Original Paper

Correlation of Angiogenesis and Inflammation with Post-Operative Complications in Patients with Fatty Liver Disease Undergoing Liver Resection

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ABSTRACT: Background: Hepatic steatosis has been identified as an independent risk factor for post-operative complications. The aim of our research was to assess how inflammation and neoangiogenesis associated with different stages of hepatic steatosis are related to post-operative complications in patients who undergo hepatic resection. Methods: Our study included 19 patients with hepatic steatosis undergoing liver resection for primary or secondary tumors. For every patient we performed immunostaining using a panel of 5 primary antibodies (CD3, CD20, CD68, CD31, CD34) to highlight inflammation and neoangiogensis in the non-tumoral hepatic parenchyma. Results: Taking into consideration the number of vessels as well as the signal area and integrated optical density (IOD) forCD3, CD20, CD68, and also the degree of steatosis, the univariate analysis with a log-rank (Mantel-Cox) test revealed that patients with higher values of CD31 and CD34 had a higher rate of post-operative complications on a 30-day follow-up period. Also, we used a Mann-Whitney U and Kruskal-Wallis H tests for group distributions. We noticed thatCD34 was significantly increased in patients diagnosed with steatosis compared to the control group and there was a statistically significant difference between CD31 median values of S0 (27.6) and S1 (55.8) grades. Conclusion: Patients with steatosis that presented higher values of CD31 and CD34 had a higher rate of post-operative complications. Further studies should assess the value of pre-operative evaluation of angiogenesis in patients with liver steatosis submitted to liver surgery.

KEYWORDS: *Hepatic steatosis, fatty liver disease, angiogenesis, inflammation.*

Introduction

Fatty liver disease, a common form of chronic liver disease has become quite a "silent pandemic" [1] with an increasing prevalence over the last decades.

Worldwide, about 2 billion people are diagnosed with non-alcoholic fatty liver disease (NAFLD) [2], often co-existing with other metabolic and cardio-vascular disorders such as type 2 diabetes, obesity, hypertension.

NAFLD is considered a benign disorder when only simple steatosis is present but it can progress to a more aggressive form, steatohepatitis (NASH) which can lead to more complex liver pathology such as cirrhosis and hepatocellular carcinoma.

In this context, NAFLD is considered a real public health issue: a risk factor for individual disability and increased morbidity.

Considering its high and increasing prevalence, a significant number of patients undergoing liver resection for benign or malignant tumors have some degree of steatosis, or even steatohepatitis [3].

Although the impact of hepatic steatosis on post-resection liver function and regeneration is still incomplete elucidated, it has been identified in some studies as an independent risk factor for post-operative complications [4-6].

Thus, the complex pathogeny of NAFLD has been thoroughly investigated for the past two decades in order to identify possible modifiable risk factors and potential treatment options.

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A better understanding of the mechanisms that initiate steatosis and further disease progression can connect the dots and represent a starting point for future management strategies.

The pathogeny of NAFLD progression consists of interconnected events where angiogenesis and chronic inflammation play a key role.

On one hand, the formation of new blood vessels is thought to be triggered by hypoxia, endothelial dysfunction, inflammation and hepatic stellate cell activation [7].

On the other hand, angiogenesis promotes inflammation and the process of capillarization has been demonstrated to activate hepatic stellate cell and promote fibrogenesis [8].

It is already known that hepatic regeneration in patients undergoing liver resection is partially linked to angiogenesis [9].

Thus, in an experimental study conducted on mice, it has been stated that the presence of steatosis can influence liver regeneration following hepatectomy, by delaying the angiogenic response [10].

Regarding the inflammatory process, some authors claim that steatohepatitis and not only simple steatosis is associated with increased morbidity after hepatic resection, including hepatic and surgical hepatic complications [11].

Under these circumstances, the aim of our study was to investigate the degree of inflammation and neoangiogenesis associated with different stages of hepatic steatosis and their correlation with postoperative complications in patients with hepatic resection for primary or secondary liver tumors.

Material and Methods

The study included 19 patients who underwent partial liver resections for primary or secondary liver tumors in the 2nd Surgery Clinic, Emergency County Hospital of Craiova and 2nd Surgery Clinic, Clinical Hospital "Sf. Maria", Bucharest, selected over a period of 5 years (2016-2020).

