

ORIGINAL PAPER

ROLE OF MAST CELLS IN THE PATHOGENESIS OF LIVER FIBROSIS IN NONALCOHOLIC FATTY LIVER DISEASE

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Nonalcoholic fatty liver disease (NAFLD), a very common comorbidity of obesity, may progress from simple steatosis to nonalcoholic steatohepatitis and liver cirrhosis. The aim of this study was to determine whether mast cells (MCs) participate in the pathogenesis of liver fibrosis in patients with NAFLD.

Liver specimens collected either from obese subjects or the control group were prepared for histological examination. The patients were divided into groups depending on inflammation and fibrosis grade, and the mean total number of mast cells/mm² and those located only in portal areas/ fibrous septa or within lobules were calculated for each grade. Mast cells were detected by picro-cyanol erythrosinate staining.

There was a strong positive correlation between the number of mast cells, especially those located within portal areas and fibrous septa, and the liver fibrosis grade ($r = 0.736$, $p < 0.0001$). No similar dependency between the number of mastocytes within lobules and liver fibrosis grade was identified.

The results from this study suggest the involvement of mast cells from portal areas and fibrous septa in the pathogenesis of liver fibrosis in patients with NAFLD.

Key words: mast cells, liver fibrosis, NAFLD, obesity.

Introduction

Obesity, especially its abdominal type, carries an increased risk of many comorbidities including nonalcoholic fatty liver disease. NAFLD affects approx. 75% of obese people and comprises subtypes such as simple steatosis, nonalcoholic steatohepatitis (NASH), and cirrhosis, which can evolve to hepatocellular carcinoma (HCC) [1]. Liver cirrhosis is a late stage of organ fibrosis characterised by the presence of regenerative nodules surrounded by fibrous septa that distort the natural structure

of the liver [2, 3, 4, 5]. These changes in liver histology lead to portal hypertension and organ failure [5]. In the United States, NASH is the third leading cause of liver cirrhosis, after alcohol abuse and HCV infection [6]. Due to both a high incidence of NAFLD in the world and the risk of its progression to cirrhosis, it is imperative to look for causes of the aforementioned phenomenon in order to implement an effective anti-fibrotic therapy.

It is an established fact that fibrosis results from an imbalance between deposition and degradation of extracellular matrix (ECM) [7]. Some ECM proteins

that are secreted in excessive amounts feature type I and IV collagen as well as fibronectin, elastin, and laminin [8]. Hepatic stellate cells (HSCs) play a major role in the production of extracellular matrix constituents in the liver and are activated by an inflammation process, resulting from damage to hepatic parenchyma. Once activated, HSCs transform into cells that resemble myofibroblasts in terms of phenotype and are responsible for an increased protein deposition [9]. Moreover, it has been shown that activated HSCs release factors that reduce the degradation of extracellular matrix, which, in turn, further impairs the balance between the synthesis and degradation of matrix components. Apart from hepatic stellate cells, also portal fibroblasts and bone marrow-derived myofibroblasts show profibrogenic properties in the damaged liver [10, 11]. The activation of cells that stimulate fibrogenesis predominantly occurs by means of the transforming growth factor β (TGF- β) secreted by inflammatory cells infiltrating the affected liver [9, 12].

Among cells that may be involved in the process of liver fibrosis are mast cells (mastocytes, MCs), which originate from haematopoietic progenitor cells [13]. Mastocytes contain many mediators and cytokines in their cytoplasmic granules such as TGF- β , which is responsible for hepatic stellate cell activation. Mastocyte degranulation affects their environment due to the induction of inflammation and the attraction of other inflammatory cells [14]. Several studies indicate the role of mast cells in the fibrosis of various organs [15, 16, 17, 18, 19, 20, 21]. On the one hand, numerous studies have shown that the number of mast cells in portal areas and fibrous septa increases in late stages of liver fibrosis [22, 23, 24, 25]. On the other hand, research on mast cell-deficient rodent models suggest that MCs do not play a central role in the pathogenesis of the fibrosis of the organ in question [26].

