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Endothelium Derived Microparticles: A Reliable Biomarker for Detection of Portal Hypertension in Chronic Hepatitis C Patients

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ABSTRACT

Vascular endothelium plays a pivotal role in the pathogenesis of numerous chronic disorders. Cirrhosis is associated with enhanced endothelial dysfunction and increased intrahepatic vascular tone due to imbalance in equilibrium between endothelium derived relaxing and contracting factors. We aimed to clarify the contribution of endothelial dysfunction in the mechanism of portal hypertension in chronic liver disease [CLD] patients and to evaluate the clinical utility of endothelial dysfunction markers for detection of portal hypertension. Forty five patients were the subject of this study classified according to Child-Pugh classification into 3 groups (15 each).Patients were further classified into 2 groups according to the presence or absence of portal hypertension (30 and 15 patients respectively) in addition to 15 healthy subjects serving as a control group. Endothelial dysfunction was assessed by measurement of soluble thrombomodulin (sTM), von Willebrand factor (vWF), soluble E-selectin (sE-selectin) and quantitative assay of CD31+/CD42- endothelial microparticles (EMPs). Results revealed evidence of endothelial dysfunction reflected by increased levels of sTM, vWF, sE-selectin and EMP which were more evident with progression of the disease, particularly in Child C group. Moreover, statistically significant higher levels of sTM, vWF and EMP were identified in patients with portal hypertension compared to those without portal hypertension. Statistical analysis proved that EMPs assay is the most sensitive (96.67%) and specific (93.33%) biomarker for prediction of portal hypertension. In conclusion endothelial dysfunction is a major determinant of portal hypertension in CLD patients. EMPs have been identified as a reliable non invasive laboratory marker for the detection of portal hypertension in those patients.

KEYWORDS: Endothelial dysfunction, cirrhosis, portal hypertension, endothelial microparticles, thrombomodulin, von Willebrand factor, E-selectin.

INTRODUCTION

Portal hypertension is a frequent and dreadful complication of chronic liver disease and its presence is a hard endpoint for clinically relevant outcomes in terms of varices, ascites, hepatorenal syndrome and encephalopathy [1, 2]. In cirrhosis, portal hypertension is initiated by an increase in intrahepatic vascular resistance which is the primary factor in the pathophysiology of portal hypertension [3]. Then, it is exacerbated by changes in the systemic and splanchnic circulation that increases the portal inflow. Increased intrahepatic vascular resistance (IHVR) is caused not only by mechanical factors (e.g., fibrotic scars and regenerative nodules) that distort the hepatic vascular architecture, but also by a reversible dynamic components mediated by increase in vascular tone due to the active contraction of myofibroblasts around the hepatic sinusoids and the vascular septa. This dynamic component (which accounts for around 30% of increased IHVR) reflects a

functional disturbance in the liver circulation, secondary to increased production of vasoconstrictors and reduced release of endogenous vasodilators [2, 4, 5].

Endothelium is the active inner monolayer of the blood vessels, forming an interface between circulating blood and the vessel wall. It represents the largest organ in the body and plays a critical role in vascular homeostasis [6]. Endothelium has a central role in the regulation of blood flow through continuous modulation of vascular tone [7]. This is primarily accomplished by balanced release of endothelial relaxing and contractile factors including nitric oxide, arachidonic acid metabolites, reactive oxygen species, and vasoactive peptides [8, 9].

The complex morphofunctional rearrangement of the hepatic microvascular bed and intrahepatic angiogenesis also play important roles in hemodynamic disturbances in liver cirrhosis. It is characterized by endothelial dysfunction and impaired paracrine interaction between activated stellate hepatocytes and sinusoidal endotheliocytes, sinusoidal remodeling and capillarization, as well as development of the collateral microcirculation[10].

