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Keywords: transendothelial migration, leukocytes, cirrhosis, hepatic encephalopathy, systemic inflammation

Abbreviations

HE: hepatic encephalopathy

BBB: blood brain barrier

TEM: transendothelial migration

EC: endothelial cells

JAMs: junctional adhesion molecules

MMP: Matrix metalloproteinase
MELD: Model for End-stage Liver Disease
GGT: gamma-glutamine transferase
AST: aspartate amino transferase
ALT: alanine amino transferase
PT: prothrombin time
CRP: C-reactive protein
PCT: procalcitonin
PS-100Beta: protein S-100 Beta.

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Abstract

Background: Hepatic encephalopathy (HE) is a frequent neurological complication of cirrhosis. Evidence suggests a synergic pathophysiological implication of hyperammonemia and systemic inflammation. In addition, the blood-brain barrier (BBB) permeability can be impaired in cirrhotic patients, notably in those displaying HE. We hypothesized that systemic inflammation could trigger leukocytes transendothelial migration (TEM) through the BBB in cirrhotic patients and especially those with HE.

Methods: We studied the effects of patients' plasma on the TEM of the leukocyte U937 cell line *in vitro,* using a validated BBB model (hCMEC/D3 cell line). We compared TEM of U937 leukocytes across hCMEC/D3 monolayer incubated with the plasma of i) patients with cirrhosis without HE, ii) patients with cirrhosis and HE, iii) healthy controls.

Results: We show that the plasma of cirrhotic patients with HE enhances TEM of U937 leukocytes across hCMEC/D3 BBB model. We found a correlation between U937 TEM on the one hand, the West-Haven score and ammonemia on the other one. A trend towards a correlation between U937 TEM and PS-100Beta in plasma, a marker of BBB solute's permeability increase, was also found.

Conclusion: These findings suggest that circulating factors could increase leukocytes TEM in cirrhotic patients and contribute to the increased BBB permeability that has been described in cirrhotic patients with HE.

Introduction

Hepatic encephalopathy (HE) is defined as the neurological or neuropsychological symptoms caused by liver failure, a chronic liver disease and/or a portosystemic shunt (1). HE could be present at least in one half of cirrhotic patients in its overt form and in more than two third of patients when minimal HE is included. HE constitutes an independent risk factor of death. HE impairs the quality of life and it is associated with substantial costs related to hospitalization (2). HE represents also a major burden for families/caregivers and health care systems (3). Hepatic encephalopathy's pathophysiology is still debated. The synergic effect of hyperammonemia and systemic inflammation is now well admitted (4, 5). Modifications of the gut microbiota have also been implicated and could explain both hyperammonemia and systemic inflammation (6). Recently, we showed that the blood-brain barrier (BBB) permeability to solutes and xenobiotics was increased in cirrhotic patients with HE (7).

The BBB is a unique organization of cerebral microvessels in which endothelial cells (EC) expressing tight junction proteins (claudin-5, occludin and junctional adhesion molecules (JAMs)) lay on a basal lamina made of type-IV collagen (8, 9). Pericytes embedded in the basal lamina could be largely implicated in the cerebral vasoregulation (10). Astrocytic endfeet cover the entire microvessels providing what is called the glial limitans. This special organization confers a particularly low permeability to solutes and circulating cells explaining that specific transport systems (receptors and transporters) are needed to fuel the brain with nutrients and allow exclusion of its waste products. EC are sensitive to inflammatory stimuli. They express Toll-Like receptor 4 (TLR-4) and receptors of cytokines including TNF-alpha and IL-1beta (CD120a and IL-1R respectively), that are elevated in the circulation of cirrhotic patients with HE (4). In patients with other neurological disorders such as multiple sclerosis (11), stroke (12), or Parkinson disease (13), systemic inflammation is associated with an increased transendothelial migration (TEM) of leukocytes through the BBB (8, 12, 13). Within the brain, leukocytes cause an increase in the amount of matrix metalloproteinase -2 and -9 (MMP-2 and MMP-9) that will alter cerebral microvessels' basal lamina and thus BBB permeability.

