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ORIGINAL ARTICLE

Functional variants of *eNOS* and *iNOS* genes have no relationship to the portal hypertension in patients with liver cirrhosis

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Abstract

Objective. Nitric oxide is an important vasoactive mediator. Changes in NO production, caused by functional variants of both endothelial and inducible NO synthase (*eNOS, iNOS*), might play a role in portal hypertension. The aim was to study the significance of functional *eNOS* and *iNOS* gene variants in cirrhotic patients and their interrelationship to both inflammatory and endothelial activation parameters. ***Material and methods.*** One hundred and thirty-two patients with liver cirrhosis (age 36–72 years) and 101 controls were examined for functional variants of *eNOS* (E298D, 27bpintr4, 786T/C) and *iNOS* (R221W, S608L) genes. Inflammatory (IL6, IL8, IL10) and vasoactive (sVCAM-1, E-selectin) cytokines were measured using ELISA kits. ***Results.*** The frequency of E298D (GG 12%, GT 41%, TT 47%), 28bpintr4 (AA 6%, AB 28%, BB 66%), 786T/C genotypes (CC 17%, CT 45%, TT 38%), as well as R221W (CC 93%, CT 7%, TT 0%), and S608L (CC 65%, CT 32%, TT 3%) genotypes in cirrhotic patients did not differ from the controls ($p > 0.05$ for all comparisons). No relationship was found between the frequency of these genotypes and the severity of portal hypertension, or either inflammatory or vasoactive cytokines. A positive correlation was found between hepatic venous pressure gradient and cytokine concentration: sVCAM-1, IL6, IL8, IL10. ***Conclusions.*** Examined *eNOS* and *iNOS* variants have no relationship to pathogenesis of liver cirrhosis. Severity of portal hypertension was associated with the changes in endothelial activation.

Key Words: *cirrhosis, eNOS/iNOS functional variants, hemodynamic parameters, portal hypertension*

Introduction

Portal hypertension accounts for the majority of fatal complications of liver cirrhosis such as bleeding from esophageal varices, ascites, and hepatic encephalopathy. Increased pressure in the portal system results from both increased intrahepatic resistance and increased splanchnic vasodilation. Nitric oxide (NO) is among the most important contributing factors [1]. While the splanchnic bed is marked by NO overproduction in liver cirrhosis [2], its bioavailability is diminished in the intrahepatic circulation. With local deficiency of NO production [3] and increased production of endothelins [4] in the intrahepatic circulation, a situation similar to endothelial dysfunction in the systemic circulation [5] is induced. In fact, endothelial dysfunction is an early key event in vascular disorders such as portal hypertension in liver cirrhosis [6].

Vasoactive NO is synthesized by a family of enzymes, the NO synthases (NOS) [7]. Both constitutive endothelial NOS (eNOS, NOS3, OMIM *163729) and inducible NOS (iNOS; NOS2A-C) could play an

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Table I. Summary of characteristics of patients and healthy controls.

Parameter	Patients with liver cirrhosis (n = 132)	Healthy controls (n = 101)
Age (years)	55.5 ± 10.9	54.8 ± 5.4
Male/female (No. of patients)	89/43	64/37
Etiology of cirrhosis (alcohol/hepatitis/other)	78 (59%)/37 (28%)/17 (13%)	-
Child-Pugh score (points)	7.23 ± 2.04	-
Bilirubin (μmol/l)	*30.2 (17.1–53)	-
Albumin (g/l)	33.5 ± 6.8	-
Platelet count ($\times 10^9$/l)	*103 (65–113)	-

Data expressed as mean ± SD.
*Bilirubin and platelet count are expressed as median and 25–75 percentile.

important role in the hemodynamic abnormalities of portal hypertension [8,9].

Changes in NO and superoxide production (as the consequence of an altered eNOS reactive mechanism) can contribute to the pathogenesis of atherosclerosis, essential hypertension, septic shock, as well as tumors or neurodegenerative disorders [10,11]. E298D (G894T) variant at exon 7 of *eNOS* gene has been related to essential hypertension [12], coronary artery disease, and myocardial infarction [13].

