

CLINICAL STUDIES

Angiogenesis within the duodenum of patients with cirrhosis is modulated by mechanosensitive Kruppel-like factor 2 and microRNA-126

Karolina Kobus¹, Justyna Kopycinska¹, Anna Kozłowska-Wiechowska², Elzbieta Urasinska³, Agnieszka Kempinska-Podhorodecka¹, Tara L. Haas⁴, Piotr Milkiewicz⁵ and Malgorzata Milkiewicz¹

1 Medical Biology Laboratory, Pomeranian Medical University, Szczecin, Poland

2 Department of Endoscopy, Ministry of Internal Affairs Hospital, Szczecin, Poland

3 Department of Pathology, Pomeranian Medical University, Szczecin, Poland

4 Faculty of Health, Muscle Health Research Centre, York University, Toronto, ON, Canada

5 Liver Unit, Pomeranian Medical University, Szczecin, Poland

Keywords

angiogenesis – KLF-2 – microRNA-126 – portal hypertension – splanchnic circulation

Abbreviations

Ang-2, angiotensin II; eNOS, endothelial NO synthase; HO-1, heme oxygenase-1; KLF2, lung Kruppel-like factor; miR-126, microRNA-126; PH, portal hypertension; PIGF, placental growth factor; qPCR, quantitative real-time PCR; VEGF, vascular endothelial growth factor.

Correspondence

Dr Malgorzata Milkiewicz, Pomeranian Medical University, Medical Biology Laboratory, Powstancow Wielkopolskich 72, Room 418, 70-111 Szczecin, Poland
Tel: +48 91 466 1496
Fax: +48 91 466 1492
e-mail: milkiewm@sci.pum.edu.pl

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Abstract

Background: The mechanism involved in neovascularization in splanchnic circulation and the main trigger that induces angiogenesis in patients with cirrhosis are not fully recognized. **Aims:** To explore the involvement of flow sensitive lung Kruppel-like factor (KLF2), microRNA-126 (miR-126), angiotensin II (Ang-2) and heme oxygenase-1 (HO-1) in modulation of vascular endothelial growth factor (VEGF) signalling that have a critical effect on growth of new blood vessels. **Methods:** Duodenal biopsies from 22 patients with cirrhosis and 10 controls were obtained during routine endoscopy. The process of angiogenesis was evaluated by a measurement of CD31 concentration, immunodetection of CD34 protein and estimation of capillary densities. Messenger RNA (mRNA) and protein expressions were analysed by real-time PCR, Western blot or ELISA respectively. **Results:** Markers of angiogenesis (both, CD31 and CD34) were significantly enhanced in cirrhotic patients. In comparison to healthy controls, levels of Ang-2 and KLF-2 mRNAs as well as Ang-2, KLF-2, HO-1, VEGF protein expressions were considerably increased. Levels of sCD163, a surrogate marker of portal hypertension, correlated with levels of Ang-2, ($P = 0.021$) and VEGF ($P = 0.009$). The expression of miR-126, a KLF2-mediated regulator of the VEGF signalling was enhanced in cirrhotic patients. **Conclusions:** Our results demonstrate, for the first time in humans, that neovascularization is induced in duodenal tissue of patients with cirrhosis and proangiogenic factors such as KLF-2, Ang-2, miR-126 and VEGF can contribute to the angiogenesis induced by hemodynamic forces. Thus, cirrhosis-induced blood flow and pressure within splanchnic vessels may be important hemodynamic triggers that initiate the angiogenic signalling cascade.

Cirrhosis is associated with intrahepatic vascular bed resistance and may lead to serious hemodynamic complications such as portal hypertension (PH) that impedes portal blood flow. Changes in hemodynamic forces, which result from increased blood flow and pressure in the portal vein, play an important role in vascular remodelling within splanchnic circulation (1, 2) which may lead to formation of oesophageal and gastric varices.

It was demonstrated that angiogenesis, the growth of new capillaries, occurs in the mesentery and splanchnic territory of portal hypertensive rats and mice (3–7). Several factors such as hypoxia, oxidative stress, inflammation, and shear stress, which is the hemodynamic force

exerted by blood flow, are well characterized modulators of angiogenesis (8, 9). The same factors were reported to be potential mediators in the pathogenesis of PH (1, 4, 5, 10, 11). However, the mechanisms of PH-induced angiogenesis in splanchnic circulation in humans remain to be fully elucidated.

Vascular endothelial growth factor (VEGF) is well characterized as one of the most critical mediators in the complex angiogenic cascade (8, 12, 13). The VEGF induces endothelial cell proliferation, migration and acts as a survival and/or permeability inducing agent. Moreover, during angiogenesis, VEGF cooperates with the family of angiotensin molecules (i.e. Ang-1 and Ang-2) that bind to the Tie-2 receptor. The Ang-1 inhibits

endothelial apoptosis and stabilizes blood vessels (14), whereas, the induction of Ang-2, a natural antagonist of Ang-1, leads to the formation of a proliferative phenotype of the vessel (15). In the presence of VEGF, Ang-2 induces destabilization of blood vessels and increases sensitivity to angiogenic stimuli. Induction of Ang-2 and VEGF correlate with new robust angiogenesis (15). Nevertheless, the expression of VEGF in the splanchnic circulation of patients with PH has not yet been thoroughly investigated.

Lung Kruppel-like Factor (KLF2), a subclass of the zinc finger family of transcription factors, is exclusively expressed in endothelial cells of the adult vasculature and has distinct functions in vasomotor regulation, inflammation, homeostasis and angiogenesis (16, 17). In cardiovascular biology, KLF2 is described as a crucial molecular transducer that converts hemodynamic forces into the induction of downstream gene expression (18).