Of the 19 patients, 10 were diagnosed with a type of digestive cancer (oesophageal, gastric, colorectal, pancreatic cancer) with hepatic metastases and 3 patients with hepatocellular carcinoma.

The biological samples embedded in paraffin blocks were obtained from the archives of the pathology departments of the two hospitals.

The paraffin blocks were processed and stained for the immunohistochemical study, techniques performed at the Centre for Studies of Microscopic Morphology and Immunology at the University of Medicine and Pharmacy, Craiova.

The immunohistochemical study was performed on serial slides, using a panel of 5 markers to highlight inflammation and the degree of capillarization.

To reveal angiogenesis, we performed immunostaining with CD31 and CD34 monoclonal.

As for inflammation markers, CD3 antibody was used to reveal T lymphocytes, being known as a highly effective T cell marker, while CD20 antibody was used to identify B-lymphocytes.

In order to reveal the monocyte-macrophage population, CD68 was the antibody of choice.

Briefly, 5μm-thick seriate sections were cut on a rotary microtome, and slides were processed for individual immunohistochemical detection of CD31 [Dako (Glostrup, Denmark), mouse, 1:100], CD34 (Dako, mouse, 1:100), CD68 (Dako, mouse, 1:200), CD20 (Dako, mouse, 1:200), CD3 (Dako, rabbit, 1:100).

The sections were deparaffinized, rehydrated in decreasing alcohol series, processed for antigen retrieval by microwaving in 0.1M citrate buffer pH6 for 20 minutes, incubated in 1% hydrogen peroxide in distilled water for 30 minutes in order to block the endogenous peroxidase activity, and then kept for another 30 minutes in 3% skimmed milk in PBS for blocking unspecific antigen sites.

The primary antibodies were incubated on the slides at 4°C for 18h, utilising optimised dilutions, and the next day the signal was amplified for 60 minutes utilizing a species-specific peroxidase polymer-based system adsorbed for human immunoglobulins (Nikirei Bioscience, Tokyo, Japan).

The signal was then detected with 3,3'-diaminobenzidine (DAB) (Nikirei-Bioscience) and the slides were coverslipped in DPX (Sigma-Aldrich, St. Louis, MO, United States) after a haematoxylin counterstaining. Negative controls were obtained by omitting the primary antibodies.

Slides were viewed and images taken on a Nikon Eclipse 55i microscope (Nikon Europe BV, Amsterdam, the Netherlands) equipped with a 5Mp CCD camera and the Nikon NIS-Elements AR image analysis package.

A number of 5 images have been captured randomly on each slide utilizing constant exposure and illumination settings, and the 20×objective.

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All images have been next analyzed utilizing the Image-Pro Plus AMS 7 image analysis software (Media Cybernetics, Bethesda, MD, United States).

Vascular signals (CD31 and CD34) have been manually counted as number of elements and quantified as area densities for the 20×objective area.

Macrophages, T and B lymph cells have been quantified as the expression area and integrated optical density (IOD) of the DAB-stained signal.

The retrospective study also included the clinical data of the patients (age, sex, environment of origin, postoperative evolution and complications) as well as the histopathological diagnosis, data collected from the accompanying files of patients.

Clinical features and all the data obtained from the software analysis were exported in an electronic database using Microsoft Excel for Windows.

Initially, the patients' data was regrouped in Microsoft Excel and then we used Statistical Package for Social Sciences (SPSS), version 20 (IBM Corp.) to convert inputs into numerical or categorical parameters, and to perform a statistical analysis upon the acquired values.

We used Mann-Whitney U and Kruskal-Wallis H tests for group distributions, when there is no data normality.

Associations were analyzed based on Kendall's tau-b correlation and point-biserial correlation tests.

For each group of markers (neoangiogenesis and inflammation) we used binominal logistic regression to analyze the relation between the presence of postoperative complications and markers' values, adjusting the result with the degree of steatosis.

Results

The histopathological study revealed that steatosis was present in the liver parenchyma at distance from the tumor bearing tissue in 16 of the patients and was mild (S0-S1) in 10 patients and moderate in 6 (S2), with no severe steatosis cases.

We compared the results with 3 normal liver histology cases.

Immunostaining for vascular endothelial marker CD31 showed a tendency for higher vascular densities in peritumoral liver parenchyma associated with steatosis, regardless of the type of tumor (i.e. primary hepatocellular carcinoma or metastases), compared to control liver tissue (Figure 1 A,B).