To our knowledge, only three papers concerning *eNOS* gene variations in patients with liver cirrhosis have been published so far. One paper, on patients with primary biliary cirrhosis [14], showed that the TT genotype of E298D gene variant might be related to a worse prognosis, and two papers on Chinese patients suggested that the presence of both allele T in E298D and allele A in a repetitive sequence in intron 4 e*NOS* might correlate with the severity of portal hypertension [15,16].

The aim of the present study was to investigate the role of functional *eNOS/iNOS* gene variants in patients with liver cirrhosis and portal hypertension, and their relationships to the parameters of endothelial activation and inflammation. We hypothesized that *eNOS/iNOS* gene variations could aggravate portal hypertension via an alteration of intrahepatic circulation. Secondly, we aimed to assess the parameters of endothelial activation and inflammation to the degree of portal hypertension.

Material and methods

Patients

A group of 132 patients with proven liver cirrhosis and portal hypertension were included in the study. A diagnosis of liver cirrhosis was made on the basis of clinical examinations, laboratory parameters, and verified by liver biopsy. The etiology of liver cirrhosis is given in Table I. Hepatic vein catheterization and a hepatic venous pressure gradient (HVPG) measurement [17] were performed in all patients.

The control group consisted of 101 age- and sex-matched healthy individuals without liver disease, coronary artery disease, or other chronic diseases. The basic characteristics of these groups are provided in Table I.

The study was approved by the local Ethics Committee, and the protocol conformed to the latest ethical guidelines of the Declaration of Helsinki. Informed consent was obtained from all subjects.

Methods

Measurement of HVPG. A measurement of HVPG was performed using the classical wedged technique [18]. Shortly after overnight fasting, patients were transferred to the catheterization room. Under local anesthesia, a 7F catheter introducer was placed in the right jugular vein using the Seldinger technique. Under fluoroscopic control, a 7F balloon-tipped catheter (B. Braun Melsungen AG, Germany) was advanced into the right hepatic vein in order to measure both the free hepatic venous pressure and the wedged hepatic venous pressure. All measurements were performed in triplicate. HVPG was calculated as the difference between the wedged hepatic venous pressure and free hepatic venous pressure.

Laboratory analyses. Biochemical and hematology examinations were done using routine techniques on automatic analyzers. The severity of liver disease was evaluated by Child-Pugh scoring, and then evaluated separately according to serum levels of albumin, bilirubin, and platelet count, presence of esophageal varices, ascites, and hepatic encephalopathy.

The serum levels of TNFα, interleukin-2, interleukin-6, interleukin-8, interleukin-10, E-selectin, intercellular adhesion molecule-1 (ICAM-1), and soluble vascular adhesion molecule (sVCAM) were established using commercially available ELISA kits (BioSource Europe, S.A., Belgium). The detection limits, as well as intra- and inter-assay precision, were as follows: TNFα: detection limit = 1.7 pg/ml, intra-assay precision coefficient of variability (CV) = 4.4%, inter-assay precision CV = 7.5%; IL-2: detection limit = 4 pg/ml, intra-assay precision CV = 5.9%, inter-assay precision CV = 7.2%; IL-6: detection limit = 2 pg/ml, intra-assay precision CV = 6.2%, inter-assay precision CV = 7.9%; ICAM-1: detection limit = 0.5 ng/ml, intra-assay precision CV = 5.6%, inter-assay precision CV = 7.8%; E selectin: detection

Table II. Frequency of specific *eNOS* genotypes in patients with liver cirrhosis.

Genotype	Liver cirrhosis (n = 132)	Control group (n = 101)	p-value
E298D			
GG (wild type)	62 (47)	49 (49)	0.92
GT	54 (41)	39 (39)	
TT	16 (12)	13 (12)	
27bp intr4			
BB (wild type)	87 (66)	72 (71)	0.6
BA	37 (28)	26 (26)	
AA	8 (6)	3 (3)	
786 T/C			
TT (wild type)	50 (38)	45 (44)	0.65
TC	59 (45)	39 (39)	
CC	23 (17)	17 (17)	

Data expressed as number of patients (%).

limit = 0.5 ng/ml, intra-assay precision CV = 5.4%, inter-assay precision CV = 6.0%.