Recently, microRNAs, which are a class of small non-coding RNAs, have emerged as important modulators of endothelial cell biology. In particular, the endothelial cell specific microRNA, i.e. microRNA-126 (miR-126), is a key modulator of VEGF-dependent angiogenesis that regulates endothelial cell proliferation, cord formation and blood vessel integrity (19, 20). Interestingly, the transcription of miR-126 is regulated by *klf2* (a zebrafish homolog of KLF2) and this micro-RNA was reported to promote flow-induced angiogenesis by directly repressing the inhibitors of VEGF signalling such as *spred-1* and *PIK3R2/p85* (20, 21).

The ability to inhibit pathological angiogenesis could be important for developing therapies for conditions associated with PH. However, the precise mechanisms of angiogenic response in cirrhotic patients remain to be defined. To our knowledge, the expression of KLF-2, Ang-2 and miR-126 in the duodenum of patients with cirrhosis has never been explored. We hypothesized that KLF2 could be a significant factor that modulates the formation of portosystemic collateral vessels in response to the obstruction to portal blood flow by means of the VEGF signalling pathway. Therefore, in the present study, we investigated the expression of pro-angiogenic and mechanosensitive factors such as KLF-2, Ang-2 and VEGF, in the duodenum of patients with cirrhosis.

Materials and methods

Patients

Twenty-two consecutive and clinically stable patients with histologically proven cirrhosis were included in the study. Ten subjects, without cirrhosis, who underwent endoscopy for dyspeptic symptoms and had no significant macroscopic changes served as controls. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of Pomeranian Medical University. Written informed consent was obtained from all subjects.

Histological assessment

Histological analysis of duodenal tissue was performed by a pathologist blinded to the clinical and laboratory data of included patients. Six histological features were assessed: (i) Mucosal capillary congestion; (ii) mucosal extravasation; (iii) mucosal oedema; (iv) mucosal inflammation; (v) villous atrophy and (vi) mucosal fibrosis. Features i–iii and vi were assessed as present/absent; feature iv as absent/mild/moderate/severe; and feature v as absent/mild/marked.

Tissue samples

Four duodenal biopsies were obtained from each patient. Biopsies were: (i) placed into liquid nitrogen and then stored at -75°C (two samples); (ii) put in *RNAlater* solution and (iii) immersed in formalin and later embedded in paraffin. Blood samples were obtained from all included subjects. After centrifugation (10 min at 3000 g), serum was immediately separated and then transferred to -75°C .

RNA extraction and cDNA synthesis

The RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and cDNA synthesis was carried out using the SuperscriptTM II RT kit

Table 1. Clinical and serological characteristics of the patients and control subjects

	Patients with cirrhosis ($n = 22$)	Controls ($n = 10$)
Gender (M/F)	13/9	5/5
Mean age (\pm SD)	54 \pm 3	59 \pm 5
Beta-blockers (yes/no)	8/14	0/10
Presence of oesophageal varices (yes/no)	14/8	0
Aetiology of cirrhosis (no. of patients)		
alcoholic liver disease (ALD)	11	N/A
autoimmune hepatitis (AIH)	2	N/A
primary biliary cirrhosis (PBC)	2	N/A
primary sclerosing cholangitis (PSC)	1	N/A
other	6	N/A
CTP	6 (5–8) ^a	N/D
MELD	9 (7–16) ^a	N/D
Albumin (38–48 g/dl)	38 (26–48) ^a	N/D
INR (08–12)	12 (10–16) ^a	N/D
Total bilirubin (02–10 mg/dl)	12 (05–30) ^a	N/D
ALT (3–30 IU/L)	37 (12–78) ^a	N/D
Creatinine (05–10 mg/dl)	07 (04–10) ^a	N/D
Platelets ($150\text{--}450 \times 10^3/\text{m}^3$)	123 (51–362) ^a	N/D

^aData presented as median (range) ALT, alanine aminotransferase; CTP, Child-Turcotte-Pugh Classification; INR, international normalized ratio; MELD, Model of End-Stage Liver Disease; N/A not applicable; N/D not done; Both groups were not statistically different in terms of age and gender.

(Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantification of gene expression using real-time PCR

Quantitative real-time PCR (qPCR) was performed using a 7500 Fast real-time PCR System (Applied Biosystems, Carlsbad, CA, USA). The FAM-labelled probes for KLF2 (Hs00360439_g1); Ang-2 (Hs01048042_m1); VEGF (Hs00900058_m1); VEGFR2 (Hs00911700_m1); placental growth factor (PlGF; Hs01119262_m1); heme oxygenase-1 (HO-1; Hs00157965_m1), endothelial NO synthase (eNOS; Hs00167166_m1) and control GAPDH (Hs99999905_m1) were obtained from Applied Biosystems. The PCR reactions were performed in duplicate in a total volume of 20 μ l containing 10 μ l of TaqMan[®] Gene Expression PCR Master Mix (Applied Biosystems,

Foster City, CA, USA), 2 μ l diluted first strand cDNA and 1 μ l of the assay. The fluorescence data were analysed with 7500 Software v2.0.2 (Applied Biosystems). The expression of target genes was calculated using the $\Delta\Delta$ Ct method of relative quantification.

Protein expression analysis

For Protein extraction frozen duodenal tissues (0.15 cm³) were homogenized in an ice-cold RIPA buffer containing protease inhibitors (Sigma, Steinheim, Germany). Proteins were quantified using the Micro BCA[™] Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). For enzyme-linked immunosorbent assays (ELISA): Concentrations of VEGF, Ang2 and sCD-163 proteins in tissue lysates and/or serum samples were measured using colorimetric sandwich ELISA (R&D,

