Association between hepatic angiogenesis and serum adipokine profile in non-obese chronic hepatitis C patients

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It is unclear whether angiogenesis merely represents a homeostatic mechanism aimed at ensuring an adequate oxygen supply or one that exerts an additional pathogenic role leading to liver damage in chronic hepatitis. Chronic hepatitis C (CHC) patients present a proangiogenic profile of angiogenic markers. Adipokines not only regulate adipose tissue and glucose metabolism, but also influence inflammation, fibrogenic process and production of proangiogenic factors.
On the basis of this evidence we aimed to assess the number of new blood vessels in lobules and portal tracts in the liver and evaluate the relationship between angiogenesis intensity and serum adipokine concentrations in CHC.
Our study showed a positive association between serum vaspin and angiogenesis intensity in portal tracts and lobules in CHC patients ($r = 0.41$, $p = 0.04$; $r = 0.46$, $p = 0.03$; respectively). Serum visfatin was found to be negatively related to angiogenesis in portal tracts and lobules but only in females ($r = -0.76$, $p = 0.03$; $r = -0.95$, $p < 0.001$; respectively).
In conclusion, the role of some adipokines in liver angiogenesis seems to be different in females than in males. Serum vaspin concentration seems to reflect intensity of liver angiogenesis in CHC. Further studies are necessary to better determine the role of adipokines in new blood vessel formation in CHC.

Key words: adiponectin, adipokines, angiogenesis, chemerin, chronic hepatitis C, fibrosis, leptin, liver, vaspin, visfatin.

Introduction

Angiogenesis, the formation of new vascular structures from pre-existing vessels, occurs in chronic liver diseases (CLDs) [1]. It is still unclear whether angiogenesis merely represents a homeostatic mechanism aimed at ensuring an adequate oxygen supply or one that has an additional pathogenic role that leads to liver damage [2, 3].

From a mechanistic point of view, angiogenesis in CLDs can be interpreted according to two main pathways. Firstly, neo-angiogenesis is stimulated in hepatic

tissue by progressive tissue hypoxia. Accumulation of inflammatory cells and development of fibrosis may increase resistance of liver tissue to blood flow and oxygen supply, resulting in hypoxia [4]. Under these circumstances, angiogenesis switch occurs, leading to an increase of proangiogenic factors contributing to vascular remodelling and formation of new vessels [5]. Secondly, the process of liver chronic wound healing typical of fibrogenic CLDs is characterized by an overexpression of the same growth factors, cytokines and metalloproteinases (MMPs) with an inherent proangiogenic action [6]. Angiogenesis in liver is characterized by capillarization of the sinusoids [7]. Structures responsible for proliferation and maturation of new blood vessels in the liver are hepatic stellate cells (HSCs), Kupfer cells, regenerating hepatocytes and existing endothelial cells (ECs) [2]. A key area in the study of cellular and molecular relationship existing between fibrogenesis and angiogenesis concerns the proangiogenic role of activated HSCs [8].

Adipose tissue-derived cytokines, termed adipokines have been shown not only to be regulators of fibrogenesis, metabolic and inflammatory processes but also as potent regulators of angiogenesis. They may influence structures and regulate synthesis of agents responsible for modulation of angiogenesis [9, 10]. Leptin, visfatin, chemerin and resistin have been found to promote, whereas adiponectin to attenuate angiogenesis [11-16]. Up to now there has only been one study describing vaspin (visceral adipose tissue-derived serine protease inhibitor) influence on endothelial cells, and no studies relating to the involvement of vaspin in regulation of angiogenesis [17].

Amongst viral hepatitides, hepatitis C virus (HCV) infection has been documented to show more evident angiogenesis [18]. Chronic hepatitis C (CHC) patients present a proangiogenic profile of angiogenesis markers such as vascular endothelial growth factor (VEGF), angiopoetin-2 (Ang-2), receptor of Ang-2 (Tie-2), hepatocyte growth factor (HGF) and CD 31 [2, 19]. In CHC angiogenesis intensity was positively associated with the stage of fibrosis and grade of inflammatory activity and steatosis [20, 21]. The studies regarding adipokine profiles in CHC have shown conflicting results. Some of them point to the pivotal role of adipokines in fibrosis progression, inflammatory activity and steatosis. There are no data about the relationship between serum adipokines and hepatic angiogenesis intensity in CHC. In respect to the relationship existing between fibrogenesis and inflammation and angiogenesis we hypothesize that adipokines influence angiogenesis in CHC and their serum concentrations may reflect its intensity.

CD34 is a cell surface, sialomucin-like glycoprotein which is expressed on hematopoietic progenitor cells, normal vascular endothelium and fibroblasts [22]. 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 [23].

The aim of the study was to assess the number of new blood vessels in lobules and portal tracts in the liver and evaluate the relationship between angiogenesis intensity and serum adipokine concentrations in non-obese CHC patients before antiviral therapy.

Material and methods

The study was performed on 40 non-obese patients with CHC (23 M/17 F), infected with the HCV genotype 1b, with persistently elevated alanine aminotransferase (ALT) activity for at least 6 months, aged between 23 and 60 years (average 42.5 $\pm$11.1), body mass index (BMI) 25.0 $\pm$0.6 kg/m^2 (range 19.3–30.0 kg/m^2), 20 of whom had BMI below 25 kg/m^2. Exclusion criteria included other virus genotypes, drug or alcohol abuse and autoimmune, neoplastic, thyroid and psychiatric diseases, hepatitis B or HIV coinfection, diabetes mellitus, and renal or heart failure. Six of the CHC patients had hypercholesterolemia and five had impaired glucose tolerance.

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) and viral load by signal amplification nucleic acid probe assay for the quantitation of human hepatitis C viral RNA [Bayer Versant™ HCV RNA 3.0 Assay (bDNA), Berkeley, USA].