Genotyping of the eNOS and iNOS sequence variants. Three variations of the *eNOS* gene and two variations of the *iNOS* gene were analyzed. The missense Glu298Asp variant in exon 7 (E298D; rs1799983), 27 base pairs (bps) variable number of tandem repeat in intron 4 (eNOS4a4b; CG962921), and 786 T/C (rs2070744) in the promoter of *eNOS* gene were ascertained, as well as S608L in exon 16 (rs2297518), and R221W (rs3730017) the promoter of the *iNOS* gene. Genomic DNA was isolated from peripheral blood leucocytes and the polymorphic sites of *eNOS/iNOS* genes were detected using both the polymerase chain reaction (PCR) and PCR restriction fragment length polymorphism technique, as previously described [19–21]. In brief, the presence of the Glu298Asp sequence variant was determined using the oligonucleotide primers (the forward primer 5′-GAG ATG AAG GCA GGA GAC AGT-3′ and the reverse primer 5′-TCC ATC CCA CCC AGT CAA T-3′). The PCR fragments were digested with the *Mbo*I restriction enzyme, separated by electrophoresis using 3.8% agarose gel, and visualized by ethidium bromide staining. For detection of the 27-bp repeat variations we used the primers and PCR conditions by Ichihara et al. [21] with slight modification (a PCR total volume 25 µl; sense primer 5′-AGG CCC TAT GGT AGT GCC TTT-3′; antisense primer 5′-TCT CTT AGT GCT GTG GTC AT-3′). Each PCR method was revised by direct sequencing technique.

Statistical methods

The data are expressed as the mean ± SD, or median (IQ range) when the data were non-normally distributed. Allele frequency was evaluated by the Fisher's exact test. Linear regression analysis was used to find the relationships between the analyzed parameters. A multiple logistic regression was used for multivariate analysis of independent variables. A p value of ≤0.05 was required for statistical significance. The statistics were computed using STATISTICA CZ v. 8 (StatSoft, Prague, Czech Republic).

Results

Comparison of patients with liver cirrhosis and the control population

The frequencies of the examined gene variations are presented in Tables II and III. No statistical difference between the cirrhotic and control population regarding the frequencies of analyzed genotypes was found. Similarly, no difference was found in the frequency of the above-mentioned gene variations in relationship to a person's gender. The distribution of the examined gene variations was in the Hardy–Weinberg equilibrium, indicating that the results are unlikely to be biased by population stratification.

Relationship of eNOS/iNOS gene variants and clinical parameters and inflammatory cytokines

Within the entire group of patients, no significant correlation was found between the clinical and laboratory parameters and the *eNOS* and/or *iNOS* sequence variants. As the E298D *eNOS* variation, the presence of genotype GG in women was associated with lower bilirubin levels compared to individuals with the allele GT (p = 0.087, Figure 1). Similarly, women with genotype GG had a tendency to present with higher serum albumin concentrations and higher platelet counts, but without any statistical significance.

No relationship of the examined *eNOS/iNOS* variations to the degree of portal hypertension was found.

The relationship between *eNOS/iNOS* variations and the inflammatory or vasoactive cytokines was not found.

Table III. Frequency of specific *iNOS* genotypes in patients with liver cirrhosis.

Genotype	Liver cirrhosis (n = 132)	Control group (n = 101)	p-value
S608L			
CC (wild type)	86 (65)	67 (67)	0.88
CT	42 (32)	31 (30)	
TT	4 (3)	3 (3)	
R221W			
CC (wild type)	123 (93)	97 (96)	0.64
CT	9 (7)	4 (4)	
TT	0 (0)	0 (0)	

Data expressed as number of patients (%).