Aim of the study

Only limited data are available in the literature as regards the interdependence between the number of mast cells and the advancement stage of liver fibrosis and inflammation in nonalcoholic fatty liver disease. Therefore, it is essential to examine whether the progression of fibrosis is associated with an increased number of MCs and/or with intensified inflammation in the liver parenchyma in patients with NAFLD. The aim of this study was to determine whether the MCs participate in the pathogenesis of liver fibrosis in patients with NAFLD, and which of the mastocytes have a greater impact on the process: cells located in the portal areas/fibrous septa or those within the liver lobules. It was also interesting for us to investigate which of the two following

factors affects the development of liver fibrosis to a greater degree: mast cell content or stage of inflammation (portal and intralobular). The study complies with up-to-date global standards of histological scoring and grading systems approved for NAFLD.

Material and methods

The study material comprised liver specimens collected from obese subjects who underwent bariatric surgery in Norbert Barlicki Memorial Teaching Hospital No. 1 of the Medical University of Lodz. Indication for surgery was class III obesity (BMI > 40) or class II obesity (BMI 35-40) with obesity-related complications (such as type II diabetes, hypertension, or lipid disorders) and insufficient weight loss despite the therapy undertaken.

Forty-seven patients with features of NAFLD were enrolled in the study (32 females and 15 males). The average BMI in the study group was 45.8 ± 5.84 , and the mean age of the patients was 41 years. The control material was collected during routine autopsies (without signs of lesions to the organs), carried out at the Department of Forensic Medicine of the Medical University of Lodz. The exclusion criteria included: overweight, obesity, and alcohol poisoning as the cause of death since the latter implied alcoholism. Ten specimens of liver were included in the control group. All specimens were obtained from males. The average BMI in the control group was 21.5 ± 2.9 , and the mean age amounted to 48 years.

Methods

Staining and specimen assessment

Immediately upon collection, tissues were fixed in 10% buffered formalin and prepared for histological examination. Sections were stained with haematoxylin and eosin, picro-cyanol erythrosinate [27], and Mallory trichrome stain.

Histological specimens were analysed by means of a computer image analysis system consisting of an Authentic ADM computer with an AMD Duron TM processor and a graphics processor combined with a Sony SSC-DC58AP video camera and a Carl Zeiss Jena Jenamed2 optical microscope. The specimens were assessed at various magnifications: 50 \times , 200 \times , 400 \times , and 1000 \times . The microscopic images were collected by a video camera that generated an analogue output signal. After the signal was converted from analogue to digital, it was transmitted for morphometric analysis. An analysis was performed using the program MultiScan Base v. 11.06.

The number and location of mast cells in the organ

Pina-cyanol erythrosinate (PE)-stained specimens were used for mast cell identification [27]. For each specimen, the mean number of mast cells/mm² was calculated based on the evaluation of 30 images magnified by 200 \times . Then the percentage of MCs located (a) in the portal areas and fibrous septa and (b) in the liver lobules was calculated. Mast cells were identified by virtue of their purple or violet-blue colour as a result of pina-cyanol erythrosinate staining as well as by virtue of the presence of cytoplasmic granules visible at large magnifications (400 \times , 1000 \times).

Grade of portal inflammation, intralobular inflammation, and fibrosis

The progress of portal and lobular inflammation was assessed at a magnification of 200 \times , while 50 \times , 200 \times , and 400 \times magnifications served for the evaluation of the advancement of fibrosis. For the assessment of each parameter, ≥ 30 images were analysed. The results were classified in accordance with the standards set out in the Table I.

Statistical methods

The obtained results were analysed in statistical terms. Patients were divided into four groups depending

on the fibrosis grade, in order to determine the mean number of mast cells/mm² for each grade and the mean number of MCs/mm² restricted to portal areas and fibrous septa or within lobules. One-factor analysis of variance (ANOVA) was used to test the differences. The relationships between following parameters: MC count, fibrosis grade, portal inflammation grade, and lobular inflammation grade, were determined using Spearman rank correlation coefficient. The level of statistical significance was set at $p \leq 0.05$.