All patients were naïve of the antiviral treatment. At the beginning of the study, on the day of liver biopsy, a single blood sample was drawn in the morning from all patients subjected to fasting. The samples were centrifuged and serum was aliquoted and frozen at –70°C until further processing. All patients underwent an oral glucose tolerance test for diagnosis of diabetes mellitus or impaired glucose tolerance.

Adipokine serum concentrations were assessed in duplicate by immunoenzymatic method with the commercially available EIA or ELISA kits: visfatin EIA kit [Visfatin C-terminal (Human) EIA Kit, Catalogue No. EK-003-80, Phoenix Pharmaceuticals, Inc., USA; minimal detectable concentration 2.83 ng/ml]; vaspin (Human Vaspin ELISA Kit, Catalogue No. V0712TP AdipoGen Inc., Seoul, Korea; with sensitivity of 12 pg/ml); chemerin (Human Chemerin ELISA Kit, Catalogue No. E0945h, Wuhan Uscn Sciences Co., Ltd, China); leptin (Human Leptin ELISA Kit, Catalogue No. RD191001100, BioVendor-Laboratorni medicina a.s., Modrice, Czech Republic, analytical limit of detection 0.17 ng/ml), adiponectin (Human Adiponectin ELISA, BioVendor-Laboratorni medicina a.s., Catalogue No. RD195023100, analytical limit of detection

7 ng/ml) and resistin (Resistin ELISA, Catalogue No. RD191016100, BioVendor-Laboratorni medicina a.s., Modrice, Czech Republic, analytical limit of detection 0.033 ng/ml). The study evaluated full-length form of chemerin. Insulin concentration was measured by DiaMetra Insulin EIA Kit, Catalogue No. DKO076, DiaMetra S.r.l. headquarters: via Garibaldi, Foligno (PG), Italy.

The remaining biochemical parameters were measured using routine methods. The upper limit of ALT activity was set at 38 IU/l and aspartate aminotransferase (AST) at 40 IU/l. The degree of insulin resistance was calculated according to the homeostasis model assessment for insulin resistance (HOMA-IR) by the formula: fasting insulin level (mUI/l) × fasting glucose level (mg/dl)/405.

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.

Liver histology

All patients had a liver biopsy performed with the Hepafix kit (B. Braun, Melsungen AG, Germany) before antiviral therapy as part of the diagnostic routine. Biopsy samples included at least 6 portal tracts and were examined by two pathologists. 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 features were assessed according to Scheuer (inflammatory activity, fibrosis) and Brunt (steatosis) scales [24, 25]. Histopathological examination was carried out retrospectively by two experienced pathologists using double-headed microscope.

Immunohistochemistry for CD34

Formalin-fixed paraffin-embedded sections were deparaffinized, dehydrated and demasked in a microwave oven for 20 minutes in 0.01M sodium citrate buffer (pH 6.0). Monoclonal anti-CD 34 (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 the 6 portal spaces and lobules (magnification 400×). Analysis included portal tracts irrespective of their shape and size. All CD34 positive structures with lumen observed in portal tracts were estimated. Subsequently, the median value of vessel counts in portal tract and lobules was calculated (CD34 index). The number of vessels was estimated with "hot-spot" technique according to Vermeulen criteria [26]. In statistical analysis, the amount of blood vessels was expressed by CD34 index in portal tracts (CD34pt) and CD34 index in lobules (CD34lob).

Statistical analysis

The values were expressed as the mean and standard error of the mean (± SEM). The Shapiro-Wilk test was used to evaluate the distribution. Because of the non-Gaussian distribution, nonparametric tests were used. Differences in studied variables between groups were tested using U Mann-Whitney and ANOVA rang Kruskal-Wallis tests for independent groups. Correlations were analyzed with the Spearman rank correlation coefficient. $p < 0.05$ was considered to be statistically significant. The statistical analysis was performed with STATISTICA 7.0 (StatSoft Polska Sp. z o.o., Kraków, Poland).

Results

Demographic, biochemical and morphological characteristics of analyzed patients and comparison of analyzed parameters in males and females are presented in Table I. Comparison of males and females showed a significant difference in levels of adiponectin (8.9 ±0.9 vs. 18.4 ±3.8 ng/ml, $p = 0.002$) and leptin (11.0 ±2.5 vs. 22.0 ±4.7 ng/ml, $p = 0.03$). Body mass index (24.0 ±1.1 vs. 26.2 ±1.3 kg/m^2) and serum levels of remaining adipokines did not differ between men and women (chemerin 25.9 ±6.9 vs. 37.3 ±7.2 ng/ml, $p = 0.11$; visfatin 39.9 ±2.8 vs. 42.7 ±4.9 ng/ml, $p = 0.24$; vaspin 0.69 ±0.1 vs. 0.75 ±0.1 ng/ml, $p = 0.71$). The mean number of newly formed blood vessels – CD34pt was 10.1 ±1.2 and CD34lob 2.9 ±0.6 in CHC patients. There was no difference between males and females ($p = 0.51$; $p = 0.56$) with respect to newly-formed blood vessels in portal tracts and lobules. There was a mutual correlation between CD34pt and CD34lob expression ($r = 0.65$, $p < 0.001$).