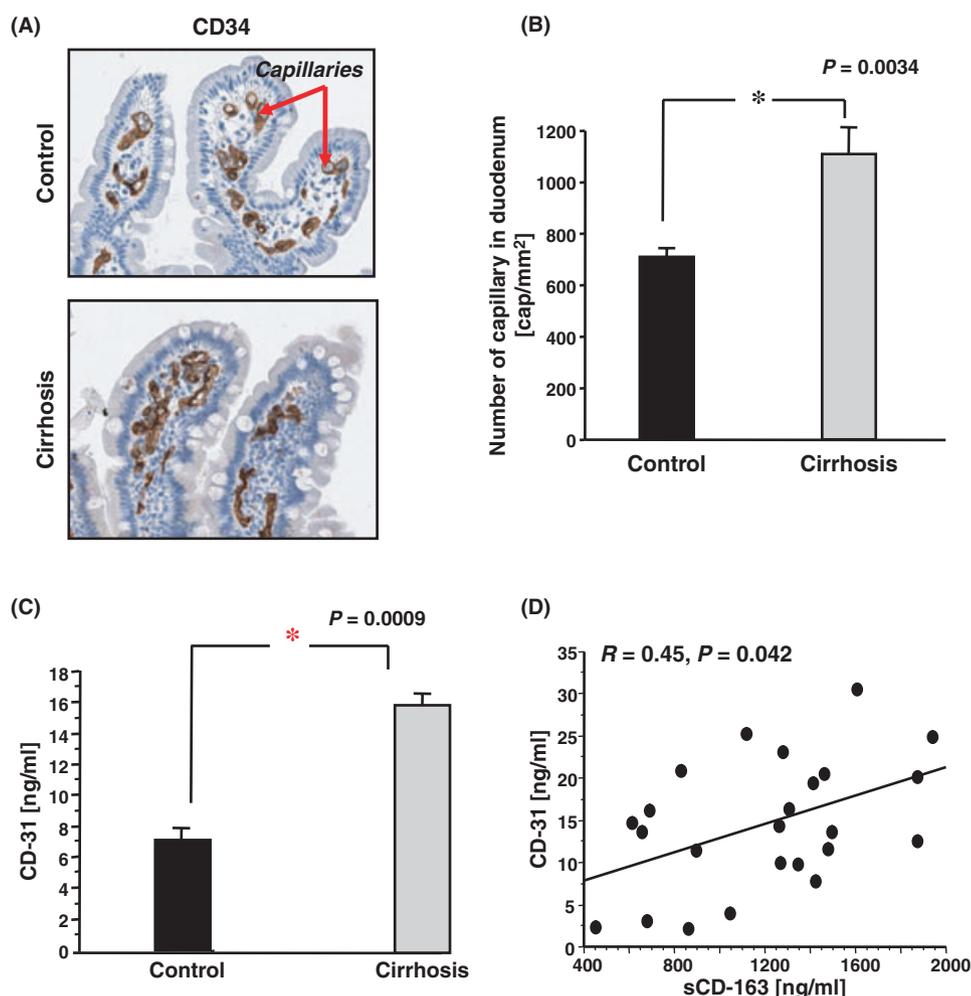


Fig. 1. The process of angiogenesis was significantly enhanced in duodenal tissue of patients with cirrhosis (A) CD34 staining in the duodenum showed pronounced immunodetection in patients with cirrhosis compared to controls (magnification $\times 400$) (B) The density of capillaries per mm² in duodenal tissue of patients with cirrhosis was higher than that in controls; capillaries were visualized by anti-CD34 antibody (C) Quantitative analysis by ELISA demonstrated increased levels of CD31 protein in the duodena of patients with cirrhosis (D) Linear regression analysis between sCD163, a surrogate marker of PH, and the concentration of CD31 protein in human duodenum ($R = 0.45$; $P = 0.042$). Correlations between variables were analysed by using Spearman's rank correlation. Values are means \pm SEM. * $P < 0.05$.

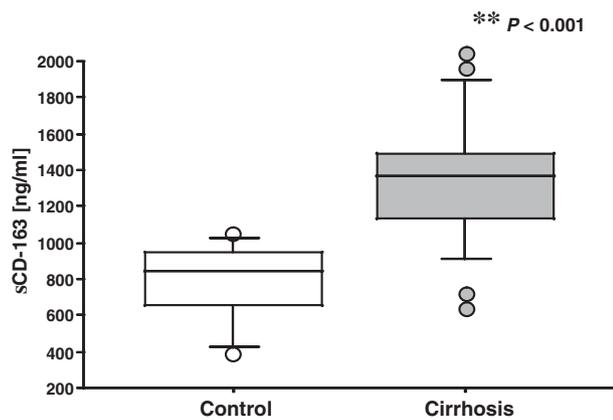


Fig. 2. Assessment of the serum levels of sCD-163 showed a significant increase in patients with cirrhosis compared to control subjects. Values are means \pm SEM.

Minneapolis, MN, USA); while, the levels of CD-31 and HO-1 proteins in duodenal tissue extracts were determined using ELISA Kits from Abcam (Cambridge, MA, USA) and Stressgen (Farmingdale, NY, USA) respectively. An indirect assessment of nitric oxide concentration in serum samples was carried using the Total Nitric Oxide and Nitrite/Nitrate Assay (R&D). Protein expression of KLF-2 was quantified by using Western blot analysis as previously described (22).

MicroRNA extraction and quantification

Total RNA was extracted from formalin-fixed, paraffin-embedded tissue samples using the RecoverAll™ Total Nucleic Acid Isolation kit (Applied Biosystems) following the manufacturer’s protocol. Reverse transcription of miR-126 was performed using the TaqMan micro RNA reverse transcription kit (Applied Biosystems). Briefly, cDNA were reverse transcribed from total RNA