Results**Fibrosis grade and content of MCs/mm²**

A significantly higher mean number of MCs/mm² were found (10.49 ± 5.90 MCs/mm²) in patients with features of NAFLD as opposed to the control group (2.11 ± 1.76 MCs/mm²; $p < 0.0001$).

The features of fibrosis were found in 93% of patients with NAFLD. In three cases no signs of fibrosis were noted in accordance with the Kleiner *et al.* scoring system [29]. Most patients showed fibrosis grade 3 and 2 (40.4% and 31.9%, respectively). Perisinusoidal and periportal fibrosis (grade 1) was observed in 21.3% of patients, whereas cirrhosis was not

Table I. List of criteria applied for the histological evaluation of liver inflammation and fibrosis

ITEM	SCORE AND DEFINITION	STAINING
Portal inflammation	Scale of Brunt EM, Kleiner DE, Wilson LA, <i>et al.</i> [28]	HE
	0 (none) No inflammation or only rare inflammatory cells in any portal area;	
	1 (mild) A few mononuclear cells, usually, but not always, in more than one portal tract;	
	2 (more than mild) Moderate to marked density of inflammation in at least one portal area, and/or the presence of lymphoid aggregates.	
Lobular inflammation	Scale of Kleiner DE, Brunt EM, Van Natta M, <i>et al.</i> [29]	HE
	0 No foci	
	1 < 2 foci	
	2 2-4 foci	
	3 > 4 foci	
Liver fibrosis	Scale of Kleiner DE, Brunt EM, Van Natta M, <i>et al.</i> [29]	Mallory trichrome
	0 None	
	1 Perisinusoidal or periportal	
	2 Perisinusoidal and portal/periportal	
	3 Bridging fibrosis	
	4 Cirrhosis	