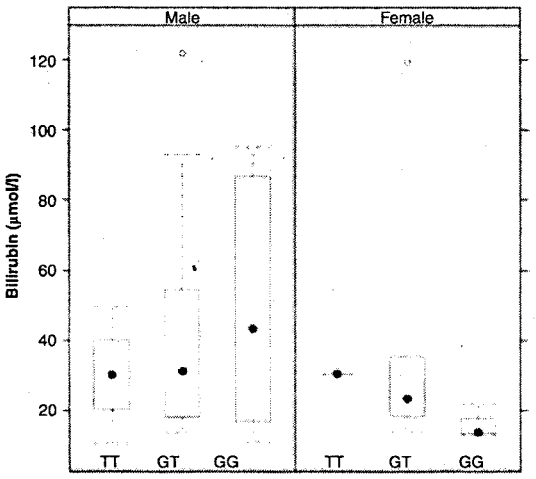

Figure 1. Association of serum bilirubin and *eNOS* E298D genotype in patients with liver cirrhosis.

Relationship of hemodynamic, inflammatory, and vasoactive parameters to portal hypertension

The hemodynamic parameters are given in Table IV. Significant differences between Child-Pugh class A, B, and C patients were found for heart rate, mean blood pressure, and HVPG (Table IV). HVPG value correlated positively with the portal vein diameter and negatively with portal vein flow velocity (Figure 2A, B).

The values for the inflammatory and vasoactive parameters in both the cirrhotic patients and controls are given in Table V. Significant differences in these parameters were found between the patients and the control population. A positive relationship was found between HVPG and cytokine concentrations: sVCAM-1 ($p < 0.05$), interleukin-6 ($p < 0.01$), interleukin-8 ($p < 0.05$), interleukin-10 ($p < 0.05$). A negative correlation was found between HVPG and E-selectin concentrations ($p < 0.05$) (Figure 3A–E).

No relationship between inflammatory and vasoactive parameters and noninvasive hemodynamic parameters (portal vein diameter, portal vein flow velocity, spleen size, spleen vein diameter) was found.

Discussion

Nitric oxide plays a role in a wide spectrum of physiological and pathological processes in a human body. Several sequence variants of the eNOS gene, leading to greater susceptibility of eNOS to proteolytic cleavage, have been reported, and the correlations between these genotypes and cardiovascular diseases have been identified [11,12]. The presence of a specific gene variation may also predict the response to antihypertensive treatment [19]. Thus, it seemed possible that specific *eNOS* or *iNOS* gene variations, associated with negative impacts on cardiovascular diseases, could also play an inverse role in portal hypertension.

But in contrast to essential hypertension, the situation in liver cirrhosis is more complicated. Vasodilatation occurs in the arterial bed, mainly splanchnic, due to the increased production of NO. The splanchnic vein system is also dilated, and the vasoconstriction is coupled to the intrahepatic circulation (i.e. to the terminal branches of the portal vein and hepatic sinusoids), and eventually to the renal circulation [5].

In fact, in our study, the allele frequencies of the examined sequence variants of *eNOS* an *iNOS* did not differ between the patients and controls. This is in concordance with the study on primary biliary cirrhosis [14] but, however, in contrast to the data published in China [15,16]. The explanation for this discrepancy is not clear, as the detailed data from China are not widely available. One reason might be the etiology of cirrhosis (hepatitis B in the Chinese reports). Our study is large enough to conclude that there is no relationship between the presence of candidate functional *eNOS* and *iNOS* gene variations and the risk for cirrhosis, at least in Caucasian population.

Table IV. Hemodynamic characteristics of patients with liver cirrhosis.

		Child-Pugh		
Parameter	All patients	A	B	C
No. of points	132	55	49	28
HVPG (mm Hg)	15.6 (±5.9)	14.5* (±4.8)	17.3* (±5.5)	19.5* (±4.7)
Diameter of VP (mm)	13.3 (±2.2)	13.5 (±2.2)	13.4 (±2.3)	12.9 (±2.1)
Flow velocity VP (cm/s)	13.3 (±2.2)	16.8 (±6.4)	14.6 (±4.8)	23 (±6.6)
Spleen length (mm)	143 (±20.1)	147 (±18)	137 (±21.9)	144 (±19.8)
Mean BP (mm Hg)	90.6 (±11.2)	93.7° (±10.2)	91.2° (±12.5)	87.2° (±9.9)
Hearth rate (beats per min)	79.4 (±13.1)	73.9⁺ (±10.6)	82.2⁺ (±13.6)	83.5⁺ (±12.2)

*A vs. B: $p = 0.013$, A vs. C: $p < 0.001$, B vs. C: ns ($p = 0.098$).
°A vs. B: ns ($p = 0.37$), A vs. C: $p = 0.019$, B vs. C: ns ($p = 0.22$).
⁺A vs. B: $p = 0.008$, A vs. C: $p = 0.003$, B vs. C: ns ($p = 0.55$).