The histopathological evaluation of liver biopsy specimens revealed no fibrosis (F0) in 3 (7.5%), fibrosis stage 1 (portal fibrosis, F1) in 19 (47.5%), stage 2 (periportal fibrosis, F2) in 14 (35%) and stage 3 or 4 (septal fibrosis/cirrhosis, F3/4) in 4 (10%) CHC patients. In males,

Table I. Demographic, biochemical and morphological characteristics of analyzed patients and comparison of analyzed parameters in males and females. (Results presented as mean ± SEM)

PARAMETER	CHC PATIENTS (N = 40)	MALES (N = 23)	FEMALES (N = 17)	P
Weight (kg)	76.6 ±2.2	83.4 ±3.6	64.6 ±2.8	< 0.001
BMI (kg/m^2)	25.0 ±0.6	24.0 ±1.1	26.2 ±1.3	0.06
Adiponectin (ng/ml)	12.6 ±1.4	8.9 ±0.9	18.4 ±3.8	0.002
Leptin (ng/ml)	13.7 ±1.8	11.0 ±2.5	22.0 ±4.7	0.03
Chemerin (ng/ml)	30.3 ±5.1	25.9 ±6.9	37.3 ±7.2	0.11
Visfatin (ng/ml)	41.1 ±2.6	39.9 ±2.8	42.7 ±4.9	0.24
Vaspin (ng/ml)	0.71 ±0.11	0.69 ±0.11	0.75 ±0.10	0.71
Resistin (ng/ml)	14.8 ±0.7	16.3 ±0.9	14.0 ±1.5	0.12
Glucose (mg/ml)	106.9 ±2.7	106.1 ±4.0	107.0 ±3.8	0.80
Insulin (μUI/ml)	11.6 ±1.2	13.8 ±2.1	9.4 ±0.8	0.11
HOMA-IR	3.1 ±0.4	3.7 ±0.7	2.5 ±0.2	0.27
CD34pt	10.1 ±1.2	10.8 ±1.6	8.7 ±1.7	0.51
CD34lob	2.9 ±0.6	2.8 ±0.8	3.1 ±0.8	0.56
ALT (UI/l)	82.2 ±5.5	102.1 ±10.6	65.5 ±8.8	0.02
AST (UI/l)	53.0 ±3.5	58.0 ±6.3	52.6 ±8.6	0.19

p – value according to U Mann-Whitney test
BMI – body mass index
HOMA-IR – homeostasis model assessment for insulin resistance
CD34lob – CD 34 index in lobules
CD34pt – CD 34 index in portal tracts
ALT – alanine aminotransferase
AST – aspartate aminotranspherase

F0 was found in 1 (4.35%), F1 in 9 (39.13%), F2 in 10 (43.48%) and F3/4 in 3 (13.04%) persons whereas in females, F0 in 2 (11.76%), F1 in 10 (58.82%), F2 in 4 (23.53%) and F3/4 in 1 (5.88%) subject. Necroinflammatory activity grade 1 (minimal, A1) was observed in 7 (17.5%), grade 2 (mild, A2) in 26 (65%), grade 3 (moderate, A3) in 7 (17.5%) patients. There were no patients with severe necroinflammatory activity (grade 4). In males, A1 was found in 1 (4.35%), A2 in 15 (65.22%) and A3 in 7 (30.43%) subjects, whereas in females, A1 was found in 6 (35.29%) and A2 in 11 (64.71%) subjects. Steatosis was observed in 16 (40%) CHC patients. In 13 (32.5%) individuals it encompassed less than 33% (grade 1, S1) and in 3 (7.5%) patients – between 33% and 66% of the lobule area (grade 2, S2). In males, steatosis was observed in 10 (43.5%) patients. S1 was assessed in 7 (30.43%) and S2 in 3 (13.0%) men. In females, steatosis was observed in 6 (35.29%) patients and in all cases it encompassed less than 33% of lobule area (S1).

Vaspin serum concentration was positively related to both CD34pt and CD34lob in analyzed CHC patients ($r = 0.41$, $p = 0.04$; $r = 0.46$, $p = 0.03$, respectively) (Figs. 1 and 2). There was no relationship between the number of newly-formed blood vessels in lobules (CD34lob) and adiponectin ($p = 0.81$), leptin ($p = 0.79$), chemerin ($p = 0.31$) and resistin ($p = 0.13$) in analyzed patients. Similarly, there was no association between levels of these adipokines and CD34pt ($p = 0.61$; $p = 0.98$; $p = 0.48$; $p = 0.29$, respectively).

The separate analysis of the relationship between adipokines and angiogenesis in males and females revealed some differences. Vaspin serum concentration was positively related to both CD34pt and CD34lob only in men ($r = 0.40$, $p = 0.05$; $r = 0.51$, $p = 0.04$, respectively) (Figs. 3-6). On the other hand, serum visfatin was negatively associated with CD34pt and CD34lob only in females ($r = –0.76$, $p = 0.03$; $r = –0.95$, $p < 0.001$) (Figs. 7-10). Despite differences in leptin and adiponectin serum concentrations between men and women, there was no association between angiogenesis intensity and these adipokines in separately analyzed males and females.

Expression of CD34pt and CD34lob was positively associated with the fibrosis stage in the entire group ($r = 0.45$, $p = 0.03$ and $r = 0.62$, $p = 0.002$) and in males ($r = 0.50$, $p = 0.04$ and $r = 0.76$, $p = 0.001$). CD34pt and CD34lob differed significantly between patients with fibrosis stage 1 and stage 2-4 (7.6 ±4.0 vs. 12.6 ±5.3, $p = 0.04$ and 1.8 ±1.7 vs. 4.0 ±3.0, $p = 0.02$, respectively). CD34pt and CD34lob were also associated with the inflammatory grade in females ($r = 0.75$, $p = 0.03$ and $r = 0.76$, $p = 0.02$) but not in males. CD34 expression in portal tracts and lobules was significantly higher in patients with steatosis ($p = 0.034$; $p = 0.021$; respectively). CD34pt and CD34lob differed significantly between patients with

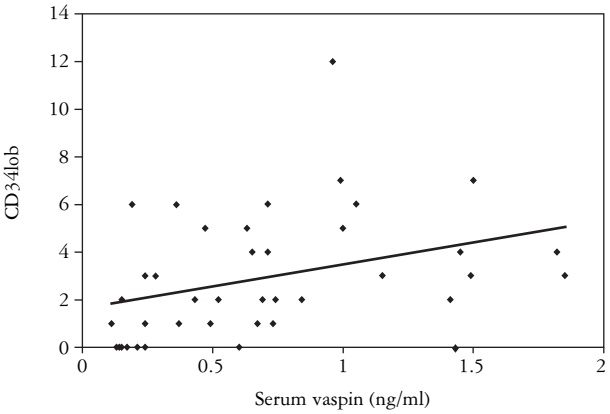

Fig. 1. Association between serum vaspin and CD34lob in all analyzed CHC patients (p = 0.03)