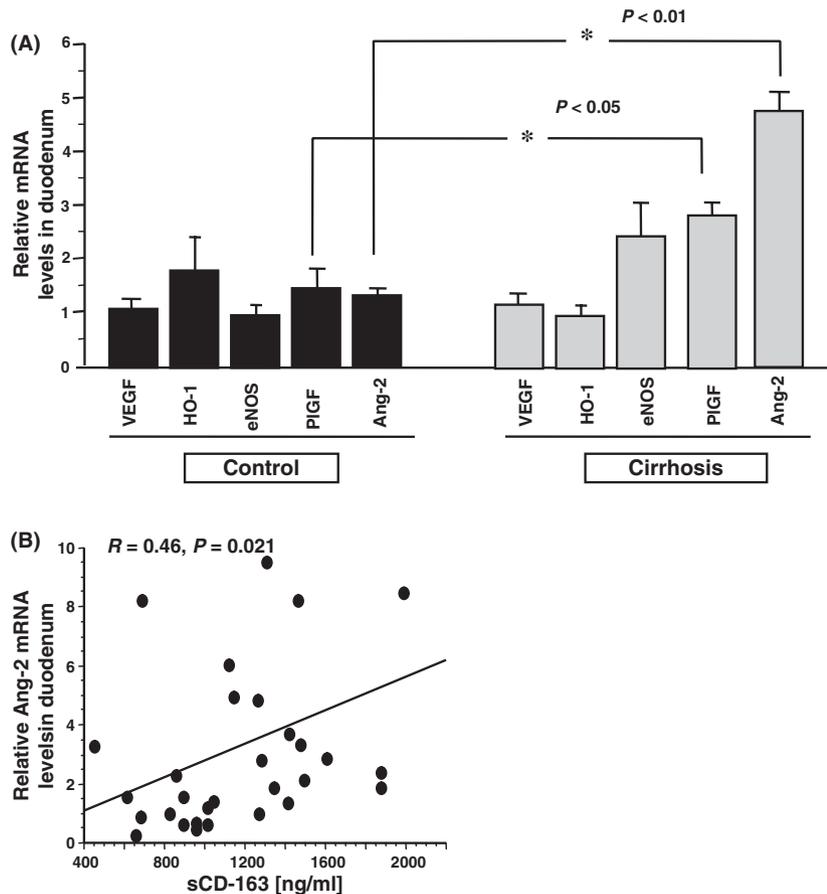


Fig. 3. (A) Comparison of relative mRNA levels of the examined factors in duodena of control subjects and patients with cirrhosis mRNA. Expression was analysed by real-time PCR, with values normalized to the housekeeping gene GAPDH. Expression of PIGF and Ang-2 mRNA was considerably enhanced in patients with cirrhosis ($P < 0.05$ and $P < 0.01$ vs control respectively). No statistically significant differences were seen for the other factors. Values are means \pm SEM (B) Linear regression analysis between serum soluble CD-163 and mRNA levels of Ang-2 in duodenal tissue ($R = 0.46$; $P = 0.021$).

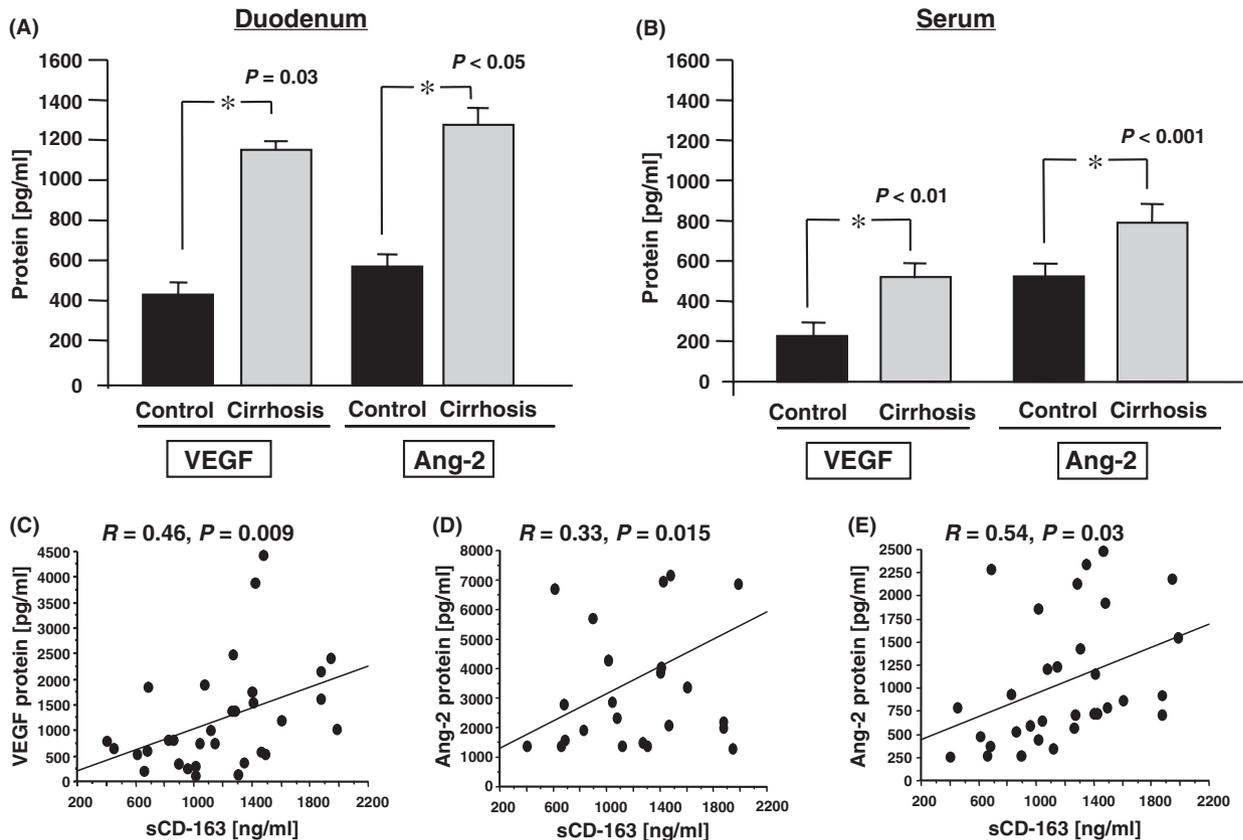


Fig. 4. Assessment of VEGF and Ang-2 protein concentrations in the duodena (A) and sera (B) showed a significant increase in patients with cirrhosis compared to controls. Values are means \pm SEM. Linear regression analysis between serum soluble CD-163 and (C) VEGF protein expression in the duodenum ($R = 0.46$; $P = 0.009$). (D) Ang-2 protein expression both in the duodenum ($R = 0.33$; $P = 0.015$) and (E) in serum ($R = 0.54$; $P = 0.03$).