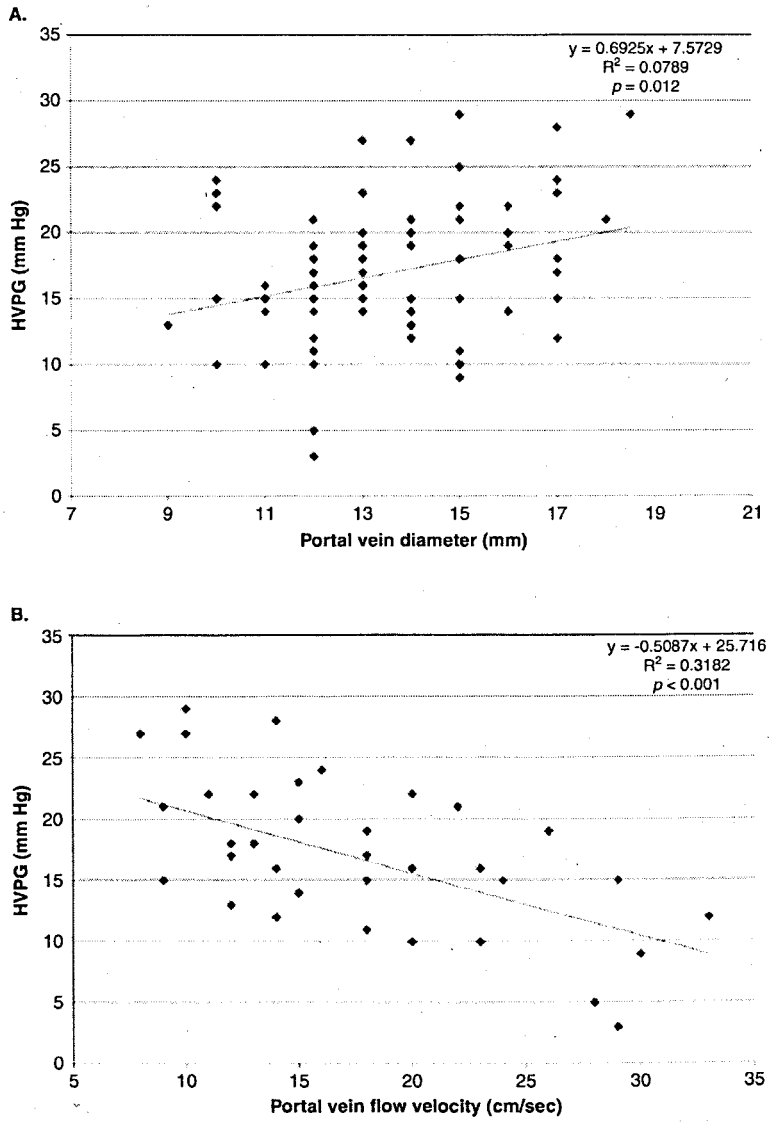

Figure 2. Association of HVPG and ultrasonographic noninvasive portal hemodynamic parameters: A. Portal vein diameter. B. Portal vein flow velocity.

In our group of cirrhotic patients, the presence of *eNOS* variations was marginally related to different clinical parameters in a subgroup of the patients. Women homozygous for the G allele in E298D had a tendency to present with more favorable laboratory parameters regarding liver function. This might be in accord with the study by Selmi et al. [14], in which more than 90% of the patients were female. One explanation for this finding could be that gender plays a role in the regulation of eNOS activity [22]. NO production and activity have been shown to vary according to changes in estrogen levels [23]. Secondly, a correlation between E298D *eNOS* genotype, gender, and hemodynamic response to angiotensin II has been observed. Page et al. [24] found that glomerular filtration decreased after angiotensin II infusion in men with the T allele, while men with a GG genotype or women irrespective of this variation did not show a decrease in glomerular filtration rate. The authors suggested that the presence of the T allele correlated with decreased NO activity. To explain the possible differences in laboratory parameters, we could

Table V. Serum concentrations of inflammatory cytokines in patients with liver cirrhosis.