We hypothesized that leukocytes TEM through the BBB is possible in HE cirrhotic patients due to the systemic inflammation and that this could exacerbate altered BBB permeability. We took advantage of the unique human hCMEC/D3 cell line that resumes human BBB characteristics (14), to test this hypothesis *in vitro.*

Materiel and methods

Patients

This study was approved by local ethic's committee of La Pitié-Salpêtrière Hospital. All participants were recruited in the Intensive Care Unit of Hepatology in La Pitié-Salpêtrière University Hospital, Paris, France and they all gave written informed consent. Inclusion criteria were: 1) cirrhosis, defined by clinical biological and/or morphological parameters and 2) age between 18 and 70 years. Exclusion criteria were: 1) a past history of neurological disease; 2) current evolutive neurological disease; 3) hepatocellular carcinoma; 4) expected survival of less that 3 months; 5) refusal to participate. HE was evaluated with the West Haven score (15). A score ≥2 defined overt HE. The cohort of cirrhotic patients was divided into 2 groups, *i.e.*, with or without overt HE. A control group included subjects without any history of liver or neurological disease, who also gave their written informed consent.

Blood samples

Blood samples were collected on EDTA tube and placed on ice. Then, 2 centrifugations were performed (10 min, 800 *g*, 4°C) to obtain plasma that was immediately stored at -80°C. Biological analyses were performed using routine biochemical methods.

Human cerebral endothelial *cells*

We used the hCMEC/D3, a stable model of human EC and a largely validated *in vitro* BBB model (14). EC were cultured in Petri dish coated with collagen I (Rat Tail Collagen I, RDS, France), and were seeded each 3 days, as previously described (14, 16).

Monocytic cell line U937

We used the U937 monocytic cell line, that resumes leukocytes property to cross the BBB by TEM (17). For all experiences of TEM, *U937* monocytes were used and labeled with the tracer Deep-Red Dye (Molecular Probes, C34565, OR, USA). Monocytes were labeled on the day of TEM experience.

Transendothelial leukocytes migration experiments

hCMEC/D3 cells were cultured as a monolayer on 8µm porous Transwell inserts (Corning) previously coated with collagen IV. At Day 6, *i.e.*, when hCMEC/D3 are confluent, monocytes (U937 cell-line) were added to the upper chamber in SVF-free RPMI 1640 medium (Molecular Probes) containing 1% of plasma from patients or healthy controls (Fig 1A) and were incubated at 37° for 16h. In each experiment, 25 ng/mL of the CCL-2 chemokine was added to the lower chamber to create a chemotactic gradient. The confluence of hCMEC/D3 cells was controlled by the immunofluorescence of PECAM-1 (CD31) (Fig 1B). Patient's plasma samples were diluted (endoGRO 1% with U937 labeled): 10^6 monocytes in 100 µL of plasma were placed in the upper compartment. After 16 hours, TEM was stopped and the upper compartment was analyzed using flow cytometry.

Cell counting by flow cytometry

All U937 cells were marked before experiments with a fluorescent cell tracker (Deep-Red Dye, Molecular Probes). The content of the lower chamber was harvested and cell counting was directly performed by flow cytometry using counting beads (Gallios, Beckman Coulter). Counting beads (CountBright, Molecular Probes) were added to each sample immediately before assay. The concentration of beads was precisely known and enabled us to estimate the absolute number of U937 cells that had migrated. The beads were easily separated from cells during flow cytometry assays on the basis of their physical properties (FSC/SSC, Fig 2A) and their high fluorescence in each channel (Fig 2B). Doublets were eliminated through an FSC-A/FSC-H assay (Fig 2B and 2E). The cell-tracker on U937 cells was only fluorescent in the FL-6 channel (Fig 2C). The efficacy of the cell tracker was also checked, comparing the cytometric profile of dyed U937 (red histogram) with undyed U937 (black histogram) and undyed hCMEC/D3 cells (blue histogram) (Fig 2D). To confirm beads' identity, an analysis was performed in the FL-1 channel (Fig 2F). The accuracy of the counting beads was checked comparing a wide range of expected concentrations of cells that were obtained by serial dilutions and the calculated ones, showing excellent correlations (Fig 2G).

Statistical analyses

Results were expressed in median and interquartile range for quantitative variables and in absolute value and percentage for each group. Qualitative variables were compared using Fisher exact test and quantitative variables with non-parametric tests Wilcoxon and Kruskal-Wallis. All analyses were performed with JMP 9.0 software (CA, United States).

Results

Patients' and healthy controls' characteristics

Twenty-eight cirrhotic patients and 7 healthy controls were included. Twelve patients had no HE and 16 displayed HE. Baseline characteristics of patients and controls are depicted in Table 1.