In response to various agents, such as bacterial endotoxin, viruses, drugs and ethanol, liver sinusoidal endothelial cells experience oxidative stress [11,12]. These results in the activation of inflammatory pathways, which causes the liver sinusoidal endothelial cells to become dysfunctional, thus, may contribute to portal hypertension [13]. Furthermore, mechanical stimuli, such as shear stress, change the gene expression pattern in cirrhotic livers, may worsen liver sinusoidal endothelial cell dysfunction [14]. Snowdon *et al.* [2], reported that endothelial dysfunction is a major determinant of the increased intrahepatic vascular tone observed in cirrhosis.

In response to various injurious stimuli, vascular endothelial cells express a broad variety of proteins [15] but only few of these have been studied in serum or plasma in patients with chronic liver disease. Currently, vWF, TM and sE-selectin are best described [16,17]. La Mura *et al.* [18], reported that in patients with portal hypertension, vWF levels significantly correlated with hepatic pressure venous gradient. However, it must be noted that several factors may influence the levels of these circulating proteins. For example, thrombomodulin undergoes renal excretion. Hence, serum levels are influenced by renal function. Other confounding factors, such as liver function, clotting or fibrinolysis changes may also influence these proteins. Finally, these soluble markers do not distinguish between endothelial activation and damage [19]

Microparticles have recently received more attention as markers of activation in eukaryotic cells. Resulting from exocytic budding, these vesicles consist of cytoplasmic components and phospholipids. They carry markers of the parent cell, including those induced by activation or apoptosis [20].

Endothelial microparticles (EMPs) are novel marker of endothelial dysfunction. Circulating membrane-shed microparticles participate in the regulation of vascular tone, permeability and haemostasis [21]. Higher levels of EMP was found in several cardiovascular and atherothrombotic diseases [22] and have been shown to correlate with loss of flow-mediated dilatation, arterial stiffness and severe hypertension [23]. Circulating EMPs affect both proinflammatory and proatherosclerotic processes in endothelial cells. In addition, they can promote coagulation and inflammation or alter angiogenesis and apoptosis in endothelial cells [22].

Cirrhosis is associated with increase in circulating subpopulation of microparticles, likely resulting from systemic inflammation and liver cell damage [21] and are considered to reflect the severity of vascular injury [20]. The gradual increase in the severity of portal hypertension was observed to be correlated with the deterioration of the endothelial functional condition in patients with alcoholic steatohepatitis [24].

The aim of this study was to assess the relevance of different markers of endothelial cell dysfunction, namely sTM, vWF, sE-selectin and EMPs, to determine the most specific and sensitive endothelial biomarker, in attempt to identify the most reliable noninvasive laboratory marker for detection of CHC patients at high risk of developing portal hypertension, as this may launch new targets for treatment of such condition.

MATERIALS AND METHODS

Study population:

The study was conducted on 45 patients with CLD (18 females, 27 males, ages ranged between 31-62 years) admitted to Gastroenterology and Hepatology Department (Theodor Bilharz Research Institute, Giza, Egypt). All patients were positive for hepatitis C virus and negative for hepatitis B virus. Patients were classified according to modified Child Pugh classification [25] into Child A, B and C groups (15 patients each). Patients were further classified into 2 groups according to the presence or absence of portal hypertension (30 and 15 patients respectively), 18 out of 30 patients of portal hypertension (60%) has esophageal varices. Fifteen age and sex matched healthy subjects selected from medical and paramedical staffs were included in the study to serve as a control group. Informed consents were obtained from all patients in accordance with the Declaration of Helsinki. This study was approved by the local ethical committee of TBRI-IRB (FWA 00010609) with approval number 01/14.

Diagnosis of patients was based on thorough clinical examination, abdominal ultrasonography, liver function tests and hepatitis markers. Upper gastrointestinal endoscopy and liver biopsy were done when

indicated.Portal hypertension was defined by the presence of varices and/or increased portal vein diameter (PVD)>13mm and spleen size >13cm [26]. None of the patients had active variceal bleeding or encephalopathy at time of investigation. Patients with fever, overt infectious disease (septicemia, pneumonia, urinary tract infection) or renal insufficiency were excluded.

