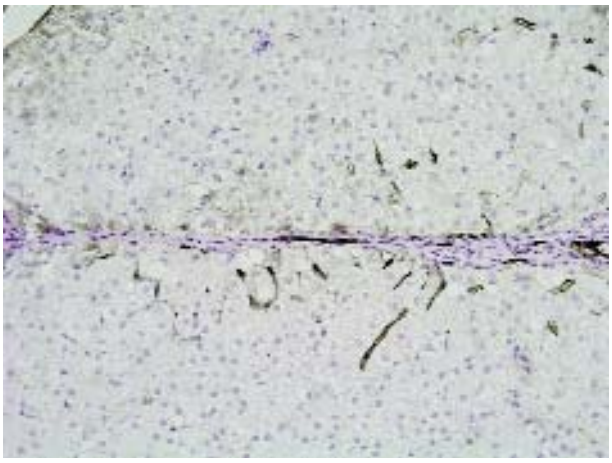

Fig. 8. CD34 expression arranged linearly, similar to that of septal structures, and accompanied by bile duct proliferation. Lack of inflammatory infiltration near these alterations

significance. However, it differed significantly between patients with a various steatosis grade.

Hepatic steatosis leads to chronic oxidative stress which influences the mitochondrial function by reduction of adenosine triphosphate synthesis and increase therefore hepatocellular vulnerability to necrosis [25]. Hepatocyte injury evokes reparative and regeneration processes. The wound healing process is strictly associated with new blood vessel formation and is characterized by an over-expression of several factors, cytokines and metalloproteinases with an inherent pro-angiogenic action [19, 26]. A key area in the study of the cellular and molecular relationships existing between fibrogenesis and angiogenesis concerns the proangiogenic role of activated hepatic stellate cells [19]. Steatosis is positively associated with increased liver cell apoptosis. In the absence of steatosis, increased liver cell apoptosis was not related to HSCs activation. In contrast, in the presence of steatosis, increased apoptosis was associated with HSCs activation [27]. Activated HSCs are able to produce VEGF, angiopoietin-1 (Ang-1) and their receptors stimulating angiogenesis [28]. Finally, they produce increased levels of TGF-β which in the final stage promotes mesenchymal cells differentiation into pericytes which are required for new vessel maturation [11]. TGF-β expression in the liver both lobular and portal was found to be higher in CHC patients with steatosis [29]. A more evident expression was observed in the lobule area of steatotic hepatocytes as compared with portal tracts [29]. These observations suggest that the activity of HSCs is likely to be enhanced by fatty changes.

CD34 expression both in portal tracts and lobules was significantly more evident in CHC patients with steatosis compared to subjects with no steatosis. A higher expression of CD34 in portal tracts and lobules was associated with a more advanced grade of steatosis. Evident increase of CD34 expression in lob-

ules and portal tracts was observed in the case of steatosis grade 2 or 3. Angiogenesis in CHC patients with grade 1 of steatosis was similar to that in patients without steatosis. This observation suggests that new blood vessel formation may be stimulated by more advanced steatosis. Our earlier study showed that CD34 expression was positively related to the inflammatory activity grade and fibrosis stage. This relationship was evident in the portal tracts, fibrous septa and periportal zones of lobules. More evident angiogenesis in the periportal lobular zone is strictly co-localized with periportal hepatitis in CHC [30].

In conclusion, our results showed that steatosis of hepatocytes was positively associated with CD34 expression and fibrosis stage in CHC patients. It seems that only more advanced steatosis of liver parenchyma is associated with angiogenesis development. Hence, understanding the relationship between steatosis, fibrosis and angiogenesis may influence on therapeutic approaches to chronic hepatitis C and in the evaluation of disease progression.

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