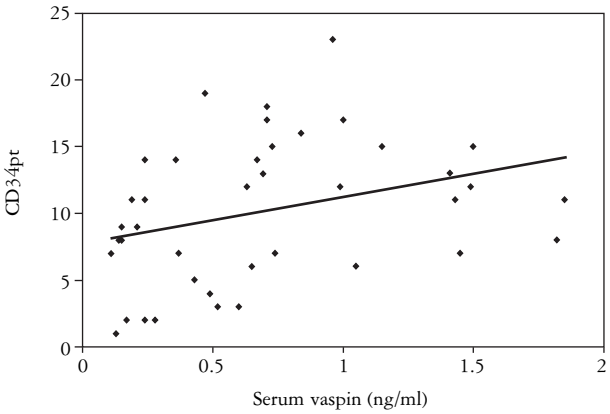

Fig. 2. Association between serum vaspin and CD34pt in all analyzed CHC patients (p = 0.04)

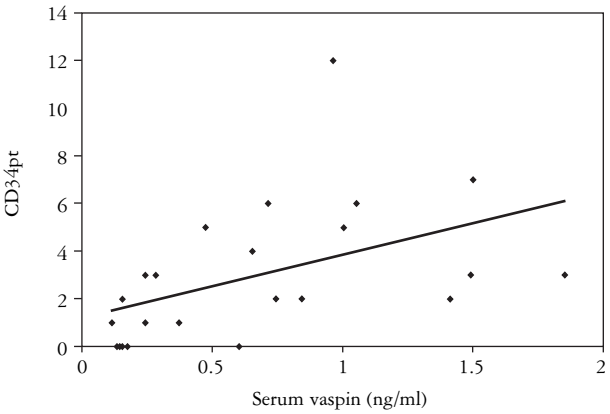

Fig. 3. Association between serum vaspin and CD34lob in males (p = 0.04)

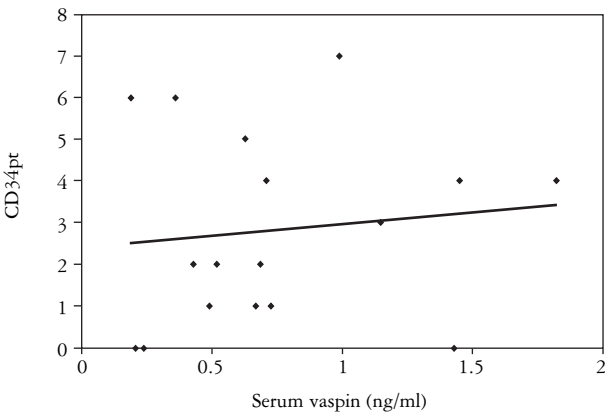

Fig. 4. Association between serum vaspin and CD34lob in females (p > 0.05)

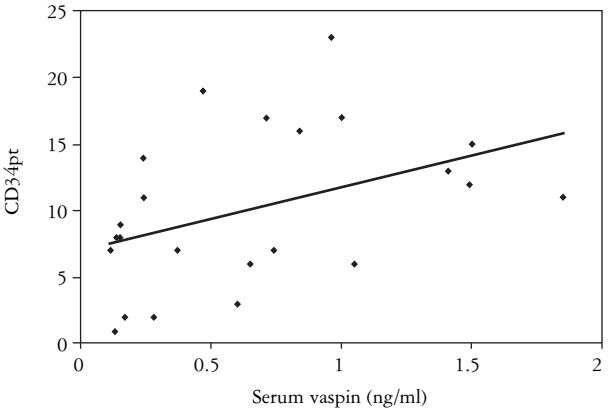

Fig. 5. Association between serum vaspin and CD34pt in males (p = 0.05)

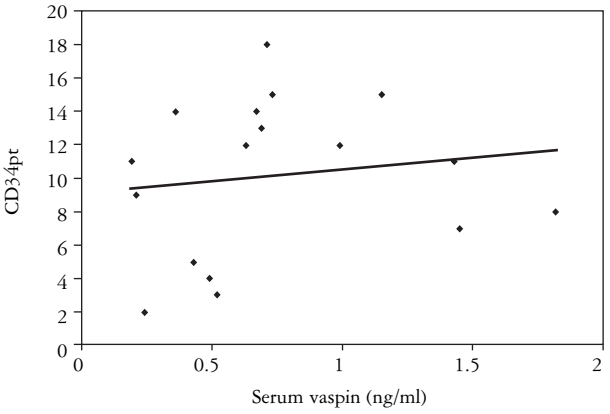

Fig. 6. Association between serum vaspin and CD34pt in females (p > 0.05)

various steatosis grade (p = 0.009; p = 0.013, respectively).

Insulin resistance (HOMA-IR) was positively associated with the fibrosis stage (r = 0.33, p = 0.03) but not with CD34pt and CD34lob (p = 0.49; p = 0.47, respectively). Also steatosis grade was positively related to HOMA-IR (p = 0.36, p = 0.04). There was no relationship between HOMA-IR and serum levels of adipokines.