Sampling:

A sample of 6ml blood was collected from each subject into sterile endotoxin- free vacuum blood collection tubes, distributed as follows:

- Two ml were collected on EDTA for hemogram by automated hematology analyzer ACT Differential (Beckman Coulter, France) and for assay of endothelial microparticles using flowcytometry (Bekhman Coulter, USA).
- Two ml were collected on trisodium citrate to measure prothrombin time (PT) using Thromborel S reagent (Behring werke AG) and serum endothelial markers including: TM, vWF, and E-selectin using enzyme linked immunosorbent assay (ELISA) technique.
- Two ml were withdrawn into a plain tube and left to clot. Sera obtained were collected for liver function tests {Alanine aminotrasferase (ALT) and aspartate aminotransferase (AST)} using autoanalyzer (Hitachi 736, Hitachim Japan). The rest of the sera were aliquoted, stored and kept frozen at -70° to measure the hepatitis markers (HBs-Ag and HCV-Ab) by (ELISA) technique.

Assay methods:

-Quantitative analysis of endothelial markers:

- Plasma TM level was assayed by ELISA technique using Asserachrom TM kit (DiagnosticaStago, France).
- Plasma vWF antigen level was assayed by ELISA technique using AsserachromvWF kit (DiagnosticaStago, France).
- Plasma E-selectin level was assayed by ELISA technique (R&D Systems, Minneapolis, USA).

-Assay of endothelial microparticles (EMPs):

Fifty µl of EDTA blood was incubated with 5 µl anti CD31 monoclonal antibody labeled with fluorescinisothiocyanate (FITC) (Bekhman Coulter, USA) and 5 µl anti CD42b monoclonal antibody labeled with phycoerythrin (PE) (Bekhman Coulter, USA) for 30 min. One ml of phosphate buffered (PBS) saline was added. Then 100 µl of flow count flurospheres (Flow beads of standard size, Beckman Coulter, USA) were added and the samples were analyzed by flowcytometry. For each sample, matched control tube (without CD31 and CD42b) was used to correct for non specific binding. EMPs were characterized by size <1µm and bound to CD31 but not to CD42 (CD31positive/42b negative). Data acquisition was stopped after 1000 fluorospheres were counted. To count EMPs, the following calculation was used (sample count – control count) x (blood dilution) x (Proportion of flow count fluorospheres counted to total amount added). Results were reported as $x10^3$/ml [27].

Statistical Analysis:

All statistical analyses were performed using the SPSS for Windows, version 12 (software). Results were expressed as means ± standard deviation. Comparing means was performed by one-way ANOVA test. To evaluate correlations among the variables, a Pearson correlation test was used as appropriate. Receiver operating characteristic (ROC) curves giving a graphical display of the performance of a test was used to determine the sensitivity and specificity of different parameters. Test sensitivity was plotted vs 1 – specificity, with each point of the curve representing a different cut-off level. The area under the curve (AUC) described the test's overall performance and was used to compare different tests. For the ROC curves, AUCs were calculated and compared using Medcalc software. This program applies the Hanley and McNeil method [28] for the non-parametric estimation of the AUC.

Results:

The results of all parameters studied in different groups classified according to the Child Pugh classification and the presence of portal hypertension are shown in Tables 1, 2 and 3 respectively (Figs1&2). Sensitivity and specificity of endothelial dysfunction markers in relation to portal hypertension in patient groups specified by the ROC curve are illustrated in table4.

Our study revealed a significant increase in the mean plasma concentrations of markers of endothelial dysfunction. sTM and EMPs were significantly increased in all diseased groups compared to control group and with the progress of the disease. vWF was also significantly increased with the progress of the disease but there was no significant difference on comparing child B and C groups. E– selectin increased significantly in all diseased groups compared to control group but there was no significant difference between the diseased groups

(Fig 1). All parameters were significantly increased in patients with portal hypertension compared to those without portal hypertension except E –selectin (Fig 2).