samples using specific miRNA primers from the TaqMan microRNA assay (Applied Biosystems) and reagents from the TaqMan microRNA reverse transcription kit (Applied Biosystems). The reverse transcription reaction mixture contained 7 μ l MasterMix, 3 μ l miRNA-specific primers and 5 μ l of total RNA. The reaction was carried out at 16°C for 30 min followed by 30 min at 42°C; finally, mixtures were incubated at high temperature (85°C) for 5 min. Real-time quantification of miRNA expression was performed using the TaqMan microRNA assays kit (Applied Biosystems) specific for hsa-mir-126 (002228). The 20 μ l reaction included 1.33 μ l RT product, 10 μ l TaqMan[®] Universal PCR Master Mix with no UNG (P/N: 4324018, Applied Biosystems), and 1 μ l TaqMan[®] MicroRNA assay. The real-time PCRs for each miRNA were run in duplicate and RNU44 (001094) was used as an endogenous control. The results were analysed using 7500 Software v2.0.2.

Immunohistochemistry

Immunohistochemical detection of CD-34 and KLF2 protein was carried out in paraffin-embedded sections

of duodenal tissues. The sections were exposed to anti-KLF2 antibody (1:2000 dilution; Millipore, Tamecula, CA, USA) or anti-CD34 antibody (1:50 dilution; Dako, Glostrup Denmark). Reactions were visualized using Vector Laboratories ABC Vectastain and DAB kits (Vector, Burlingame, CA, USA). All stained slides were digitalized. Changes in duodenal mucosa microvasculature were assessed by the estimation of capillary densities. The location of capillaries visualized by CD34 staining were examined and counted at $\times 400$ in the same 10 fields of good cross-sectional orientation per biopsy (each 0.0063 mm² at $\times 400$) using ImageScope.

Statistical analysis

Statistical analysis was performed using chi-squared tests, chi-squared tests with Yates correction, Fisher's exact test or ANOVA with the StatView[®] Program (SAS Institute Inc. Cary, NC, USA). Correlations between variables were analysed by using Spearman's rank correlation. Results were considered statistically significant when two-sided P -values were < 0.05 .

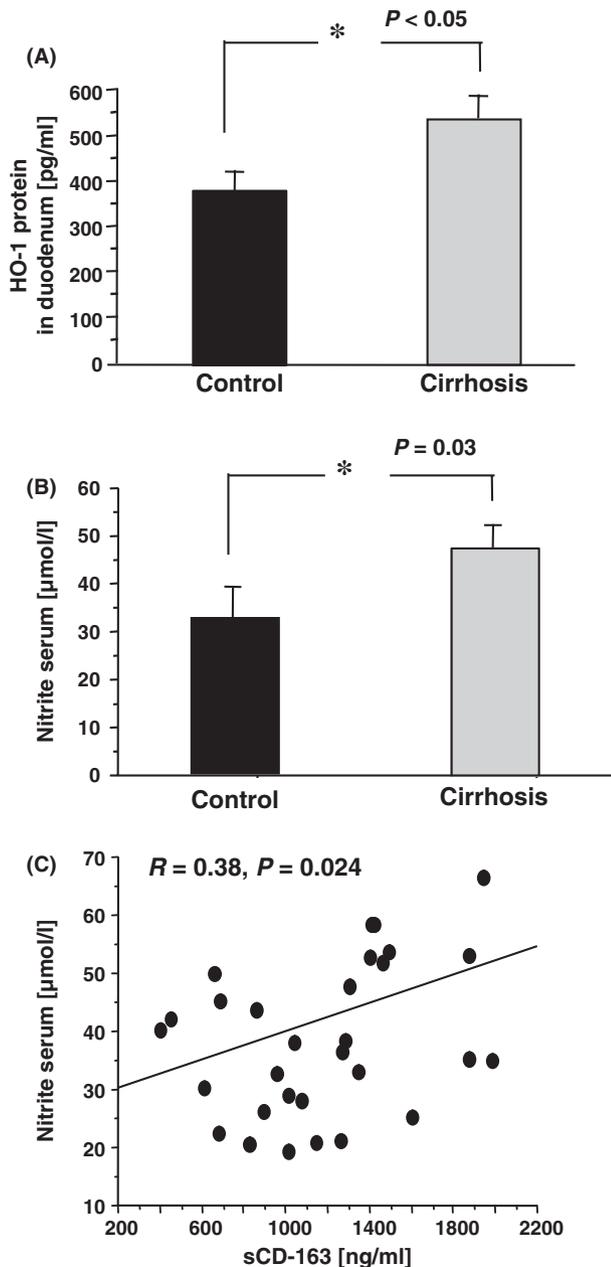


Fig. 5. Comparison of heme oxygenase protein levels in the duodena (A) and an indirect assessment of nitric oxide concentration in sera (B) showed significantly higher concentrations of both analysed factors in patients with cirrhosis in comparison to controls. Values are means \pm SEM (C) Linear regression analysis between serum soluble CD-163 and serum nitric oxide metabolite levels ($R = 0.38$; $P = 0.024$).

Results

The aetiology of cirrhosis and clinical and laboratory data on the included subjects are summarized in Table 1. Oesophageal varices were seen in 14 patients; in all patients, they fulfilled the criteria for small varices according to the recent AASLD Guidelines (23).

Histological assessment

Histological analysis showed mucosal extravasations and oedema which occurred more commonly but with no statistically significant difference in patients with cirrhosis vs controls (36% vs 10%, $P = 0.21$ and 63% vs 40%, $P = 0.26$ respectively). Moderate mucosal inflammation was observed in 36% of patients with cirrhosis compared to 0% in controls $P = 0.04$.