Parameter	Liver cirrhosis (n = 132)	Controls (n = 101)	p-value
TNFα (pg/ml)	4.52 (2.7–6.9)	3.96 (2.8–5.3)	0.405
IL-1β (pg/ml)	0.640 (0.6–0.6)	0.13 (0.1–0.2)	<0.001
IL-2 (pg/ml)	30.5 (0–50)	0.53 (0.3–1)	0.074
IL-6 (pg/ml)	6.65 (3.6–13.2)	0.71 (0.3–1.4)	**<0.001**
IL-8 (pg/ml)	15.7 (7.8–34.9)	ND	
IL-10 (pg/ml)	3.96 (1.9–9.1)	6.89 (4.3–10)	**0.014**
E-selectin (ng/ml)	32.65 (25.5–47.6)	23.2 (12.8–45.8)	0.198
ICAM-1 (ng/ml)	186.6 (162–262)	673 (516–803)	**<0.001**
sVCAM-1 (ng/ml)	1411 (1276–1653)	ND	

Abbreviation: ND = not done.
Data are expressed as the median and IQ range.
The values in bold are statistically significant.

hypothesize that those women with the GG genotype might have increased expression of *eNOS* in the hepatic bed, and could have a tendency to a milder liver disease. However, other data supporting this theory were not found, and thus it is questionable if this sequence variation has any clinical relevance.

The increased production of NO in the splanchnic bed, resulting in vasodilatation, can be induced by bacterial translocation of intestinal microflora or shear stress [25]. In fact, both markers of systemic inflammation and/or endothelium activation [26] are elevated in cirrhotic patients. Activation of the immune system in patients with cirrhosis and portal hypertension has been described, even in the absence of overt infection [27], as a consequence of chronic endotoxemia. It has been suggested that the presence of endotoxemia would induce, directly or *via* cytokines, the synthesis of NO in peripheral blood vessels, which is responsible for the hyperdynamic circulatory state [28]. Many investigations have demonstrated that several cytokines are elevated among patients with cirrhosis and portal hypertension [29], but the correlations between the evaluated parameters (cytokines) and the resulting consequences (hemodynamic alterations) might be very difficult to ascertain because the direct effect might be masked by a cascade of events mediated by other intermediate products. Genesca et al. [30] clearly demonstrated elevated levels of TNF-α, interferon γ, interleukin-1, interleukin-6, and endotoxin in patients with cirrhosis and portal hypertension. While TNF-α, interferon γ, interleukin-1, and endotoxin could directly induce the production of NO, interleukin-6 is probably implicated by NO-independent vasodilatory mechanisms. Vascular effect of interleukin-6 could be mediated *via* other vascular mediators, such as prostacyclines. In our study, we confirmed elevated levels of inflammatory parameters in patients with liver cirrhosis compared to controls. Moreover, we found significant relationship between serum concentrations of interleukin-6, interleukin-8, and interleukin-10, and portal hypertension, an observation supporting the role of examined cytokines in the pathophysiology of portal hypertensive vasodilatation.

Increased intrahepatic vascular resistance calls for quite a different explanation. Besides the fixed structural changes, there is an unpredictable functional component. It has been suggested that pro-inflammatory cytokines (such as TNF-α and IL-6) are implicated in the expression of cell adhesion molecules such as E-selectin on endothelial cells [31], or ICAM-1 on endothelial cells, sinusoidal cells, fibroblasts, and hepatocytes [32]. These adhesion molecules contribute to endothelial activation and modification of circulatory changes in patients with portal hypertension, probably due to increased intrahepatic vascular resistance [28].