Inflammatory activity grade was inversely correlated with serum chemerin (r = –0.48, p = 0.02), visfatin (r = –0.65, p = 0.03), adiponectin (r = –0.31,

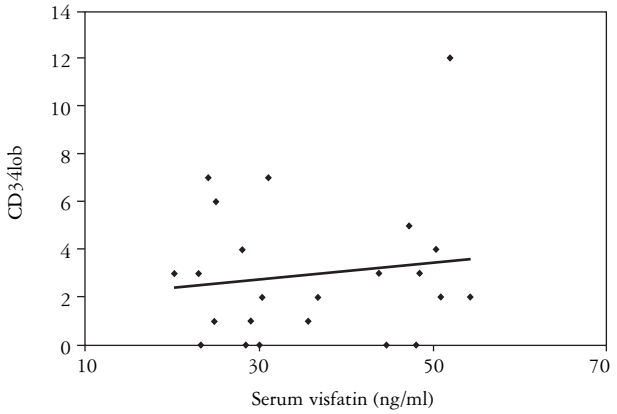

Fig. 7. Association between serum visfatin and CD34lob in males ($p > 0.05$)

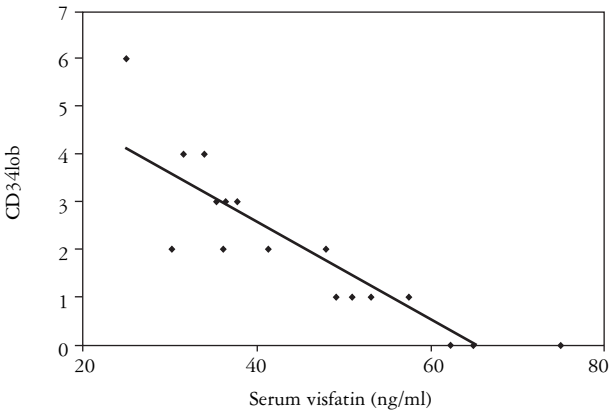

Fig. 8. Association between serum visfatin and CD34lob in females ($p < 0.001$)

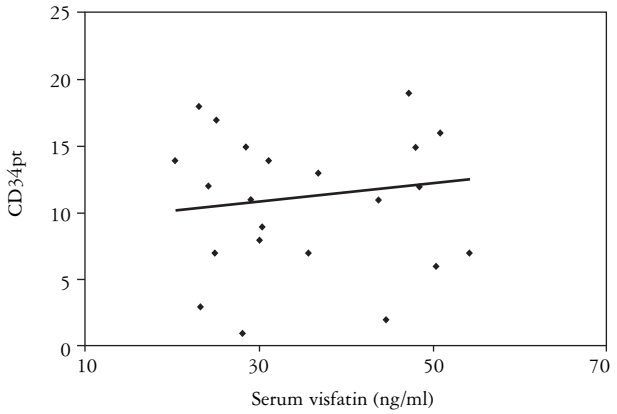

Fig. 9. Association between serum visfatin and CD34pt in males ($p > 0.05$)

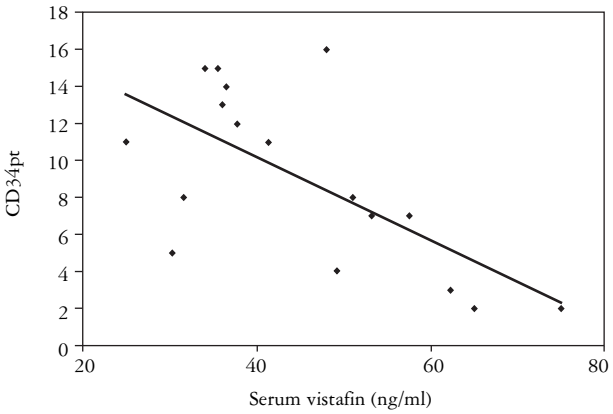

Fig. 10. Association between serum visfatin and CD34pt in females ($p = 0.03$)

$p = 0.04$) and leptin ($r = –0.30$, $p = 0.05$). There was a tendency of higher vaspin serum levels in patients with more advanced fibrosis.

Additionally, serum chemerin positively correlated with serum leptin ($r = 0.45$, $p = 0.02$).

Figures 11-18 present slides with immunohistochemical analysis of CD34 expression in the liver of CHC patients with a different stage of fibrosis, grade of inflammatory activity and steatosis.

Discussion

The study for the first time estimates the association between neo-angiogenesis and novel adipokines – visfatin, chemerin and vaspin in CHC. The data about the role of leptin, adiponectin and resistin in liver angiogenesis in CHC are very limited and unclear. Angiogenesis seems to be very important in both tissue damage as well as in wound healing processes observed in chronic inflammatory diseases of diverse tissue origin but hepatic angiogenesis may differ from homologous processes in other tissues. The main differences are related to: 1) the existence of two different types of microvascular structures in the liver – large vessels covered by a continuous endothelium and sinusoids lined by a fenestrated endothelium [27] and 2) the production of unique proangiogenic factors, such as angiopoietin-like 3 (ANGPTL3), a liver specific secreted factor showing angiogenic properties by binding to $\alpha v\beta 3$ integrin [28]; and 3) the presence of phenotypically and functionally specific HSCs-considered liver – resident pericytes, which may stimulate angiogenesis through mechanisms different from those attributed to microcapillary pericytes [8]. Additionally new factors involved in pathological neovascularisation in cirrhosis like upregulations of aquaporins (AQPs) – integral membrane water channels which enhance osmotic water permeability and FGF-induced dynamic membrane blabbing in liver endothelial cells are emerging on the horizon [29]. However a key area in the study of cellular and molecular relationship existing between fibrogenesis and angiogenesis concerns the proangiogenic role of activated HSC, like some of the adipocytokines.