For CD34, the difference seemed to be even more clear (Figure 1 C,D), however, for both markers there was an important variability even between the images of the same case.

Inflammatory markers revealed a rare population of diffusely infiltrating lymph cells, among which T lymphocytes were more abundant compared to B cells (Figure 2 A-D).

The monocyte-macrophages showed a much denser population compared to the lymph cells, as CD68 labels both Kupfer cells and migrated monocytes, as well as activated stellate cells (Figure 2 E,F).

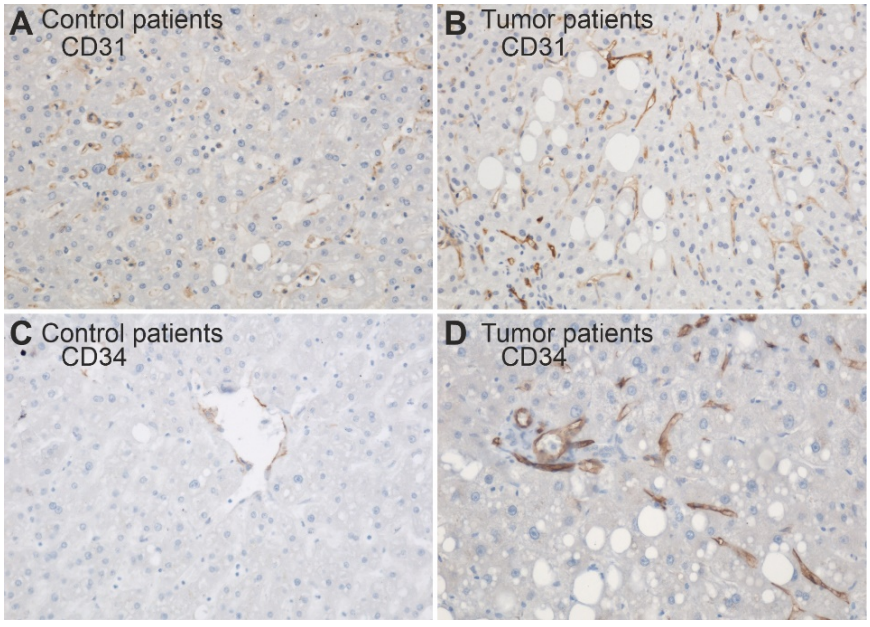

Figure 1. Vascular endothelial markers in liver control and peritumoral liver parenchyma. CD31 and CD34 tend to exhibit higher vessel densities in the peritumoral liver parenchyma with associated steatosis (B, D) compared to control tissue (A, C); objective 20×.