Angiogenesis in the human duodenum

Enhanced immunodetection of CD-34, a marker of vascular endothelium, was observed in the duodena of cirrhotic patients when compared to controls (Fig. 1A). Similarly, the capillary densities in duodenal villi were significantly higher in patients with cirrhosis (Fig. 1B). We further analysed changes in blood supply using a different marker of microvasculature i.e. CD31 protein, which is present mainly at the endothelial intracellular junctions. Quantitative analyses by ELISA showed an increased concentration of CD31 protein in the duodenal tissue of patients with cirrhosis (Fig. 1C).

Measurement of circulating soluble CD163

Changes in blood flow and pressure within the portal vein are important hemodynamic factors that may initiate the angiogenic signalling cascade within splanchnic vessels; therefore, we determined the concentration of soluble (s) CD163, a newly described surrogate marker of PH (24), in serum samples from patients with cirrhosis and healthy controls. Patients with cirrhosis had significantly higher sCD163 concentration in serum compared to controls (1365 ± 76 ng/ml vs 777 ± 70 ng/ml; $P < 0.001$ vs controls; Fig. 2).

Expression of angiogenic factors

The qPCR analysis of duodenum tissue showed no difference in the levels of VEGF, VEGFR2, HO-1 and eNOS messenger RNA (mRNA) between cirrhotic and control subjects (Fig. 3A). However, Ang-2 and PlGF mRNA levels were significantly increased (five-fold and three-fold vs controls, $P < 0.01$ and $P < 0.05$ respectively; Fig. 3A).

The VEGF and Ang-2 protein concentrations were significantly augmented compared to controls both in duodenal tissue and serum samples from cirrhotic patients (Figs 4A and B). A potential association was evaluated between hemodynamic changes in the portal vein, represented indirectly by the concentration of sCD163 in serum samples, and the expression of the modulators of angiogenesis. Significant correlations were found between sCD163 and VEGF or Ang-2 protein expression in the duodenum (Figs 4C and D) or serum (Fig. 4E). Similarly, strong correlations were

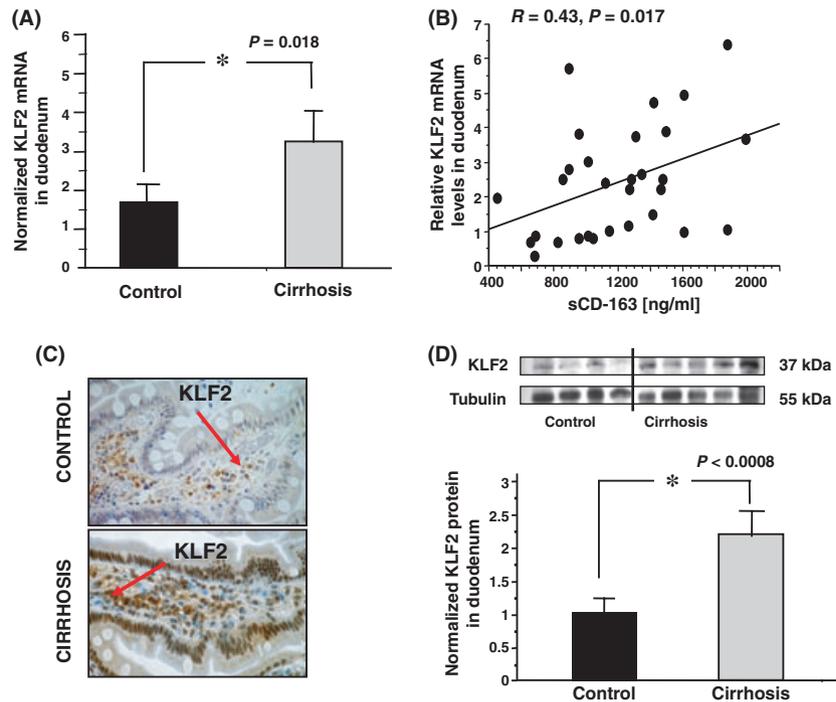


Fig. 6. (A) KLF2 mRNA expression was significantly increased in the duodena of patients with cirrhosis ($P = 0.018$ vs controls) (B) Linear regression analysis between serum soluble CD-163 and KLF2 mRNA levels in duodenal tissue ($R = 0.43$; $P = 0.017$); (C) Immunodetection of KLF2 protein showed an enhanced expression in duodenum in patients with cirrhosis when compared to controls; (D) Levels of KLF2 proteins assessed by Western blot analysis were significantly increased in the duodena of patients with cirrhosis ($P < 0.001$ vs controls). Values are means \pm SEM.

detected between sCD163 and Ang-2 mRNA and protein levels in the duodenal tissues of patients (Fig. 3B).

Furthermore, the level of heme oxygenase-1 enzyme was considerably increased in duodenal tissue from patients with cirrhosis ($P < 0.05$ vs controls; Fig. 5A). An indirect assessment of nitric oxide concentration, which was based on the enzymatic conversion of nitrate to nitrite by nitrate reductase, showed a significantly higher concentration of nitrate in serum samples from patients with cirrhosis compared to controls ($P = 0.03$ vs controls; Fig. 5B).

Kruppel-like factor 2 and post-transcriptional up-regulation of vascular endothelial growth factor via microRNA-126

The qPCR analysis demonstrated that the KLF2 mRNA levels were significantly increased in patients compared to healthy controls (2.4-fold increase; $P = 0.018$; Fig. 6A). Similarly, enhanced expression of this transcription factor was detected by immunostaining (Fig. 6C). There was a significant correlation between levels of sCD163 and KLF2 mRNA ($R = 0.43$, $P = 0.017$, Fig. 6B). Moreover, the levels of KLF2 protein in duodenal sections of patients with cirrhosis were significantly increased in comparison to healthy controls (Fig. 6D).