Transendothelial migration of U937 through the BBB in presence of sera of patients and controls

TEM of U937 cells was significantly different between the 3 groups (Figure 3A). TEM was higher in the presence of plasma of cirrhotic patients with HE compared to cirrhotic patients without HE ($p=0.0448$) and to healthy controls ($p=0.0334$) (Figure 3A). Despite the absence of statistical significance, a trend towards an increased TEM in cirrhotic patients without HE compared to healthy controls was observed. A correlation exists between the importance of TEM and West-Haven scores ($p=0.0015$) and ammonemia ($p=0.0231$) (Figure 3B&C). A similar trend was observed for PS100-Beta, a marker of BBB solute's permeability increase, but was not significant ($p=0.0981$) (Figure 3D).

Discussion

In this study, we show that the plasma of cirrhotic patients with HE enhances TEM of U937 monocytes across hCMEC/D3 BBB model compared to cirrhotic patients without HE. U937 TEM is correlated with the West-Haven score and with ammonemia and might also be correlated with PS-100Beta. This result suggests a new mechanism in the pathophysiology of HE in cirrhosis and could imply, if confirmed, new therapeutic strategies since specific blockers of TEM are already available.

Systemic inflammation in cirrhosis and elevated pro inflammatory cytokines (TNF-alpha, IL-6) were found to be necessary in patients with cirrhosis to develop HE in the presence of hyperammonemia (4). Systemic inflammation may facilitate recruitment and migration of leukocytes through endothelia in these patients. Indeed, it has been demonstrated that leukocytes and EC presented an activated state in cirrhosis (18). Pro-inflammatory cytokines such as TNF-alpha and IL-6 have an impact on BBB permeability in patients without cirrhosis (19). Moreover, their concentration correlate with the severity of HE and may promote TEM of leukocytes.

Our results reinforce the possible role of systemic inflammation to precipitate HE in cirrhosis. Several hypotheses have been raised to explain this association including an increase in benzodiazepine-like substances induced by IL-1beta and TNF-alpha (20), and a decrease in cerebral brain blood flow in response of proinflammatory cytokines (21). Our work suggests another mechanism relying of the increase in TEM. This theory is of interest, since several therapeutic strategies aiming to decrease TEM of leukocytes have been developed in different neurological diseases such as multiple sclerosis (natalizumab).

Our study included a limited number of subjects, as 28 patients were included. Moreover, HE may vary over time. Some patients may have been evaluated during improvement of HE. Finally, our results suggest, despite a non-significant difference with healthy controls, that there was an increase in BBB permeability in patients with cirrhosis, even in the absence of HE (22). One can thus infer that modifications of BBB may precede overt HE.

In conclusion, we suggest an increase in leukocytes BBB permeability in cirrhotic patients with HE. Although we used a validated model of human BBB, further studies are needed in order to evaluate TEM of leukocytes *in vivo* and validate our assumption in animal models using drugs blocking TEM like natalizumab, or even by analyzing the brain of deceased patients with cirrhosis, with or without HE.

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Table 1: Baseline characteristics of the cirrhotic patients and controls

	Healthy controls n=7	No HE cirrhotic patients n=12	HE cirrhotic patients n=16
Age (years)	29 [27-42]	55 [52-60] ###	58 [52-64] **$$**
Male gender (%)	22%	68%	58%
Etiology of cirrhosis			
Viral	NA	31%	33%
Alcoholic	NA	25%	56%
Other	NA	44%	11%
Child-Pugh score			
A	NA	25%	0%
B	NA	36%	18%
C	NA	39%	82%*
MELD Score	NA	15 [13-20]	19 [16-32]*
West-Haven Score	NA	0 [0-1]	2 [2-2]***
Ascites	0%	75%	50%
Gastrointestinal bleeding	0%	50%	50%
Previous episode of HE	0%	58%	75%
Asterixis	0%	0%	73%***
Bacteriemia	0%	0%	25%
Sodium (mmol/L)	140 [139-140]	138 [131-142]	136 [135-140 $
Bilirubin (µmol/L)	6 [5-9]	32 [19-98] ###	104 [22-476] $$$
GGT (UI/ml)	20 [14-23]	45 [30-203] ##	45 [31-102] $$
AST (UI/ml)	25 [23-28]	42 [34-54)##	63 [39-72] $$$*
ALT (UI/ml)	14 [12-23]	26 [20-40]	32 [17-45] $
Ammoniemia (µmol/L)	34 [30-37]	66 [42-84] ##	82 [58-88] $$*
PT (%)	97 [90-102]	54 [40-65] ###	42 [24-58] $$$
Albumin (g/L)	44 [42-47]	32 [27-33] ###	30 [25-31] $$$
CRP (mg/L)	5 [3-14]	14 [3-28]	11 [3-58] $
PCT (ng/ml)	0.06 [0.04-0.09]	0.23 [0.08-0.5]	0.49 [0.23-1.97] $
PS-100Beta (µg/ml)	0.06 [0.05-0.10]	0.08 [0.05-0.1]	0.1 [0.08-0.24]*
Creatinine (µmol/L)	73 [66-77]	64 [51-125]	84 [63-97)
Hemoglobin (g/dL)	12.9 [12.3-13.7]	9.75 [8.7-11.5] ##	10 [8.3-10.8] $$
Leukocytes ($10^3/mm^3$)	5.8 [5.4-6.3]	4.37 [3.92-8.39]	7.59 [4.69-10.15)*]
Neutrophiles ($10^3/mm^3$)	3.2 [2.8-4.2]	2.98 [2.15-7.27]	4.83 [3.44-8.68]*