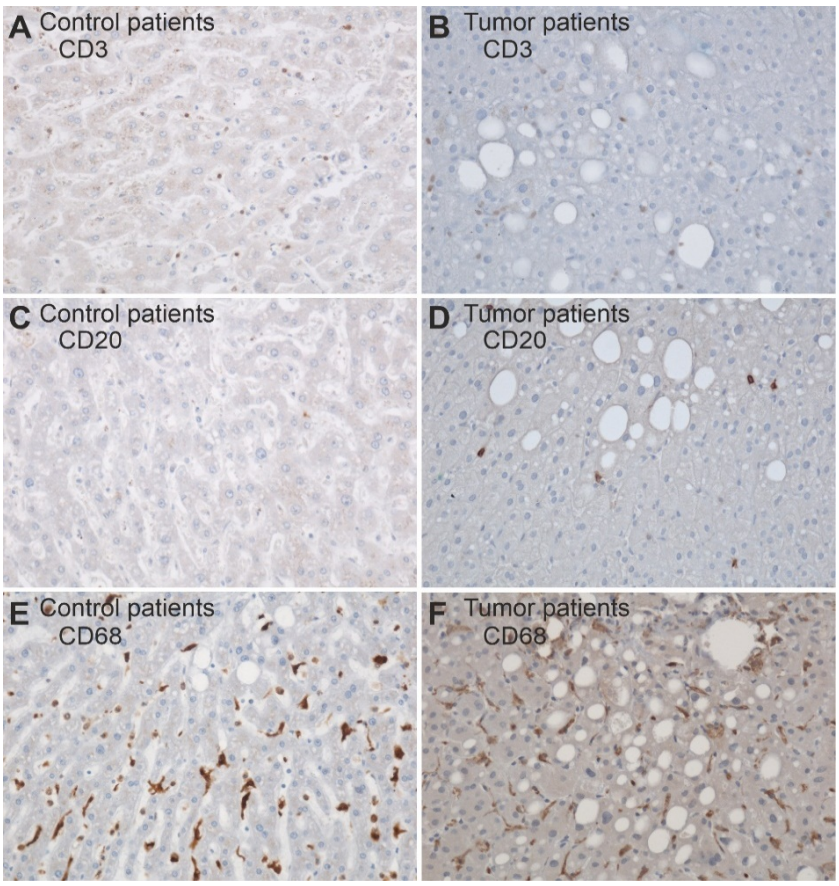

Figure 2. Inflammatory markers in liver control and peri-tumoral liver parenchyma. T lymph cells (A,B) revealed slightly higher cellular densities compared to B lymph cells (C, D) in both control and peri-tumoral liver parenchyma with steatosis, while the macrophage population (E, F) showed the highest densities in both type of tissues; objective 20×.

Group Distributions

In order to find if there are any differences in markers' areas and IOD between patients from test and control groups, we ran a Mann-Whitney U test.

There was no statistically significantly difference in markers' values between test and control groups, using an exact sampling distribution, except for CD34 (see Table 1).

Furthermore, to determine if there-is any difference in markers' values between the different stages of steatosis, S0, S1 and S2, a Kruskal-Wallis H test was run.

Visual inspection of boxplots revealed that the distributions of marker scores were not uniform across all groups. Except for CD31, the distributions of marker scores were not statistically significant different between groups (see Table 1).

Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons for CD31, which had a similar distribution of markers scores (boxplot visual inspection) and a statistically significant difference between steatosis grade.

The adjusted p-values are shown in the table below.

There were statistically significant variations in median markers values between the S0 (27.6) and S1 (55.8) groups (p=015), but not between any other group combinations, according to this post hoc analysis.

For assessing gender, environment, relapse and hepatic metastasis distribution differences, we ran a Mann-Whitney U test to determine if there were differences in markers' areas and IOD.

Distributions of these parameters were not similar, as assessed by visual inspection.

There was no statistically significantly difference in scores between patients from gender, environment and relapse groups, using an exact sampling distribution (see Table 1).

We also used a Mann-Whitney U test to determine if there were differences in markers' areas and IOD, between patients with and without hepatic metastasis.

There was a statistically significantly difference only for CD34.

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Table 1. Group distributions.

	p-values (Mann-Whitney U test)							
	CD3	CD31	CD34	CD20	CD68	CD3	CD20	CD68
Test / **Control**	0.421	0.109	**0.004**	0.712	0.712	0.421	0.79	1
	p-values (Kruskal-Wallis H test)							
S0 (4) / **S1 (6)** / **S2 (6)**	0.187	**0.012**	0.064	0.162	0.606	0.187	0.162	0.282
	p-values (Mann-Whitney U test)							
Met Yes (8) / **Met No (8)**	0.105	0.328	**0.010**	0.442	0.328	0.105	0.24	0.279

Correlations

We investigated the relationship between the post-operative evolution (favorable or not) and the presence of hepatic metastasis amongst the 16patients from the test group using a Kendall's tau-b correlation.

There was a week, negative association between favorable evolution and the presence of hepatic metastasis, which was statistically significant, τb=-0.516, p=0.046.

The following statistically significant associations were also identified:

Table 2. Kendall's tau-b correlation and point-biserial correlation between each marker and the presence of steatosis and metastases.

		Kendall's tau-b correlation		
		Cases	Tb	p
CD31	CD34	19	0.328	0.050
CD31	CD20	19	0.375	0.025
CD34	Test/Control	19	0.498	0.012
CD34	Hepatic metastases	16	0.548	0.012
Favorable evolution	Hepatic metastases	16	-0.516	0.046

Point-biserial correlation tests were run between the presence of complications and markers' scores.

Data are presented as mean±standard deviation (SD). Preliminary analyses showed there were (a) no outliers; (b) engagement score was normally distributed, as assessed by Shapiro-Wilk's test (p>05), except for two small subgroups; and (c) there was homogeneity of variances, as assessed by Levene's test for equality of variances, except for CD3.

There was no statistically significant correlation between complications and markers' values (see Table 3).