To understand how biomechanical stimuli can modulate the expression of VEGF via KLF2 in patients with cirrhosis, we examined the expression of endothelial-specific micro-RNA (miR-126), which is a KLF2-mediated regulator of the VEGF pathway. We observed enhanced expression of miR-126 in duodenal tissue from cirrhotic patients (three-fold increase vs controls, $P < 0.05$; Fig. 7A). Linear regression analysis showed significant correlations between miR-126 and either Ang-2 mRNA levels ($P = 0.027$; Fig. 7B) or Ang-2 protein concentrations ($P = 0.015$; Fig. 7C). Neither the presence of oesophageal varices nor treatment with beta-blockers had a significant effect on the expression of the angiogenic and transcription factors analysed.

Discussion

Our study provided evidence, for the first time in humans, that the process of neovascularization in response to increased hemodynamic forces within the splanchnic circulation was already initiated in the duodena of patients with cirrhosis lacking obvious macroscopic changes in endoscopy. The observed growth of new blood vessels in duodenum was associated with enhanced expression of KLF-2, VEGF, Ang-2 and HO-1 (Fig. 8).

Growing evidence collected from animal models of PH suggests that abnormal angiogenesis within splanchnic

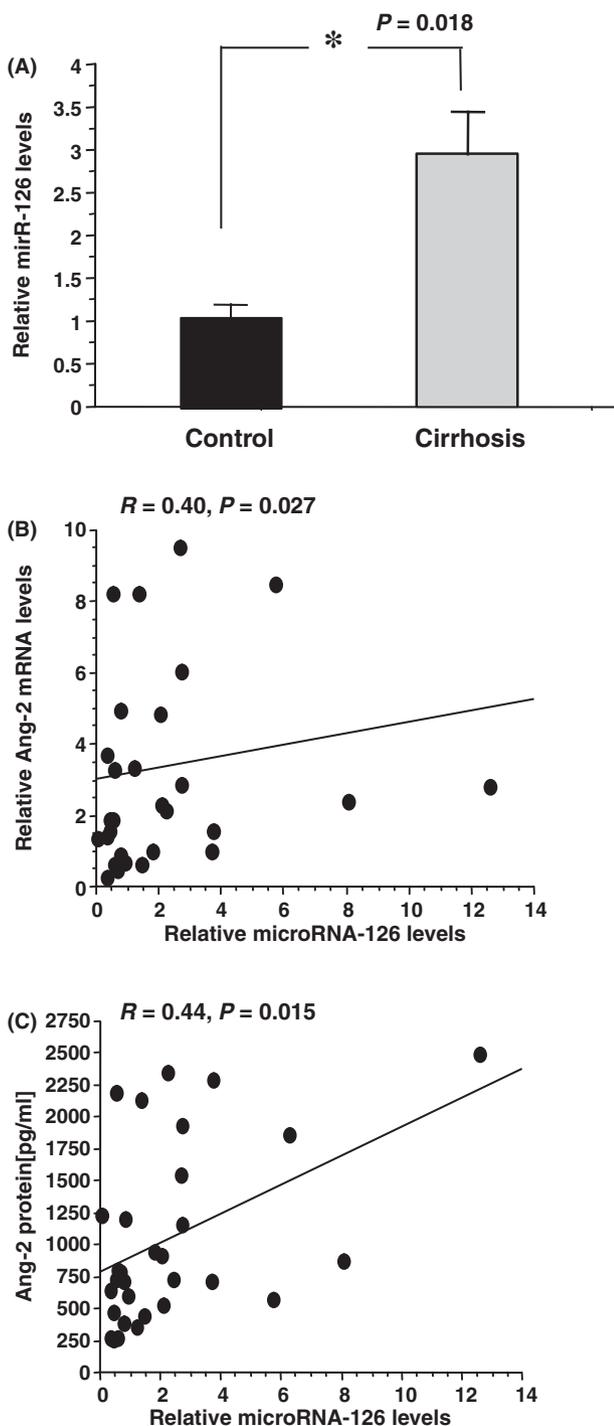


Fig. 7. (A) Expression of endothelial-specific miR-126 showed an increased expression in the duodena of cirrhotic patients. Values are means \pm SEM. Levels of sCD-163 positively correlated with (B) Ang-2 mRNA level in the duodenum ($R = 0.40$; $P = 0.027$) and (C) Ang-2 protein expression in serum ($R = 0.44$; $P = 0.015$). $*P < 0.05$.

organs plays an important role in the formation of systemic collaterals and varices (5, 25). Although VEGF-dependent angiogenesis has been demonstrated in por-

tal hypertensive animals (6, 26, 27), the reports regarding this phenomenon in human duodenum are scarce. Our observation of increased growth of blood vessels in human duodenum, assessed by two different markers of neovascularization, is a novel finding. The process was accompanied by increases in the protein expression of pro-angiogenic factors such as VEGF and Ang-2 and the changes correlate with PH, measured indirectly by evaluation of sCD163 concentration in patients' serum samples. Our data support previous reports from animal models in which molecular analyses of mesenteric tissues obtained from cirrhotic rats demonstrated increased expression of VEGF mRNA and protein levels (11, 28–30). The Ang-2 and VEGF induction correlate well with new robust angiogenesis (15). Until recently, most data on the expression of multiple factors that control angiogenesis in the liver were limited to hepatocellular carcinoma. In experimental models of cirrhosis or in mice with PH, enhanced expression of Ang-2 and PIGF in the splanchnic territory were associated with the development of portal-systemic collaterals and neovascularization (2, 5, 7, 29). The present work complements the results from portal hypertensive animals by demonstrating that the mRNA and protein levels of Ang-2 were significantly increased in duodenal tissues of cirrhotic patients. Our findings confirmed the induction of pro-angiogenic factors within the splanchnic microcirculation, which takes place in response to cirrhosis.