Table 1: Results of the clinic-laboratory data of different groups classified according to the Child Pugh classification and healthy subjects

	Control mean±SD	Child A mean±SD	Child B mean±SD	Child C mean±SD
Platelet count (x10^3/ ml)	267.46±49.15	217.26±30.55**	151.8±32.27$^{**\wedge\wedge}$	81.0±22.67$^{**\wedge\wedge\$\$}$
PT (sec)	11.03±1.26	12.90±2.80**	15.89±2.13$^{**\wedge\wedge}$	17.51±2.85$^{**\wedge\wedge\$\$}$
AST (IU/L)	25.86±6.26	57.93±27.94**	56.0±26.8**	74.33±26.58$^{**\$}$
ALT (IU/L)	27.6±4.89	56.06±28.94**	63.4±20.92**	77.73±25.34$^{**\wedge}$
PV calibre (mm)	10.40±1.12	11.52±0.92	12.47±0.83	15.73±1.33
Spleen size (cm)	10.20±0.94	11.33±0.90	12.67±1.18	15.93±2.25

*: P<0.05,.**: P <0.01 significant difference compared to control group.
^: P <0.05,^^: P <0.01 significant difference compared to Child A group.
$^{\$}$: P <0.05 ,$^{\$\$}$: P <0.01 significant difference compared to Child B group.

Table 2: Statistical comparison of endothelial dysfunction markers in different groups classified according to the Child Pugh classification.

	Control (n=15)	Child A (n=15)	Child B (n=15)	Child C (n=15)
sTM (µg/dl)	63.98±28.55	99.25±51.38^{a}	125.1±38.48^{aa}	157.63±39.80^{aabc}
vWF (mg/dl)	64.53±11.30	91.63±50.11^{a}	126.1±34.96^{aab}	136.03±38.82^{aab}
E–selectin (ng/ml)	42.12±20.26	105.80±53.26^{aa}	101.61±46.17^{aa}	107.53±51.87^{aa}
EMPs (CD 31$^+$/42b$^-$) (x10^3/ ml)	3.32±1.7	6.56±1.46^{aa}	10.93±1.58^{aab}	15.39±1.54^{aabc}

^{a}: P <0.05,^{aa}: P <0.01 significant difference compared to control group.
^{b}: P <0.05 significant difference compared to Child A group.
^{c}: P <0.05 significant difference compared to Child B group.

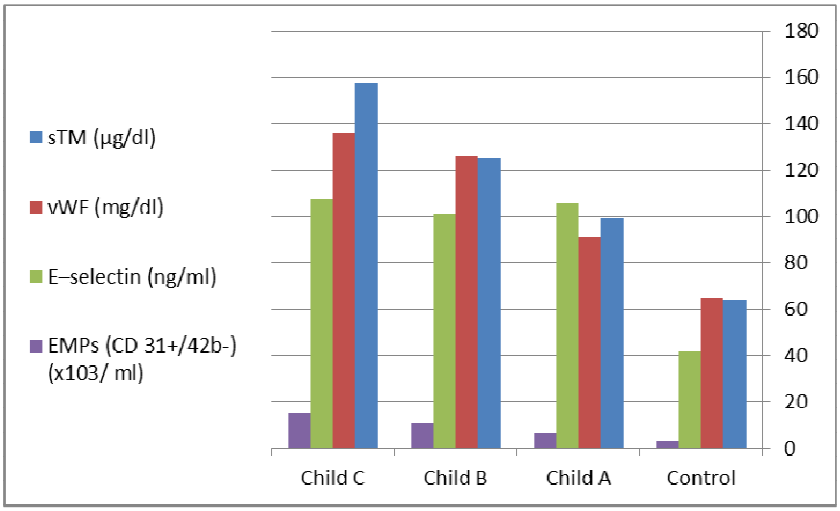

Fig. 1: Endothelial dysfunction markers in different studied groups.

Table 3: Statistical comparison of endothelial dysfunction markers in relation to the presence of portal hypertension in patients' group.