The selectin family of adhesion molecules is important in the early transient adhesion phase [31]. Firm adhesion is mediated by the binding of integrin molecules, present in inflammatory cells such as the ICAM-1, as well as the immunoglobulin gene superfamily proteins, which are present on activated endothelial cells such as vascular cell adhesion molecule-1 (VCAM-1). In fact, the adhesion molecules have been reported as increased in patients with liver cirrhosis; however, the relationship to portal hypertension is not clear.

In cirrhosis, endothelial dysfunction in the hepatic vascular bed is considered an important factor leading to the increased vascular tone, and therefore to the development of portal hypertension [6]. There is clear evidence that high levels of pro-inflammatory cytokines or adhesion molecules are associated with a poor prognosis of cirrhotics [33]. Some markers of endothelial dysfunction, such as the von Willebrand factor, have been shown to correlate with the clinical outcome of patients with liver cirrhosis [34].

In a study by Girón-González, elevated levels of markers of endothelial dysfunction were described in cirrhotic patients compared to controls with a tendency to progressively increase in patients with infectious complication [33]. Contrary to expectations, we found a negative correlation between

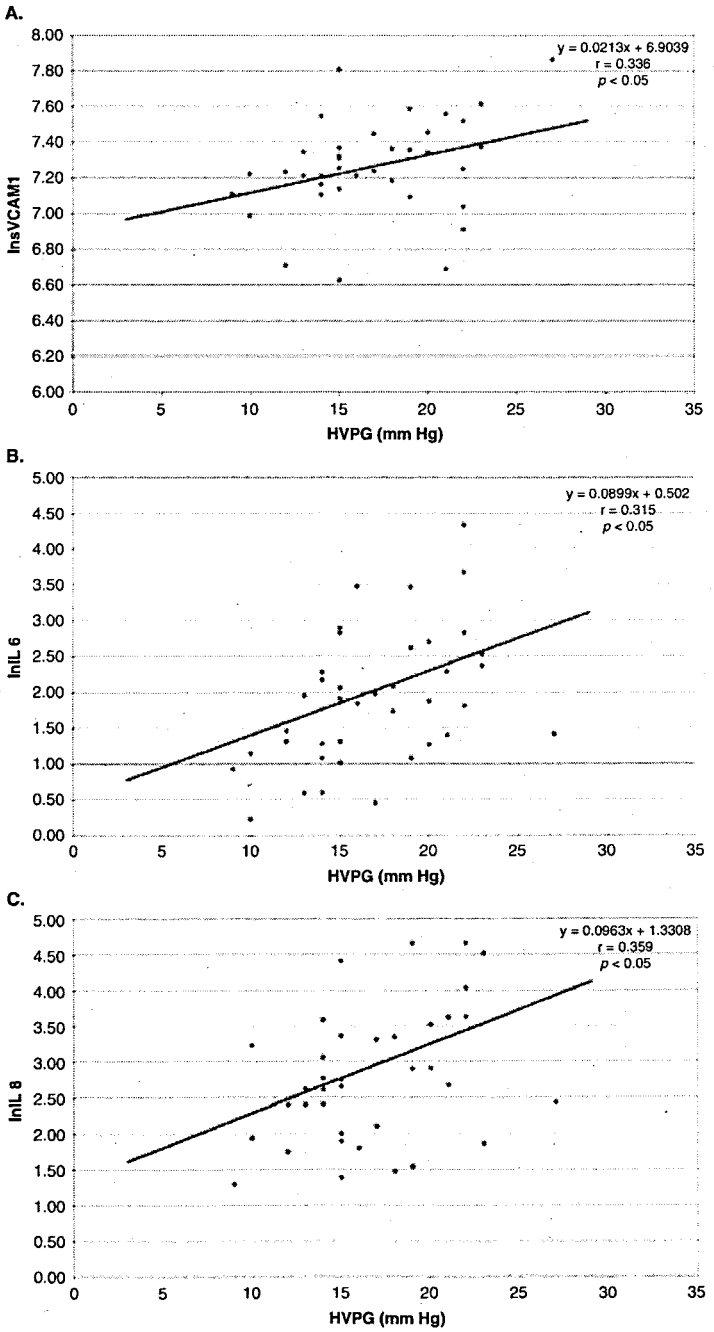

Figure 3. Association of HVPG and serum levels of A. sVCAM-1. B. IL6. C. IL8. D. IL10. E. E-selectin in patients with cirrhosis.