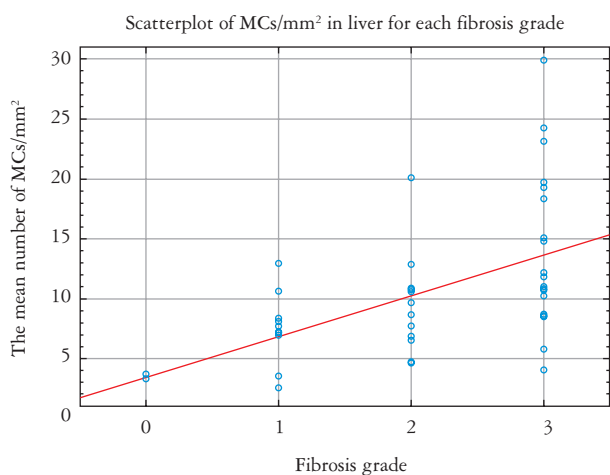


Fig. 1. Correlation between number of mast cells and fibrosis grade

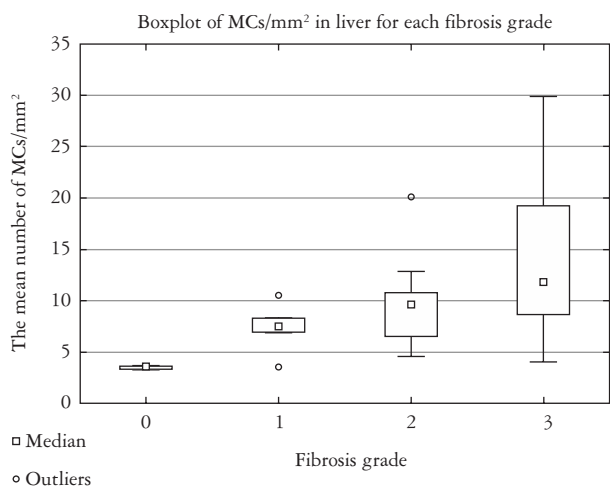


Fig. 2. The mean number of mast cells for each fibrosis grade

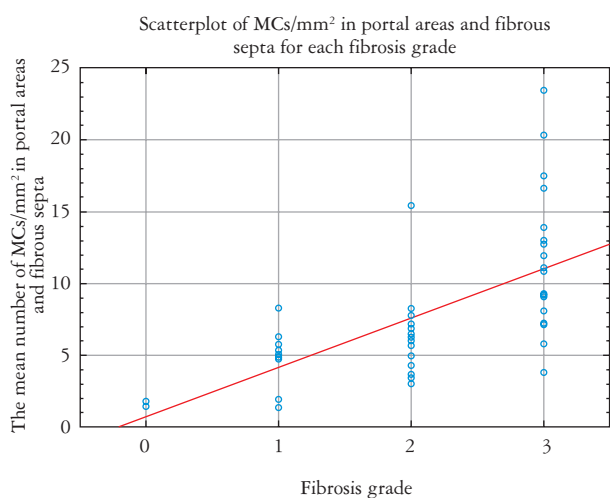


Fig. 3. Correlation between content of mast cells (restricted to portal areas and fibrous septa) and fibrosis grade

detected in any of the cases under analysis (grade 4). No features indicative of fibrosis were found in the control group.

A significant positive correlation between the mean number of mast cells and the grade of fibrosis was found in the study group ($r = 0.585$; $p < 0.0001$, Fig. 1).

The average number of MCs in patients without signs of fibrosis reached 3.54 ± 0.2 MCs/mm². The mean number of MCs increased in the case of more advanced fibrosis and amounted to: 7.52 ± 2.99 MCs/mm² (grade 1), 9.38 ± 3.82 MCs/mm² (grade 2), and 14.02 ± 6.78 MCs/mm² (grade 3), respectively (Fig. 2). The variations in MC content for the respective grades of fibrosis were statistically significant ($p < 0.001$).

Fibrosis grade vs. mast cell number in portal areas and fibrous septa

In the study group, 74.2% of all identified mast cells were found within portal areas and fibrous septa. The study showed a strong positive correlation between fibrosis grade and the count of MCs/mm² restricted to portal area and fibrous septa ($r = 0.736$, $p < 0.0001$; Fig. 3).

The mean number of MCs in portal areas and fibrous septa for each fibrosis grade amounted to: 1.53 ± 0.22 MCs/mm² (no fibrosis), 4.65 ± 2.06 MCs/mm² (grade 1), 6.40 ± 2.96 MCs/mm² (grade 2), and 11.51 ± 5.07 MCs/mm² (grade 3), respectively. The variations in the mean number of MCs in portal areas and fibrous septa for the listed grades of fibrosis were statistically significant ($p = 0.00001$).

Fibrosis grade vs. mast cell number within liver lobules

Approx. 25.8% of all identified mast cells were found within liver lobules. No statistically significant correlation between fibrosis grade and MCs/mm² in liver lobules was found in patients with NAFLD features ($r = -0.096$, $p = 0.519$).

The average mast cell number in the above location varied for the respective fibrosis grades: 2.26 ± 2.02 MCs/mm² (no fibrosis), 2.87 ± 1.28 MCs/mm² (grade 1), 2.97 ± 1.52 MCs/mm² (grade 2), and 2.52 ± 2.04 MCs/mm² (grade 3). The variations in MCs content within lobules for various fibrosis grades were not statistically significant ($p = 0.748$).

Fibrosis grade and portal inflammation grade

No portal inflammation was observed in as few as 4.3% of patients. 29.8% of cases manifested grade 1 portal inflammation, whereas grade 2 was observed in 66% of patients. In the control group, 80% of cases showed no portal inflammation, with grade 1 inflammation present in 20% of cases. There was no

case of grade 2 portal inflammation in the control group. The two groups differed significantly in terms of the above parameter ($p < 0.05$).

In patients with NAFLD, a statistically significant correlation between the grade of fibrosis and portal inflammation grade was found ($r = 0.378$, $p = 0.009$).