	Control (n=15)	Patients Without portal hypertension (n=15)	Patients With portal hypertension (n=30)
sTM (µg/dl)	63.98±28.55	99.25±51.38^{a}	134.38±38.86$^{aa\,b}$
vWF (mg/dl)	64.53±11.30	91.63±50.11^{A}	126.28±29.97$^{AA\,B}$
E–selectin (ng/ml)	42.12±20.26	105.80±53.26^{aa}	104.57±48.35^{aa}
EMPs (CD 31$^+$/42b$^-$) (x10^3/ ml)	3.32±1.7	6.56±1.46^{AA}	13.16±2.74^{AAB}

^{a}: P <0.05, ^{aa}: P <0.01 significant difference compared to control group.
^{b}: P <0.01 significant difference compared to patients without portal hypertension.

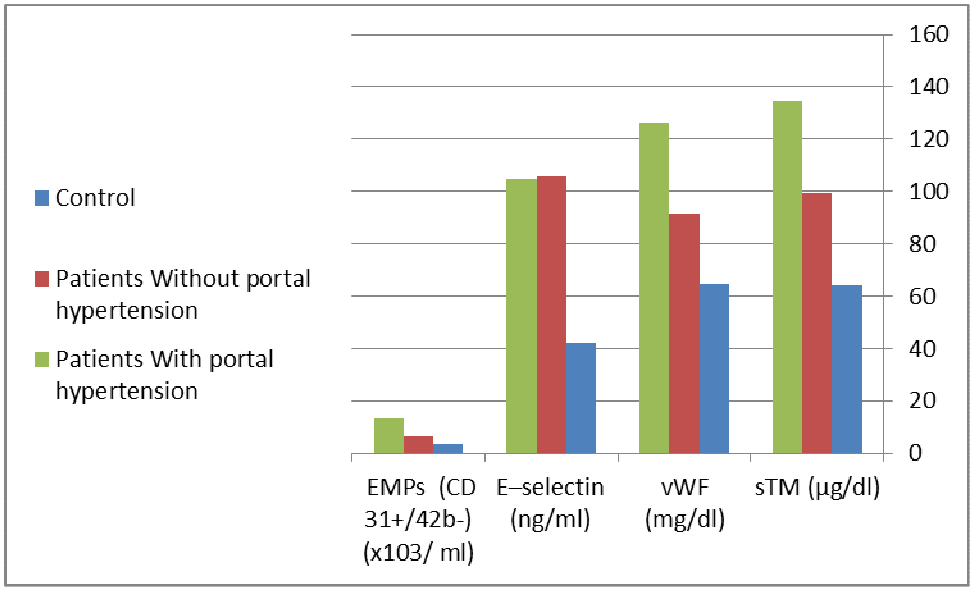

Fig. 2: Endothelial dysfunction markers in relation to the presence of portal hypertension in patients' group.

Table 4: Sensitivity and specificity of endothelial dysfunction markers in patient groups

	Area under Roc curve	Cut-off	Sensitivity (%)	Specificity (%)
sTM	0.707	74.00	90.00	46.67
vWF	0.862	91.75	93.30	73.30
E-selectin	0.514	151.45	20.00	73.30
EMPs	0.996	8.55	96.67	93.33