The first discovered and till now the most extensively studied adipokine leptin has been shown to be involved in regulation of fibroproliferative processes and an-

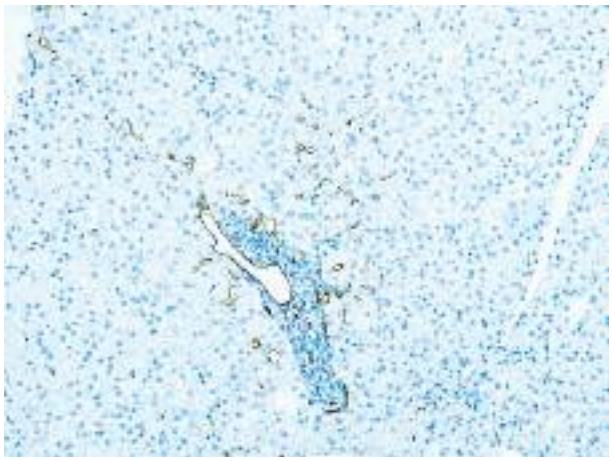

Fig. 11. Portal fibrosis. Portal and periportal expression of CD34 (CD34 immunostaining, 200×)

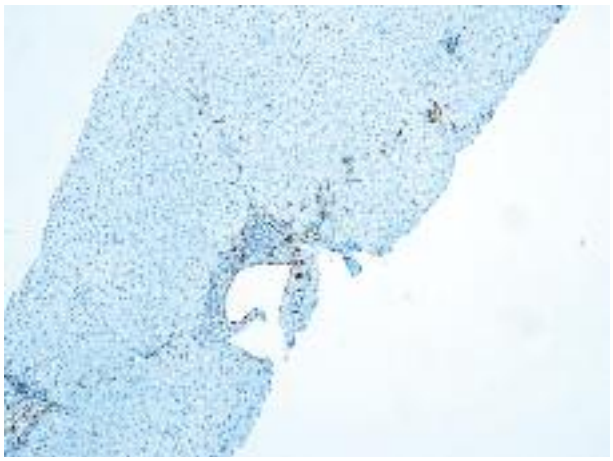

Fig. 12. Periportal fibrosis. Expression of CD34 in short fibrous septa (CD34 immunostaining, 100×)

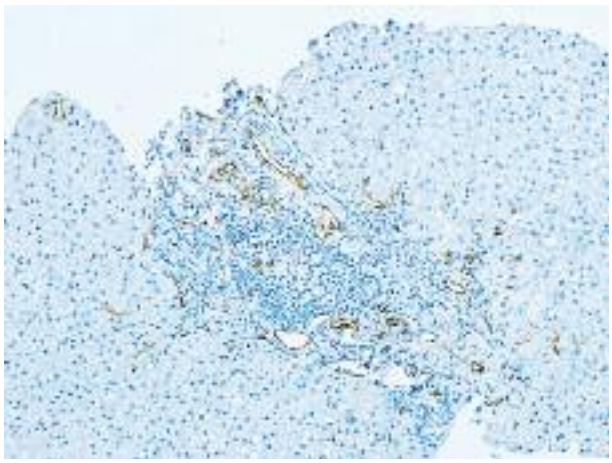

Fig. 13. Portal inflammation of moderate grade. Expression of CD34 in portal space (CD34 immunostaining, 200×)

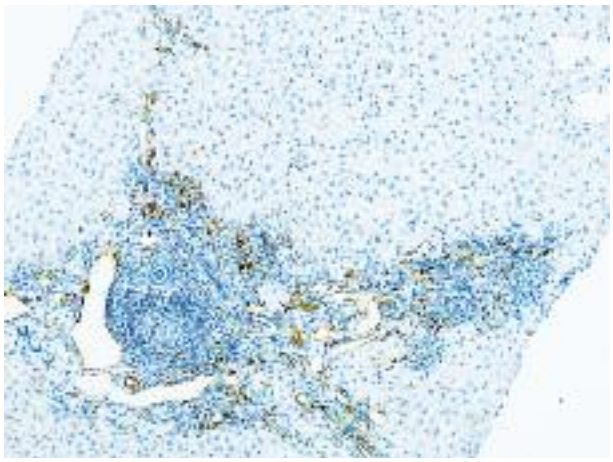

Fig. 14. Periportal fibrosis, portal inflammation of severe grade, strong expression of CD34 in portal space (CD34 immunostaining, 200×)

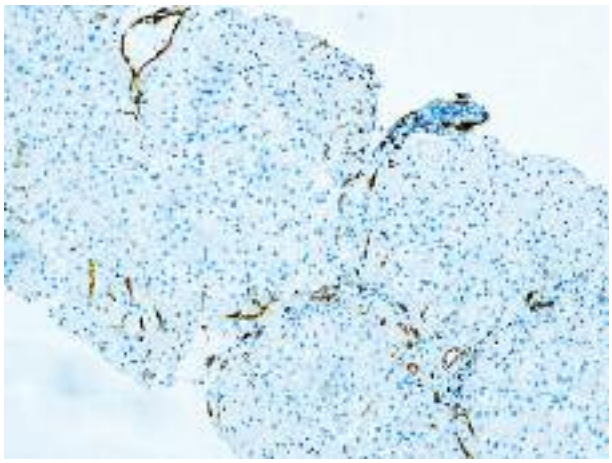

Fig. 15. Septal fibrosis, expression of CD34 in fibrous septa (CD34 immunostaining, 200×)

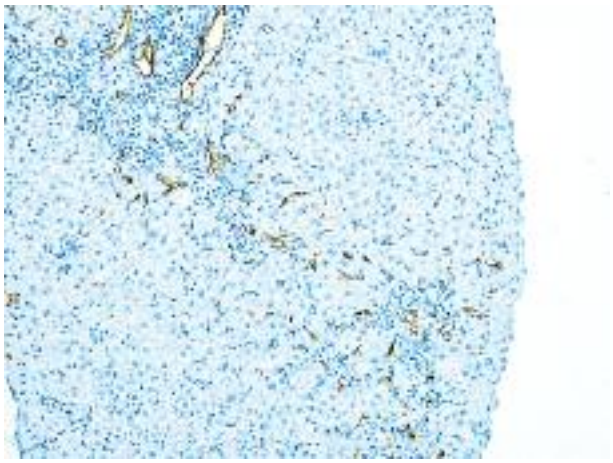

Fig. 16. Lobular inflammation, expression of CD34 in portal space and in lobules (CD34 immunostaining, 200×)