Table 3. Point-biserial correlation between the presence of complications and markers' signal area and IOD.

	Point-biserial correlation	
	$r_{pb}(19)$	P
CD31	0.336	0.204
CD34	0.086	0.753
CD3	0.055	0.840
CD20	-0.122	0.653
CD68	0.377	0.150

Binomial Logistic Regression

A binomial logistic regression was performed to ascertain the value of neoangiogenesis markers, CD31 and CD34 and inflammation markers, CD3, CD20, CD68 and the degree of steatosis on the likelihood that patients will develop complications.

The linearity of the continuous as functions on the logit of the dependent variable was evaluated via the Box-Tidwell procedure.

A Bonferroni correction was applied using all eight terms in the model resulting in statistical significance being accepted when p<00625.

Based on this assessment, all continuous independent variables (area of the signal) were found to be linearly related to the logit of the dependent variable.

The logistic regression models were not statistically significant, $\chi 2(3)=4.230$, p<0.376, respectively $\chi 2(3)=6.884$, p=0.229.

Furthermore, a similar binomial logistic regression was performed to ascertain the value of neoangiogenesis markers and the presence of hepatic metastases, as well as inflammation

markers and the presence of hepatic metastasis on the likelihood that patients will develop complications.

The linearity of the continuous as functions on the logit of the dependent variable was evaluated via the Box-Tidwell procedure.

A Bonferroni correction was applied using all six terms in the model resulting in statistical significance being accepted when p<00833.

Based on this assessment, all continuous independent variables (area of markers) were found to be linearly related to the logit of the dependent variable.

The logistic regression models were not statistically significant, $\chi 2(3)=5.511$, p<0.138, respectively $\chi 2(3)=8.584$, p=0.072.

Univariate Analysis of Prognostic Variables

We analyzed the complications that occurred postoperatively over a period of 30 days using Kaplan-Meier survival curves.

Survival time was defined as the period between surgery and the diagnosis of a major complication, whilst all cases were censored at 30 days, when the follow-up stopped.

Complications were regarded as "events" and the corresponding Kaplan-Meier survival curves were constructed based on the probability of "survival "computed as 1-probability of "decease". Beside a direct visual comparison, a logrank test (Mantel-Cox) has been computed.

The variables based on which we computed the survival functions were: the presence of steatosis, the presence of hepatic metastases, as well as immunohistochemical markers, specifically CD31 and CD34, related to the number of days until post-operative complications occurred.

We initially divided patients into 2 groups, depending on the presence or absence of hepatic steatosis.

Kaplan-Meier survival curves, stratified according to the presence of steatosis are shown in Figure 3.

A visual difference can be observed in the survival of patients with hepatic steatosis as compared to those with normal liver, but this difference was not statistically significant.

Thus, the log rank test (Mantel-Cox) had a value of 0.391 (Chi-Square=0.735).

We also divided patients into 2 groups, depending on the presence or absence of hepatic metastases.

Kaplan-Meier survival curves, stratified according to the presence of metastases are shown in Figure 4.

Again, a visual difference can be observed in the survival of patients with hepatic metastases compared to those with other underlying disease and normal liver.

However, the log rank test (Mantel-Cox) had a value of 0.066 (Chi-Square=3.371).