Increased intrahepatic resistance and enhanced portal blood flow within the vessel are important hemodynamic triggers that can initiate the angiogenic signalling cascade. Mechanosensitive and endothelial-specific transcription factor KLF2 is able to transduce biomechanical signals into changes in gene expression (18, 31, 32). However, the expression of KLF2 has never been studied in patients with cirrhosis, where microvascular vessels of the splanchnic circulation are exposed continuously to enhanced shear stress. In the present study, we demonstrated that KLF2 gene and protein expressions were significantly increased in human duodenal tissue in response to cirrhosis. Our results evidently complement the findings in animal models with advanced cirrhosis, in which overexpression of KLF2 was associated with enhanced portal vein blood flow (33). On the other hand, there are reports describing KLF2 as a potent inhibitor of VEGF-mediated angiogenesis. In cultured endothelial cells and in the nude ear mouse model of angiogenesis, KLF2 inhibited the expression of VEGF and promoted degradation of HIF-1 α , which is a key inducer of VEGF transcription in hypoxic conditions (17, 34). However, in our experiments, we observed concomitant overexpression of both KLF2 and VEGF protein. These conflicting results can be explained by the fact that the observed processes of angiogenesis occur in response to different pathological conditions. Endothelium in duodenal tissue of portal hypertensive patients is constantly exposed to increased hemodynamic forces and it is worth noting that expression and

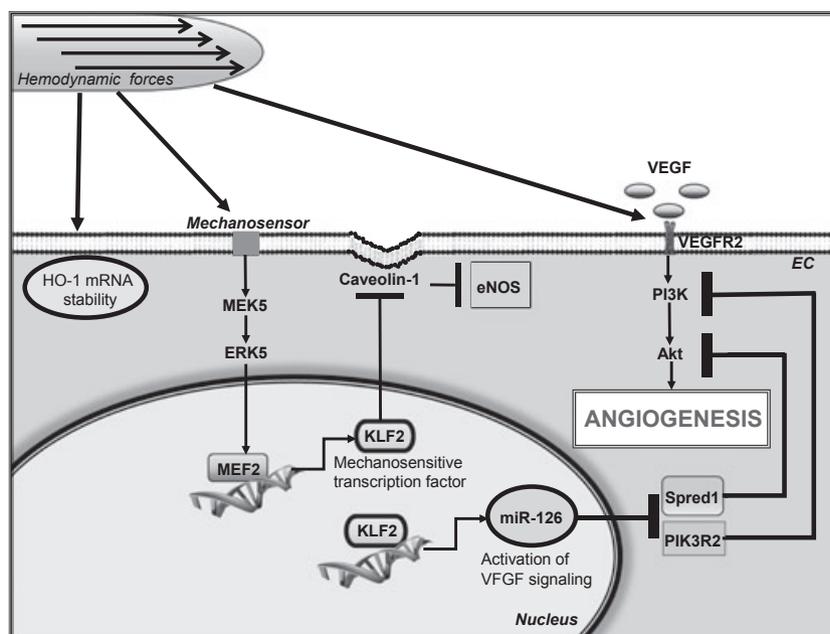


Fig. 8. Scheme of the proposed pathways responsible for flow-stimulated angiogenesis in the duodena of patients with cirrhosis.

stabilization of KLF2 mRNA is sustained by prolonged shear stress stimulation (35).

The mechanism by which KLF2 is able to integrate hemodynamic stimulation and VEGF-mediated angiogenesis is not fully elucidated. Recently, a study in zebrafish embryos showed that flow-induced blood vessel sprouting requires activation of endothelial-specific microRNA, miR-126 (36). In agreement with this report we observed an increased level of miR-126 in regions of the duodenum where growth of new blood vessels took place. The present data strongly suggest that miR-126 can be a candidate for integrating KLF2 and VEGF pathways during formation of portosystemic collaterals in response to biomechanical stimulation. However, additional investigation is needed to elucidate the miR-126/VEGF signalling pathway in duodenal neovascularization in patients with cirrhosis. (20, 21).

Heme oxygenase-1 (HO-1), an inducible enzyme that catalyses the degradation of heme in response to a wide variety of stimuli, has been recently described as a potent modulator of angiogenic response (37). However, there is a discrepancy in the role of HO-1 in angiogenesis. In inflammatory angiogenesis, VEGF-stimulated blood vessel formation requires upregulation of HO-1 (37). On the other hand, HO-1 induces VEGF expression within splanchnic vessels and attenuates the inflammation in portal hypertensive rats (38). In the present study, the expression of HO-1 protein was significantly increased in the duodenal tissue of patients. These results are consistent with reports from animal models that showed elevation of HO-1 mRNA levels in liver cells and splanchnic organs from hypertensive rats

(39, 40). Recent *in vitro* studies imply that laminar shear stress-induced HO-1 and KLF2 modulate the VEGF expression (41). The potential contribution of HO-1 and KLF2 to mesenteric neovascularization has not been assessed. Therefore, it is plausible that heme oxygenase-1 not only regulates heme degradation but also plays an additional role in modulation of angiogenesis driven by the KLF2/VEGF signalling pathway.

We observed no significant impact of the presence of oesophageal varices or treatment with beta-blockers on the expression of analysed factors. This can most likely be explained by the finding that all detected varices were small. In some patients receiving beta-blockers in the past, varices were not seen. Other patients with small varices detected for the first time were beta-blocker naïve at the time of endoscopy and obtaining duodenal tissue. Thus, this study could not precisely address the issue of the effect of varices or beta-blockers on analysed factors. For this purpose, it would be necessary to include patients with more advanced/decompensated cirrhosis with large varices. As already mentioned in this project we focused on stable and compensated patients.

Altered levels of nitric oxide have been associated with shear stimulation and hypertension (42–44). The ability of KLF2 to modulate shear-induced eNOS expression in cultured human endothelial cells was reported (16, 17). In the present study, the levels of eNOS mRNA did not change in the examined duodenal tissues but NO production, evaluated by the level of stable NO metabolites, showed a significant increase in the nitrate concentration in serum samples from cirrhotic patients.

In conclusion, the present work demonstrated that angiogenesis occurred within the duodenal tissue of patients with cirrhosis. Such an effect was associated with enhanced expression of KLF-2, Ang-2 and miR-126. Establishing the precise molecular mechanisms contributing to the pathological remodelling within duodenal and in wider context splanchnic circulation may point towards a therapeutic target for the treatment and/or prevention of major complication of PH.

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