Abbreviations: MELD, Model for End-stage Liver Disease, HE, hepatic encephalopathy, GGT, gamma-glutamine transferase, AST, aspartate amino transferase; ALT, alanine amino transferase;; NA, not

applicable; PT, prothrombin time; CRP, C reactive protein;; PCT, procalcitonin; PS-100Beta, protein S-100 Beta.

Significant differences between groups are marked as following: between healthy controls and HE patients: **$**; between healthy controls and cirrhotic patients without HE: **#**; between HE patients and cirrhotic without HE: ***;** p values are marked as following: $, #, *: $p < 0.05$; $$, ##, **: $p < 0.01$; $$$, ###, ***: $p < 0.001$.

FIGURE LEGENDS

Figure 1: Leukocytes transendothelial migration experiment across the BBB

A, Outline of TME experiment on Transwell inserts; B, anti-PECAM-1 (CD31) immunostaining of hCMEC/D3 cells.

Figure 2: Cell counting strategy by flow cytometry

Counting beads (CountBright, Molecular Probes) were added to each sample immediately before assay. The concentration of these beads was precisely known and enabled us to estimate the absolute number of U937 cells that had migrated. These beads were easily separated from cells during flow cytometry assays on the basis of their physical properties (FSC/SSC, **Fig 2A**) and their high fluorescence in each channel **(Fig 2B).** Doublets were eliminated through a FSC-A/FSC-H assay (**Fig 2B and 2E**). The cell-tracker on U937 cells was only fluorescent in the FL-6 channel **(Fig 2C**). The efficiency of the cell tracker was also checked, comparing the cytometric profile of dyed U937 (red histogram) with undyed U937 (black histogram) and undyed hCMEC/D3 cells (blue histogram) **(Fig 2D).** To confirm beads' identity, an analysis was performed in the FL-1 channel **(Fig 2F)**. The accuracy of the counting beads was checked comparing a wide range of expected concentrations of cells that were obtained by serial dilutions and the calculated ones, showing excellent correlations (**Fig 2G**).

Figure 3: U937 leukocytes transendothelial migration across the BBB in presence of plasma of cirrhotic patients and healthy controls.

A, U937 TEM across the hCMEC/D3 BBB model. Correlations between U937 TEM and B, West-Haven score; C, ammonia concentration in plasma; D, PS-100Beta concentration in plasma.