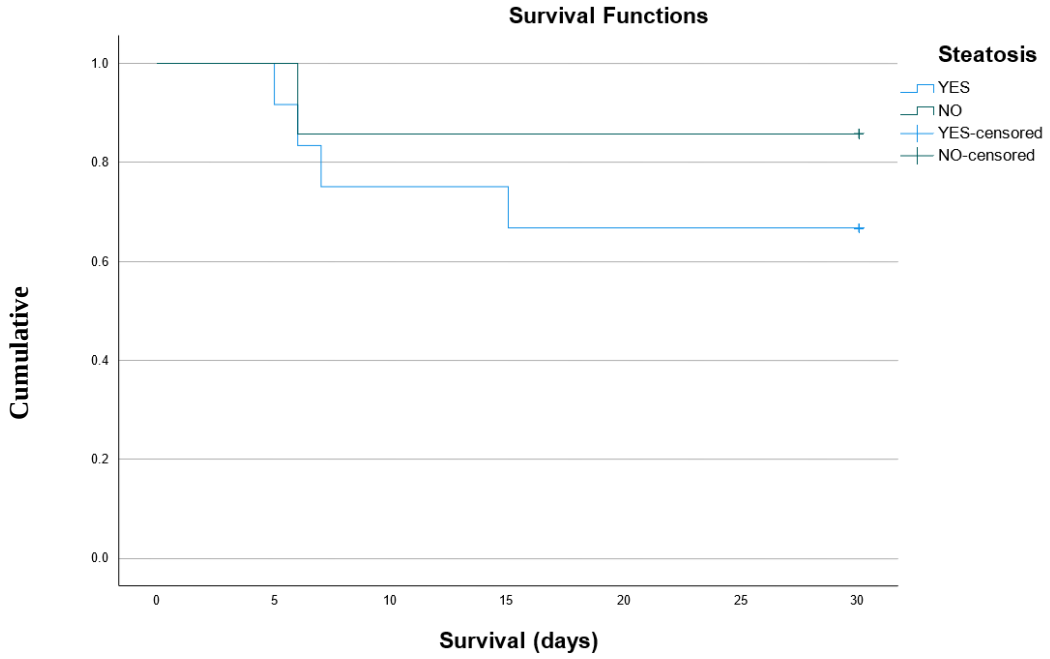

Figure 3. Kaplan Meier curves indicating the survival function of patients undergoing liver resection, followed for a period of 30 days, depending on the presence or absence of steatosis.

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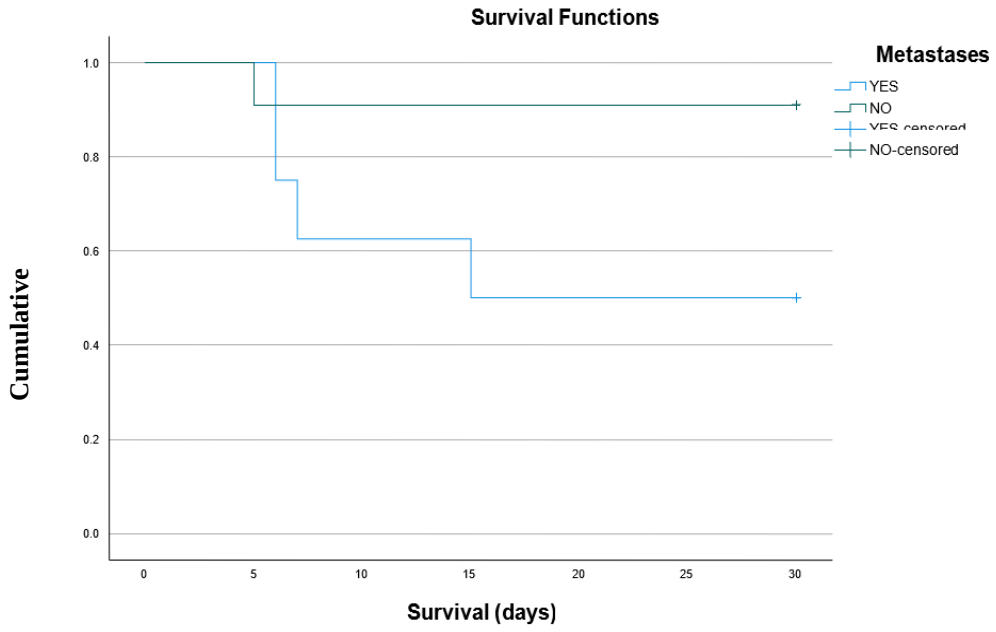

Figure 4. Kaplan Meier curves indicating the survival function of patients undergoing liver resection, followed for a period of 30 days, depending on the presence or absence of metastases.

We then divided the patients according to the mean values obtained for area of both CD31 and CD34.

The Kaplan-Meier survival curves, stratified according to the low, respectively high values for the CD31 and CD34 markers are depicted in Figure5 and 6.

Furthermore, the value of the logrank test (Mantel-Cox) was 0.051 (Chi-Square=3.816), for both markers.