E-selectin and disease progression (evaluated by severity of portal hypertension). Nevertheless, a similar paradox has been described in the literature before. Cervelo et al. [35] found that high serum levels of soluble E-selectin are associated with chronic hepatitis and liver cirrhosis. However, in the liver

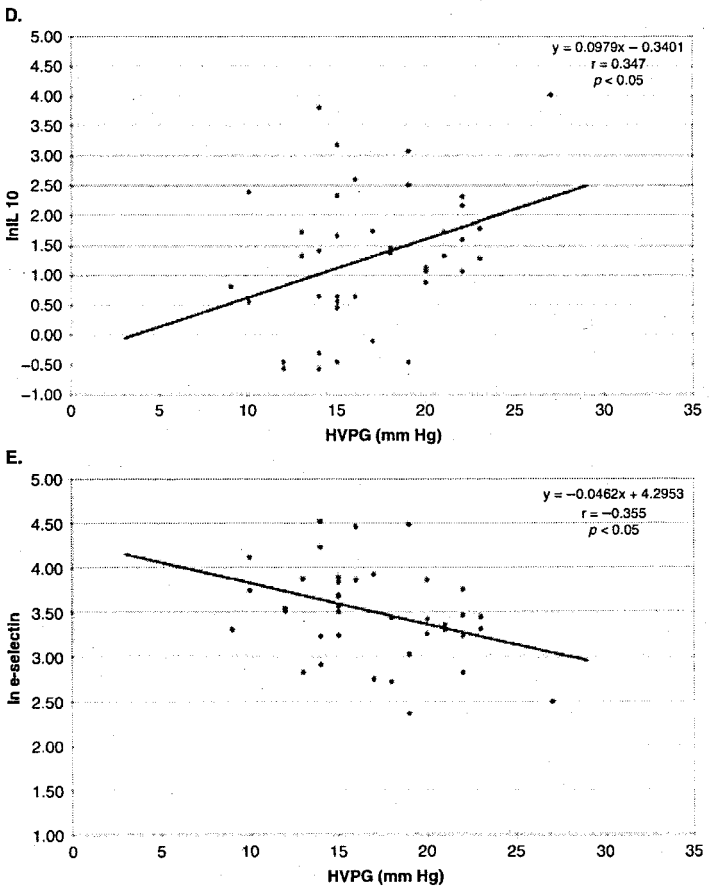

Figure 3. (*Continued*).

cirrhosis group, divided according to the Child-Pugh score, soluble E-selectin levels decreased with disease severity. Similarly, in patients with liver cirrhosis who developed hepatocellular carcinoma, soluble E-selectin concentrations decreased as the disease progressed. This paradox observation might be due to different expressions of E-selectin. It was previously described that E-selectin is expressed in the activated endothelium of inflamed tissues [36]. In the liver E-selectin is upregulated also on sinusoidal lining cells in areas of inflammation [37]. In a study by Cervelo et al. [35], immunohistochemical localization showed strong membrane staining on endothelial cells in areas rich in inflammatory cells in severe chronic hepatitis. Thus, we could speculate that high levels of E-selectin are associated rather with inflammatory changes in liver tissue than with portal hypertension, and the decrease in E-selectin levels with disease progression might follow the progressive reduction in liver mass. Thus, endothelial factors like E-selectin might play a different role in maintenance of splanchnic versus systemic vascular tone.

In conclusion, our study demonstrates that the presence of the examined e*NOS*/iNOS gene variants does not carry any increased risk of liver cirrhosis. Moreover, once cirrhosis is diagnosed, specific *eNOS/iNOS* variations do not play any role in the course or severity of the disease. No relationships were found between the *NOS* gene variations and the clinical parameters, the degree of portal hypertension, or the inflammatory or vasoactive cytokines. Portal hypertension was positively associated with the concentration of inflammatory cytokines and sVCAM-1, and correlated negatively with E-selectin.

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