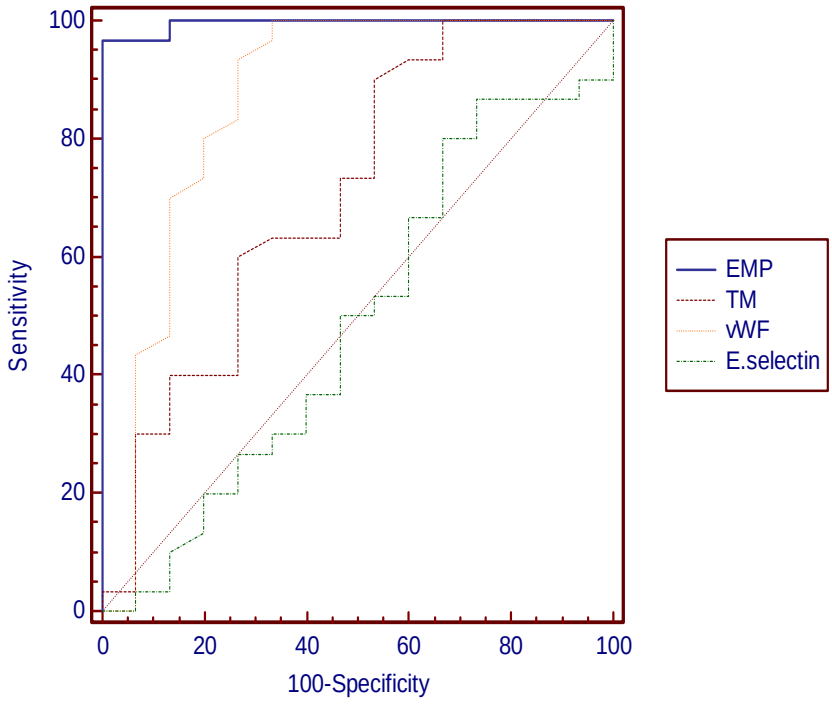

Fig. 3: Roc curve of different studied parameters.

Discussion:

Portal hypertension caused by increased intrahepatic resistance is a major consequence of chronic liver disease [13]. The syndrome of portal hypertension acquires the primary role in the prognosis for the patient's life and puts on the first place, therapeutic measures to prevent and stop the esophageal-gastric bleeding [24]. Endothelial cells play important roles in the regulation of vasomotor tone through the production of a wide variety of substances in response to various physical and chemical stimuli [29-30]. Endothelial dysfunction is

characterized by violation of endothelium-dependent relaxation of blood vessels and by increasing of endothelium adhesiveness [31].

The current study revealed a significant increase in the mean plasma concentrations of markers of endothelial dysfunction. TM is an integral membrane protein expressed on the surface of endothelial cells that converts thrombin from a procoagulant to an anticoagulant enzyme [32]. Previous studies had proved that raised sTM levels reflect endothelial injury [2,33, 34]. The present study revealed elevated plasma TM levels in different patients' groups compared to controls; which became more pronounced with advancement of the disease according to both the Child-Pugh classification and the development of portal hypertension .Similar findings were reported by Tacke *et al.* [35] and Kulwas *et al.* [36]. However, it should be noted that monocytes, macrophages, platelets and neutrophils are possible sources of plasma TM in patients with liver cirrhosis [37]. Our Results demonstrated that in patients with portal hypertension measurement of sTM as a marker of endothelial dysfunction provided a diagnostic sensitivity of 90% and specificity of 47%.

Highly significant increase in the level of vWF was noticed in all patients' groups compared to control group. A significant increase in vWF level was also noticed with the advancement of the disease and with the development of portal hypertension. These results are inaccordance withKulwas *et al.* [36] and Lisman *et al.* [38]. Moreover, La Mura *et al.* [18] and Ferlitsch *et al.* [39], recently reported that in patients with cirrhosis and portal hypertension vWF level correlated with liver function and the hepatic venous pressure gradient and is regarded as an independent predictor of clinical outcome. Plasma vWF level was found to be determined by genetic factors including ABO blood groups and vWF mutations, as well as by non-genetic factors including aging, impaired nitric acid production, inflammation and free radical production [40]. Strong correlation was reported between endotoxaemia and high circulating levels of vWF. Accordingly, high circulating level of vWF in cirrhosis might be a consequence of endothelial perturbation induced by endotoxin [41]. Moreover, it has been suggested that high vWF levels can be probably explained by increased synthesis / release of proteins since it is an acute phase reactant to tissue injury affected by inflammatory cytokines [42]. Furthermore, high vWF levels could be associated with a compensatory regulation mechanism for the haemostatic disorders of chronic liver disease [43]. Routine measurement of vWF activity in vascular patients as an index of endothelial dysfunction may have clinical importance as detection of this marker can be a noninvasive way of assessing the diagnosis of portal hypertension and indicating disease progression in chronic liver disease patients [44, 45]. Our study proved that measurement of vWF serum level yielded a 93% sensitivity and 73% specificity.