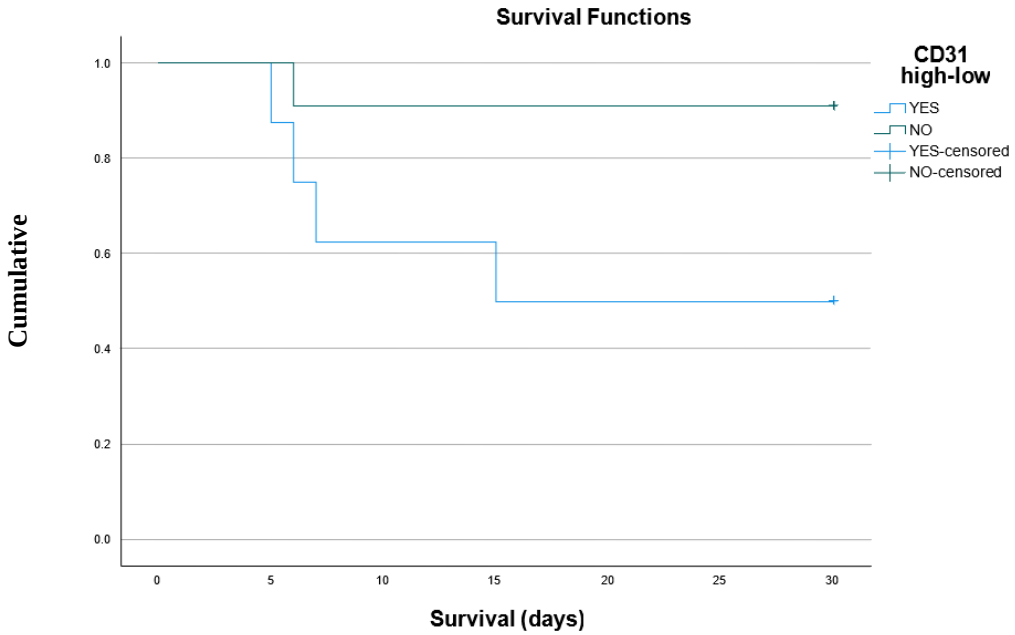

Figure 5. Kaplan Meier curves indicating the survival function of patients undergoing liver resection, followed for a period of 30 days, depending on the low and high values of CD31.

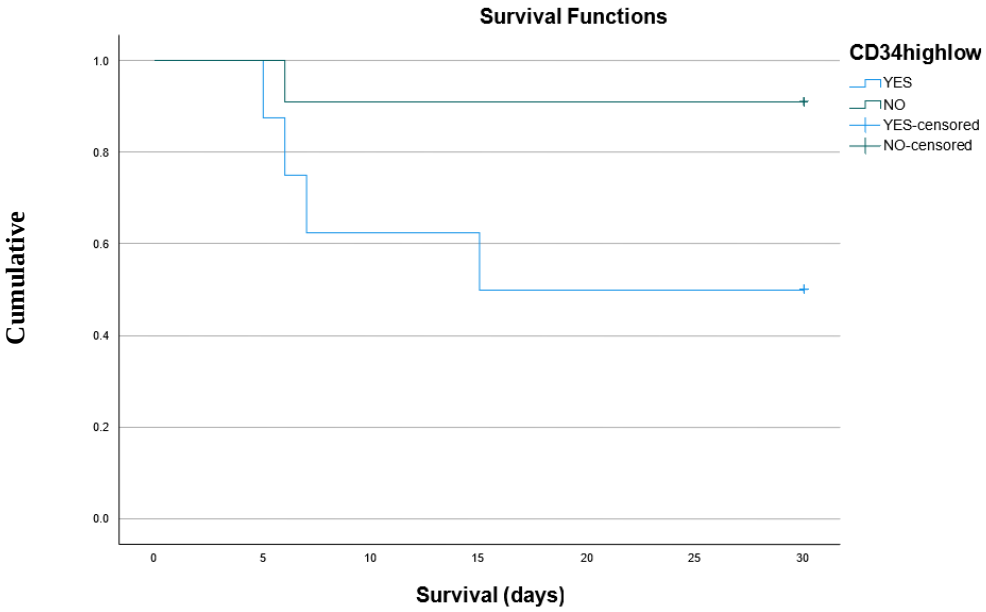

Figure 6. Kaplan Meier curves indicating the survival function of patients undergoing liver resection, followed for a period of 30 days, depending on the low and high values of CD34.

Discussions

Although our study was performed on a limited number of patients, we have identified some important aspects regarding the predictors of patient outcome in liver resections which associate steatosis.

The present study found that CD34 was significantly increased in patients diagnosed with steatosis compared to the control group, most probably associated with the tissue damage induced by the accumulation of fat in hepatocytes [12].

CD34, a marker of neovascularisation is not usually expressed by normal liver sinusoids.