Fibrosis grade and lobular inflammation grade

Only a single subject in the study was found to be free from lobular inflammation. Most cases indicated grade 1 (42.6%) and 2 (31.9%) lobular inflammation, whereas grade 3 was detected in 23.4% of patients. All subjects in the control group suffered from grade 1 lobular inflammation. The differences between groups were statistically significant ($p < 0.05$).

In patients with NAFLD, there was no statistically significant correlation between fibrosis grade and lobular inflammation grade ($r = 0.197$, $p = 0.183$).

Mast cells number vs. advancement stage of liver inflammation

The study demonstrated a significant positive correlation between MCs/mm² and portal inflammation grade ($r = 0.389$, $p = 0.007$) as well as between MCs/mm² of portal areas and fibrous septa, on the one hand, and portal inflammation grade, on the other ($r = 0.404$, $p = 0.005$). No such interdependencies occurred between MCs/mm² and lobular inflammation grade ($r = 0.00086$, $p = 0.995$) or between MCs/mm² within the lobules and lobular inflammation grade ($r = -0.090$, $p = 0.548$).

Discussion

The pathogenesis of liver fibrosis in NASH patients has been poorly understood. In search of an explanation, a two-hit hypothesis has been put forth, according to which insulin resistance is a first step in liver steatosis. Insulin resistance that enhances fat tissue lipolysis and drives hyperinsulinism contributes to an increased influx of free fatty acid into the liver and the accumulation of triglycerides within hepatocytes [30]. The second hit comes with an initiation of inflammatory process and fibrosis in the liver, resulting from the combined impact of proinflammatory agents, oxidative stress, adipokines, and mitochondrial dysfunction [31]. Numerous studies have focused on seeking the causes of the progression from simple steatosis to nonalcoholic steatohepatitis due to the severity of its consequences, of which liver cirrhosis is the gravest. MCs have been suggested to participate in the initiation of liver inflammation and fibrosis [25, 32].

Human liver contains a relatively low number of mast cells: from 1.2 to 3.9 MCs/mm² [33]. This

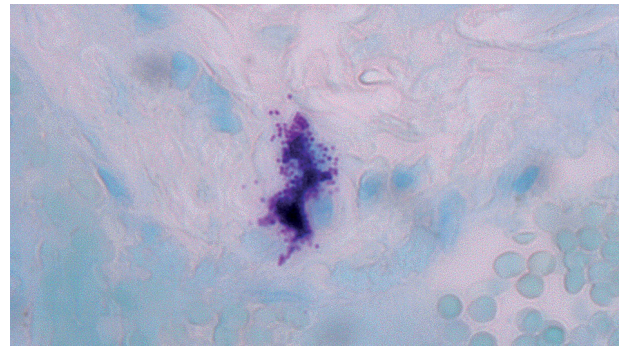


Fig. 4. Purple granules of degranulating mast cell are clearly visible at high magnification (1000 \times). Pina-cyanol erythrosinate staining

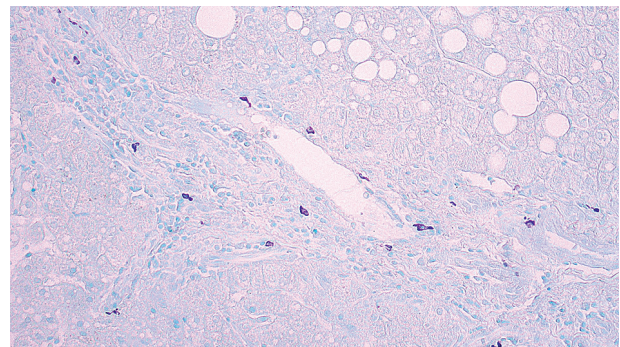


Fig. 5. Accumulation of mast cells in the portal tract. Magnification 200 \times . Pina-cyanol erythrosinate staining