Our data showed that plasma sE-selectin levels were significantly higher in all patients' groups compared to the control group. Reactive oxygen species, when present in excess, may mediate progressive endothelial damage with overproduction of adhesion molecules like E-selectin [46]. No significant difference could be detected neither with the progress from Child's A to Child C groups nor with the occurrence of portal hypertension. Similar findings were also reported by Cervello *et al.* [47], who found that high plasma levels of sE-selectin were associated with chronic hepatitis and liver cirrhosis, and that levels decreased with disease progression. These results could be explained by changes in cytokines that are involved in the progression of chronic liver disease and in response to therapy in HCV infection [48]. However, our study revealed that measurement of plasma levels of sE-selectin provided a diagnostic sensitivity of (87%),and its specificity was (73.3%).

The present study demonstrated that circulating EMPs were increased in chronic liver disease patients compared to the controls. This increase was observed using the detection of (CD31+ and CD42b-) expressing MP, in order to distinguish EMPs from (CD31+ and CD42b+) platelet microparticles [49-51]. The EMPs levels were increased with the severity of the CLD disease and the development of portal hypertension. EMPs are considered as emerging markers of endothelial repair and activation/apoptosis [52] and accentuate pre-existing endothelial dysfunction [23]. Increased circulating EMPs levels have been shown in various pathologies associated with endothelial dysfunction, such as antiphospholipid syndrome [53], preeclampsia [54], acute coronary syndromes [55] and chronic renal failure [56] and was considered as arobust independent predictor of severe cardiovascular outcome in end-stage renal failure patients with sensitivity of 63% and specificity of 82% [57]. Besides, the elevation in EMPs levels were found to be correlated to the elevation of endothelial activation markers, sTM, vWF and E-selectin in CLD. Similar correlation was previously reported in uremic patients [56]. These results may indicate that patients with CLD were found to have signs of endothelial damage, which manifests itself as excessive endothelial vesiculation and increases in the count of desquamated EMPs which represents a new marker of endothelial dysfunction in CLD. Interestingly, EMPs not only represent a marker of endothelial damage, but have been shown to mediate functional properties [58,59]. Increased EMPs in CHC patients with portal hypertension demonstrated in this study suggested that endothelial damage is a key process in the development of portal hypertension. In accordance with our results, Rautou [22], recently reported that circulating EMPs were increased in cirrhosis, likely resulting from systemic inflammation and liver cell damage increasing proinflammatory cytokines in plasma, impaired peripheral vasoconstrictor responses and decreased blood pressure, contributing to the arterial vasodilatation associated with portal hypertension. Microparticles generated from endothelial cells impair endothelium-dependent relaxation and nitric oxide production, increase

arterial stiffness, promote inflammation and initiate thrombosis [21,60]. Whether a cause or a consequence of endothelial dysfunction, our results showed that elevated levels of (CD31+ and CD42b-) expressing EMPs provided a diagnostic sensitivity of 97% and specificity of 93%.

In conclusion, our data are keeping with the evidence of significant endothelial cell dysfunction in CHC. Endothelial dysfunction is an early key event in many vascular diseases and is considered a major determinant of the increased hepatic vascular tone and the development of portal hypertension. EMPs represent a great promise as a marker of endothelial dysfunction in those patients. Measurement of EMPs is perhaps the most useful marker, because it is specific for endothelial cells, relevant for disease progression and development of portal hypertension and it is a simple non invasive assay. Measurement of EMPs could provide a more robust predictor of the occurrence of portal hypertension in CHC patients, when compared to classical markers of endothelial activation. Prospects for further investigations are the search for medications to correct endothelial dysfunction in order to improve results of treatment of patients with portal hypertension.

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