But in chronic liver disease, such as fatty liver disease, endothelial cells can alter their phenotype, express this marker and lead to capillarization of the hepatic sinusoids [13].

Some studies showed that the overexpression of CD34 was present in patients with NASH compared to simple steatosis and healthy liver [14,15].

However, we classified patients only based on the percentage of lipids within hepatocytes and did not further investigate the necroinflammatory activity.

Thus, we cannot exclude that CD34 had actually higher values in patients with a more advanced stage of hepatic steatosis, NASH.

Furthermore, CD31 is another glycoprotein with a major role in angiogenesis [16] and also a marker of sinusoidal capillarization.

We found that there was a statistically significant difference between CD31 median values of S0 (27.6) and S1 (55.8) grades.

This is consistent with other studies that reported overexpression of CD31 simultaneously with liver fibrosis progression in NAFLD [17].

Interestingly, however, is that we did not find any difference between the median values of CD31 between S1 and S2 groups.

This might be related to the small number of patients with S2 stage included in the study.

High microvessel density established by CD34 was also found in the non-tumoral liver parenchyma of patients with liver metastases.

It is well known that expression of CD34 by sinusoidal endothelial cells has been reported as an endothelial marker for hepatocarcinogenesis [18,19], but it is not clear the relationship with secondary liver tumors.

However, angiogenesis is closely related to malignant tumors, CD34 being an important indicator of neovascularization during tumor growth [20].

But as stated above, the overexpression of this marker in our patients appear to be linked to significant tissue damage and inflammation [12] in the noncancerous liver tissue, correlated to the presence of steatosis.

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We did not find any statistically significant correlations between markers of inflammation and poor patient outcome after liver resection.

It is widely recognized that intratumoral abundance of macrophages in various solid tumors is associated with a poor prognosis [21-23], a few studies have investigated the peritumoral inflammatory infiltration and its role in post-resection survival.

For example, a study found that a high expression of CD68 in the peritumoral liver parenchyma was associated with a poor prognosis in patients with HCC who underwent liver resection [24] but the study did not focus on the immediate post-operative complications.

Regarding the correlation between hepatic steatosis and post-operative complications, steatosis is widely regarded as a substantial risk factor in liver surgery, although scientific proof is scarce [4-6, 25].

A pioneer in this field, Behrns et al. investigated the outcome of patients undergoing major hepatic resections.

The perioperative morbidity and mortality, as well as longer operative time and blood transfusions rates were increased in patients with moderate to severe steatosis [26].

Similar data was reported by Belghiti et al but they found that steatosis was not an independent risk factor for hospital mortality [27].

A systematic review and a meta-analysis from 2010 that included a total of 1000 patients, revealed that the degree of steatosis and the extent of resection increases the risk of post-operative complications and mortality [28].

Paradoxically, some authors have found that associated steatosis might have a protective effect on long-term survival in patients undergoing hepatic resection of colorectal liver metastases [29].

On the other hand, a large cohort study which included 1803 liver resections, goes against earlier studies, reporting no effect of steatosis on post-operative outcome [5].

In our case series, we also did not find a statistically significant correlation between the post-operative complications and the presence of steatosis.

The heterogeneity between these studies highlights several limitations of the research in this field that, limitations that are also found in our study: a) missing information on the histopathological methods of steatosis diagnosis; b) inconsistent steatosis grading; c) non-uniform classification of surgical complications; d) clinical outcome should take into consideration other independent predictors such as underlying disease and co-morbidities (obesity, diabetes mellitus).

Analyzing survival curves, we noticed that patients that presented higher values of CD31 and CD34 had a higher rate of post-operative complications on a 30-day follow-up period.

However, our results could be difficult to interpret due to the small number of liver resections included.

Further studies with a multicentre prospective design should assess the value of pre-operative evaluation of angiogenesis in patients with liver steatosis submitted to liver surgery.

In conclusion, neoangiogenesis (as expressed by CD31 and CD34 markers), the presence of steatosis and metastases showed a positive trend for predicting post-operative complications in patients undergoing liver resections.

Despite the limitations imposed by the small number of patients, the retrospective nature of the study and the short follow-up period, the findings might be a groundwork for further research in what looks to be a potentially predictor on short-term survival in patients with hepatic steatosis undergoing liver resection.

Conflict of interests

None to declare.

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