hypothesis that MCs play a role in liver fibrosis may be corroborated by the fact that their count rises in the liver affected by late stages of fibrosis, including cirrhosis [22, 23, 24, 25]. Mastocytes might participate in the pathogenesis of fibrosis by releasing mediators from the granules. These mediators include TGF- β , which stimulates hepatic stellate cells to produce ECM components, as well as tryptase, which promotes collagen synthesis by fibroblasts and fibroblast growth [34, 35]. Other factors contained in MC granules that induce fibrosis feature PDFG (platelet derived growth factor) and GM-CSF (granulocyte macrophage colony stimulating factor) [36], histamine, heparin, and bFGF [basis fibroblast growth factor] [25]. In addition, mast cells are also a source of inflammatory factors such as TNF- α (tumour necrosis factor- α), which activates many cells and initiates the inflammatory process [14]. Further still, mast cells support the profibrogenic response of Th-2 due to the content of factors such as IL-4 or IL-10.

The study showed that MC count in patients with NAFLD increases relative to fibrosis stage, as assessed according to the NASH Clinical Research Network Scoring System Definitions and Scores [29]. This interdependence proves to be even stronger for mast cells restricted to portal areas and fibrous septa. This, in turn, may suggest that mastocytes directly affect

fibrosis progression in patients with NAFLD. The higher accumulation of MCs in portal tracts and fibrous septa might be related to an increased number of microvessels in those locations, specific to chronic liver damage [37, 38]. Mastocytes are believed to have angiogenic potential. Mast cells under both physiological and pathophysiological conditions accumulate in the close proximity of capillaries, including locations such as tumour growth, wound healing, and tissue repair – the places where angiogenesis is prominent [39, 40, 41]. MCs are also a source of angiogenic mediators such as: fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF) [42], tryptase, chymase [13], histamine [43], heparin [44], and matrix metalloproteinase-9 (MMP-9) [45]. The abovementioned factors regulate the endothelial cell proliferation and function and/or degrade ECM components to provide space for neovascular growth. Recent studies have demonstrated that angiogenesis might contribute to the liver fibrosis progression during the process of wound healing in chronic liver diseases [38, 46, 47, 48].

Moreover, our study demonstrated that the mean number of mast cells in liver with NAFLD features is significantly higher than in normal human liver. A similar observation was made in a study conducted by Armbrust *et al.*, which showed an increase in the number of MCs in portal areas and fibrous septa in human and rat liver affected by cirrhosis, thereby suggesting MC participation in late stages of liver fibrosis. However, the latter study was limited to the analysis of MC count in two polar cases: no fibrosis or cirrhosis, disregarding intermediate stages of fibrosis [22]. In effect, the correlation between various stages of fibrosis and MC content was excluded in the cited study. Furthermore, this research concerned either artificially caused cirrhosis or cirrhosis derived from diseases other than NAFLD. A positive correlation between MC content and liver fibrosis was also demonstrated in other studies [23, 24, 25], but none of them referred to NAFLD patients and none was assessed in compliance with NASH Clinical Research Network Scoring System Definitions and Scores.

Contrary to the above, Sugihara *et al.* put forward an alternative hypothesis based on the research of mast cell deficient rats Ws/Ws and mice W/W^v. In their study, liver cirrhosis was caused in the aforementioned rodents by the resection of bile duct or CCl₄ induction. Sugihara contends that mast cells do not play a central role in the pathogenesis of liver fibrosis because cirrhosis occurred despite the lack of MCs in the animals studied [26].

It is an established fact that fibrogenesis requires the retention of signals attributed to chronic liver diseases caused by infection, medicines, metabolism disorders, etc., for activation. Although, occasionally, liver fibrosis might progress radically in a few weeks

or months as a result of failure caused by hepatotoxins, HCV following transplantation [49], or concurrent HIV and HCV infections [50]; this process normally takes years [2]. In the study of Sugihara *et al.*, liver cirrhosis was artificially provoked in the assessed rodents; for instance, by CCl₄ induction resulting from acute organ injury. Cirrhosis advanced rapidly in the animals, and MC content was assessed relatively shortly after the causal factor occurred. Obesity and its comorbidities (such as liver fibrosis) develop over extended periods of time, and it is difficult to draw a comparison between the results obtained by us and the findings of Sugihara *et al.* The research of Charlton *et al.* may confirm that liver fibrosis in NASH subjects is a relatively long-term process. In the cited study, the features of progressive liver fibrosis in mice were not obtained sooner than after six months of a regular fast-food diet [51]. The study by Sugihara *et al.*, wherein liver cirrhosis was observed in rodents despite the lack of MCs, does not rule out the existence of two different mechanisms underlying liver fibrosis in acute and chronic organ injury.