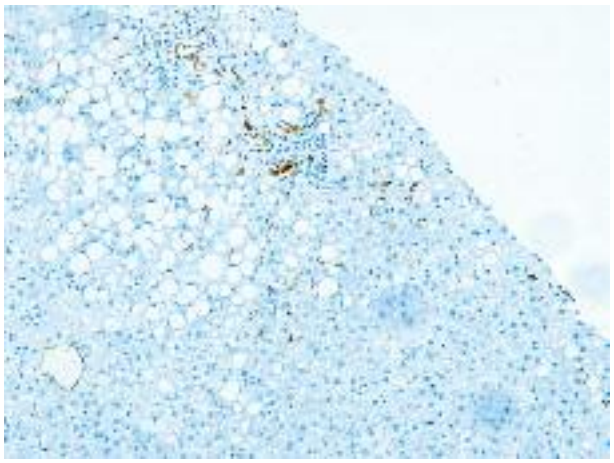

Fig. 17. Weak expression of CD34 in two small portal spaces and in steatotic area (CD34 immunostaining, 200×)

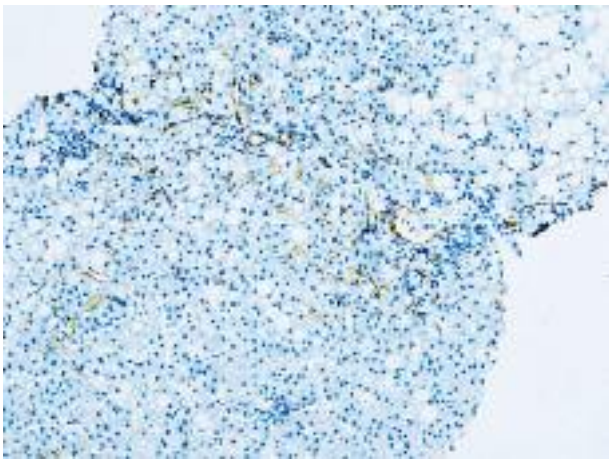

Fig. 18. Lobular steatosis and inflammation, expression of CD34 (CD34 immunostaining, 200×)

giogenesis in chronic liver diseases [9, 10, 30]. Activation of leptin receptors in HSCs leads to increase of VEGF expression [11]. It also exerts a direct angiogenic action on endothelial cells which express the functional long-term form of leptin receptor [12]. Leptin promotes endothelial cell tube formation and up-regulates VEGF mRNA expression via activation of the Jak/Stat3 signalling pathway [31]. Stimulation of neo-vessel formation in the liver by leptin is consistent with its profibrogenic role as angiogenesis is considered a relevant component of the progression of liver damage and has been described in the context of inflammation and fibrogenesis in CLDs [2, 10]. Leptin also modulates fibroblast growth factor 2 (FGF-2) and VEGF-induced vascular activity by synergistically promoting neovascularization *in vivo* [32]. These observations suggest that leptin acts not only as a direct but also as an indirect angiogenic factor or modulator of other angiogenic agents. Leptin levels were found to be elevated in hypoxia [33]. Serum levels of leptin in CHC were significantly increased compared to healthy controls and presented positive correlation with the grade of inflammatory activity [34]. Other authors reported its positive association with the fibrosis stage [35] and higher levels in the case of cirrhosis [36]. In our study there was no association between leptin and angiogenesis intensity either in portal tracts or hepatic lobules. Moreover, there was no relationship with the fibrosis stage. Surprisingly, leptin was significantly higher in patients with minimal inflammatory activity and was weakly but inversely correlated with the inflammatory grade. The lack of the association between leptin serum concentrations and hepatic angiogenesis in CHC may result from the direct influence of viral infection and ongoing inflammatory process on serum levels of this adipokine.

Adiponectin generally exerts a hepatoprotective effect in CLDs and protects against inflammation and fibrosis progression with inhibition of pro-inflammatory and induction of anti-inflammatory cytokines in leukocytes and ECs, although the correlation is not clear because there are some data indicating that serum adiponectin correlates with HCV viral load and genotype but not with histological abnormalities in patients with CHC [37]. Adiponectin has been found to activate 5'AMP-activated protein kinase (AMPK) in HSCs. The AMPK activation leads to inhibition of platelet-derived growth factor (PDGF) expression, which is essential in ECs proliferation, nascent vessel stabilization and pericytes recruitment [38, 39]. Moreover, adiponectin inhibits ECs migration and survival via activation of apoptosis [16]. There was no association between serum adiponectin and hepatic angiogenesis intensity in CHC patients in our study. However, adiponectin was inversely related to inflammatory activity. The ongoing inflammatory process which modulates serum adiponectin levels may interfere with the relationship between adiponectin and angiogenesis.

A novel adipokine visfatin/pre-B cell colony-enhancing factor (PBEF)-1 has been shown to be elevated in CHC [40]. The ability of visfatin to induce expression of genes and proteins for matrix metalloproteinases (MMP-2 and MMP-9), VEGF and its receptor (VEGF-R2) in human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner acknowledge its role in pathogenesis of chronic hepatitis and angiogenesis. Simultaneously, visfatin inhibits expression of genes and proteins for tissue inhibitors of matrix metalloproteinases (TIMP) – TIMP-1 and TIMP-2. Inhibition of VEGF and VEFG-R2 results in down-regulation of MMPs expression induced by visfatin [13]. Visfatin potentiates proliferation, migration of endothelial cells and formation of new blood vessels in a dose-dependent manner. Moreover, it decreases apoptosis of ECs. Visfatin influences the angiogenic

process by activation of phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/Akt) and $ERK_{1/2}$ (extracellular signal-regulated kinase 1/2, p42/p44 mitogen-activated protein kinase, p42/p44 MAPK) [13]. Only one study of CHC patients has found serum visfatin to be significantly increased in CHC and negatively associated with inflammatory activity [40]. In our study, there was no association between serum visfatin and angiogenesis when all CHC patients were evaluated. Surprisingly, visfatin was inversely related to angiogenesis intensity in hepatic lobules and portal tracts in females. This observation is even more interesting because there was no difference in serum visfatin concentration between men and women. The explanation of these results is difficult and requires further investigations. However, they appear to indicate that the role of adipokine in angiogenesis may differ in men and women.