FIGURE 1

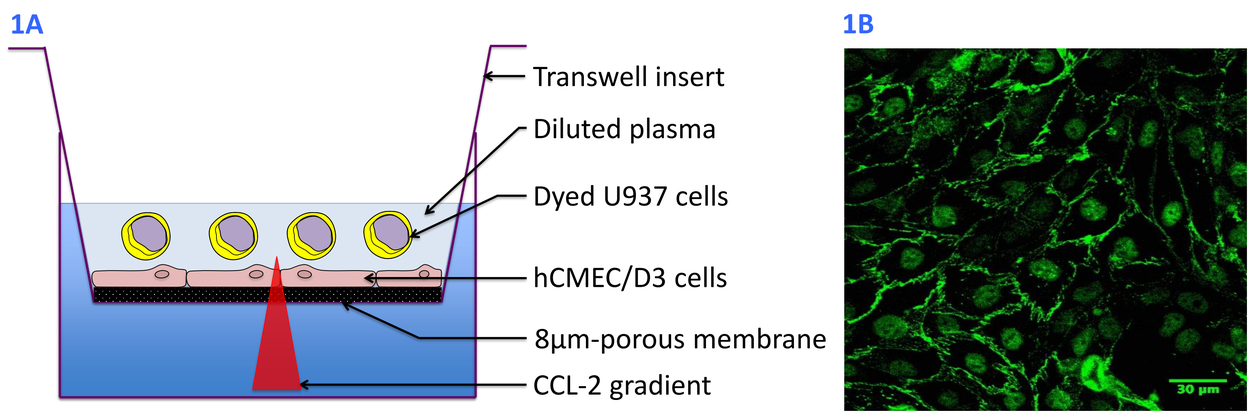

1A
Transwell insert
Diluted plasma
Dyed U937 cells
hCMEC/D3 cells
8µm-porous membrane
CCL-2 gradient
1B
30 µm

FIGURE 2

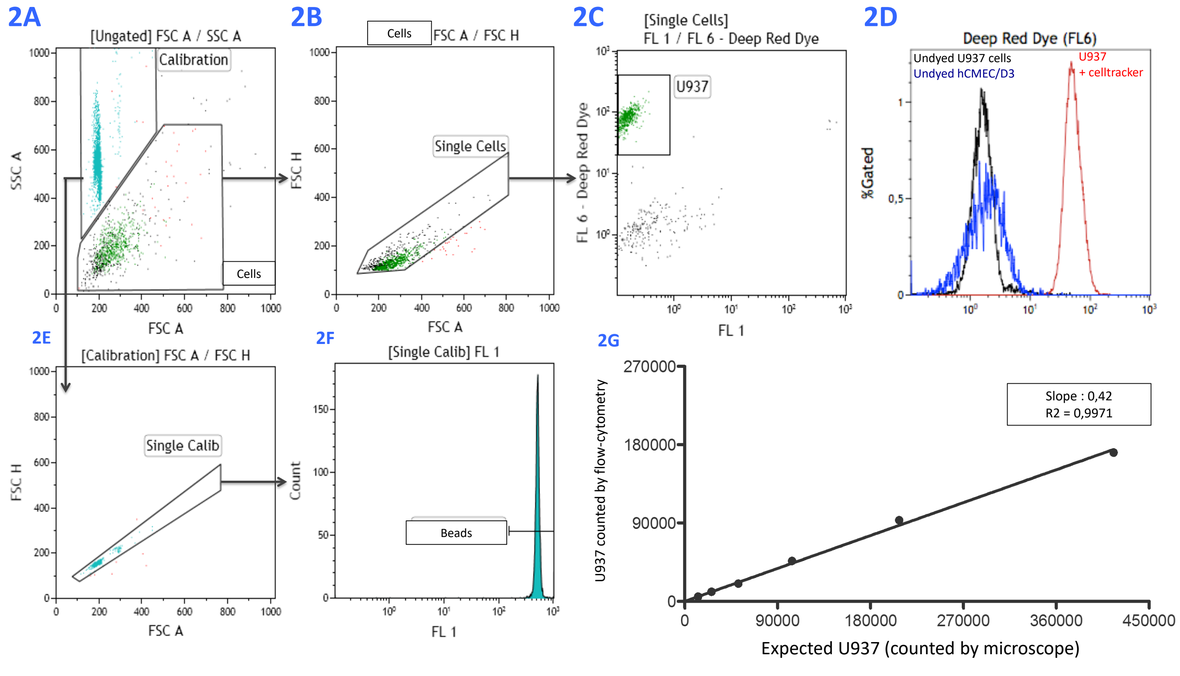
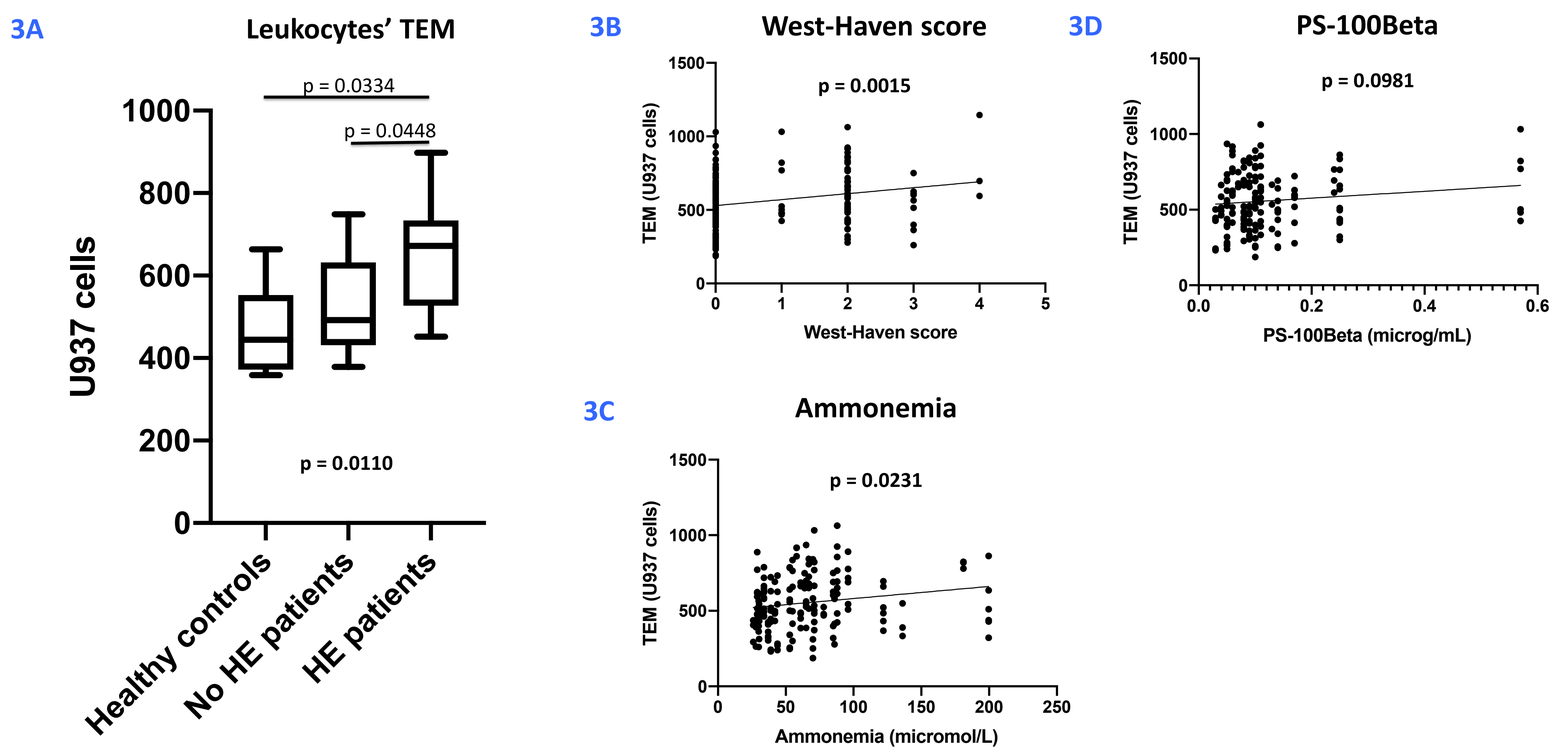

2A
[Ungated] FSC A / SSC A
Calibration
Cells
SSC A
FSC A
2B
Cells
FSC A / FSC H
Single Cells
FSC H
FSC A
2C
[Single Cells]
FL 1 / FL 6 - Deep Red Dye
U937
FL 6 - Deep Red Dye
FL 1
2D
Deep Red Dye (FL6)
Undyed U937 cells
Undyed hCMEC/D3
U937
+ celltracker
%Gated
2E
[Calibration] FSC A / FSC H
Single Calib
FSC H
FSC A
2F
[Single Calib] FL 1
Beads
Count
FL 1
2G
Slope : 0,42
R2 = 0,9971
U937 counted by flow-cytometry
Expected U937 (counted by microscope)

FIGURE 3


3A
Leukocytes' TEM
p = 0.0334
p = 0.0448
p = 0.0110
U937 cells
1000
800
600
400
200
0
Healthy controls
No HE patients
HE patients
3B
West-Haven score
p = 0.0015
TEM (U937 cells)
West-Haven score
3C
Ammonemia
p = 0.0231
TEM (U937 cells)
Ammonemia (micromol/L)
3D
PS-100Beta
p = 0.0981
TEM (U937 cells)
PS-100Beta (microg/mL)