However, our research demonstrated that mast cell content within liver lobules does not increase with the progression of fibrosis, thereby inviting the suggestion that MCs in the above location do not play an important role in liver fibrosis in patients with NAFLD.

Our study showed that liver fibrosis grade in NAFLD patients increases proportionately to portal inflammation grade, as assessed in accordance with the Brunt *et al.* system [28]. However, no similar interdependence was identified between liver fibrosis grade and lobular inflammation grade. Chronic inflammatory process in the liver typically precedes the occurrence of fibrosis [2]. If the inflammation does not subside, fibrosis may evolve into more advanced stages, including liver cirrhosis [52, 53]. Liver fibrosis begins with the stimulation of the secretion of growth factors and cytokines by inflammatory cells, which activate the hepatic stellate cells to the synthesis of extracellular matrix components [2].

In a study concerning alcoholic liver disease (ALD), Colombat *et al.* demonstrated a positive correlation between portal inflammation and liver fibrosis. The authors suggested the role of portal inflammation in liver fibrosis among patients with ALD [54]. A positive interdependence between portal inflammation and late stages of liver fibrosis in obese patients was observed in studies by Liew *et al.* [55] and Brunt *et al.*, among others. A total of 728 adults with NAFLD were enrolled in the study by Brunt *et al.* The authors indicated portal inflammation grade 1 and 2 as a marker of advanced disease [28]. The lack of correlation between lobular inflammation grade and fibrosis grade demonstrated in our study may suggest that inflammatory process within the liver

lobules has no impact on fibrosis progression, contrary to inflammation within portal areas.

We also noted in our study a positive relationship between mast cell content and portal inflammation grade. Mast cells release many inflammatory factors, which, in turn, attract other inflammatory cells to the site of mast cell degranulation. This activates inflammatory cells and initiates the inflammatory process [14], which may cause liver fibrosis, if chronic [2, 52, 53]. The positive correlation between fibrosis grade and mast cell number, on the one hand, as well as between fibrosis grade and portal inflammation grade, on the other, as demonstrated in our study, may suggest that mast cells account for fibrosis progression in patients with NAFLD together with other inflammatory cells from portal areas. However, the strong correlation between fibrosis grade and mast cells content, particularly as regards mast cells restricted to portal areas and fibrous septa, makes it highly probable that these cells play a central role in the pathogenesis of liver fibrosis in patients with nonalcoholic fatty liver disease.

Conclusions

In the study, we demonstrated a strong positive correlation between the number of mast cells located within portal areas and fibrous septa and liver fibrosis grade in patients with nonalcoholic fatty liver disease. The accumulation of mast cells in sites such as portal areas or fibrous septa contributes to fibrosis progression. This occurs because mast cells topically release various mediators that have an impact on their environment by attracting other inflammatory cells, inducing inflammation and regulating angiogenesis. Mast cells also stimulate HSCs and portal fibroblasts to produce ECM components, including collagen. The positive correlation between fibrosis grade and MC total count as well as an interdependence between fibrosis grade and portal inflammation grade suggest that portal mast cells and portal inflammatory cells contribute equally to fibrosis progression in patients with NAFLD. However, since the correlation between fibrosis grade and MC count is stronger, particularly with respect to those restricted to portal areas and fibrous septa, mast cells in the above locations may play a central role in the pathogenesis of liver fibrosis in patients with NAFLD. The lack of correlation between fibrosis grade and mast cell content within liver lobules indicates that MCs located away from portal areas and fibrous septa do not participate in fibrosis progression.

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The authors declare no conflict of interest.

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