The only available study investigating serum chemerin in CHC showed its serum concentrations to be higher in CHC patients compared to the control group [41] and inversely associated with inflammatory activity. There was no difference in CHC patients with a different fibrosis stage and steatosis grade. Recently chemerin has been shown to promote angiogenesis in endothelial cells in a dose-dependent manner [42]. Chemerin activates the pathway dependent on PI3K/Akt and MAPK in ECs, activating angiogenesis and MMPs synthesis [14]. Our study did not show any association between angiogenesis and serum chemerin in CHC.

Resistin induces *in vitro* human endothelial cell proliferation, migration, promotes capillary-like tube formation, up-regulates mRNA expression of several angiogenesis-related factors such as VEGF [43], vascular endothelial growth factor receptors (VEGFR-1 and VEGFR-2), MMP-1 and MMP-2, vascular cell adhesion molecule-1 (VCAM-1) and endothelin-1 [44], and activates $ERK_{1/2}$ and p38 pathways [45]. Resistin also induces human aortal smooth muscle cell proliferation through $ERK_{1/2}$ and Akt signalling pathways [46]. Murine resistin induces endothelial cell migration and sprouting of cellular networks via a mechanism which appears dependent upon PI3K and NF-κB activity, but independent of altered NO production [17]. Thus, resistin may play an important role in angiogenesis. Moreover resistin stimulates HSCs to secrete the proinflammatory chemokines – monocyte chemotactic protein-1 (MCP-1) and interleukin 8 (IL-8), critical mediators of intrahepatic leukocyte recruitment [47]. The results regarding resistin in CHC are conflicting. In Tsochatzis *et al*.'s study, there were higher serum resistin concentrations in patients with chronic HBV and HCV than with non-alcoholic steatohepatitis (NASH) [48]. Bertolani *et al*. found intrahepatic mRNA levels of resistin significantly higher in patients with severe alcoholic hepatitis than in controls, but not in patients with chronic HCV-related hepatitis or nonalcoholic steatohepatitis [47]. Our study did not reveal any association between serum resistin and the number of new blood vessels in the liver of CHC patients.

There has been only one study examining vaspin in CHC [41]. The study showed serum vaspin to be significantly lower in CHC patients compared to healthy controls but also revealed a tendency of higher vaspin concentration in CHC patients with more advanced fibrosis – however the study included a small number of patients with bridging fibrosis or cirrhosis [41]. There has also been only one study that has explored the influence of vaspin on endothelial cells [17]. Administration of vaspin to obese ICR mice fed with high fat and sucrose chow suppressed the expressions of tumor necrosis factor α (TNF-α), leptin, and resistin in mesenteric and subdermal white adipose tissues [49]. Although vaspin is mainly confined to the adipocytes, it may have an effect on the endothelial cells in an analogous manner to another proteinase inhibitor, plasmogen activator inhibitor-1 (PAI-1), which is derived from mesenteric fat and can inhibit endothelial cell migration and angiogenic branching [50, 51]. However, vaspin had no effects on both basal ECs morphology and TNF-α-induced morphological damages. Vaspin did not inhibit the TNF-α activation of JNK, p38 and NF-κB, but only slightly inhibited Akt. Furthermore, vaspin did not decrease the TNF-α induction of VCAM-1, intercellular adhesion molecule-1, endothelial selectin, and cyclooxygenase-2 protein expression as well as MCP-1, tissue factor, and plasmogen activator inhibitor-1 mRNA expression. Fu *et al*.'s results indicate that vaspin has no effects on normal ECs, and cannot prevent TNF-α -induced inflammatory injury [17]. Surprisingly, our study showed for the first time that serum vaspin level was significantly and positively associated with angiogenesis intensity both in lobules and portal tracts. These relationships were observed in all the patients and males but not in females. The results obtained are even more surprising given that vaspin was found to inhibit synthesis of potentially proangiogenic adipokines such as leptin and resistin. Additional studies are needed to delineate the exact role of vaspin in intrahepatic neo-angiogenesis.

We also evaluated the relationship between insulin resistance and intrahepatic neo-angiogenesis in CHC. Insulin resistance is a well-known predictor of fibrosis progression in CHC [52, 53]. Despite both HOMA-IR and angiogenesis being positively associated with the fibrosis stage, there was no relationship between the number of new blood vessels and HOMA-IR. Insulin resistance in CHC is modulated not only by adipokines and inflammatory process, but also by direct virus action on insulin signalling pathway, although the last data are somehow controversial

[54, 55]. Moreover, the ongoing inflammatory process influences adipokine production and concentration, but these associations are very complicated and seem to be reciprocal as far as BMI, adipocytes and anti-HVC immune response among the patients with CHC are concerned [56]. This may limit the relationship between serum adipokines and HOMA-IR and angiogenesis in our study.

Conclusions

The study showed for the first time that serum vaspin concentration seems to reflect the intensity of liver angiogenesis in CHC, particularly in males. Serum visfatin was negatively associated with angiogenesis intensity but only in females The role of some adipokines in angiogenesis seems to be different in males than in females. Liver angiogenesis in portal tracts and lobules was found to be positively associated with the fibrosis stage. The relationship between inflammation and angiogenesis may be dependent on gender. Further studies are necessary to better determine the role of adipokines in new blood vessel formation in CHC. Understanding the process of angiogenesis might suggest an effective therapeutic target that could prevent disease progression.

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