SUPPLEMENTARY MATERIAL

Plasma extracellular vesicles play a role in immune system modulation in minimal hepatic encephalopathy

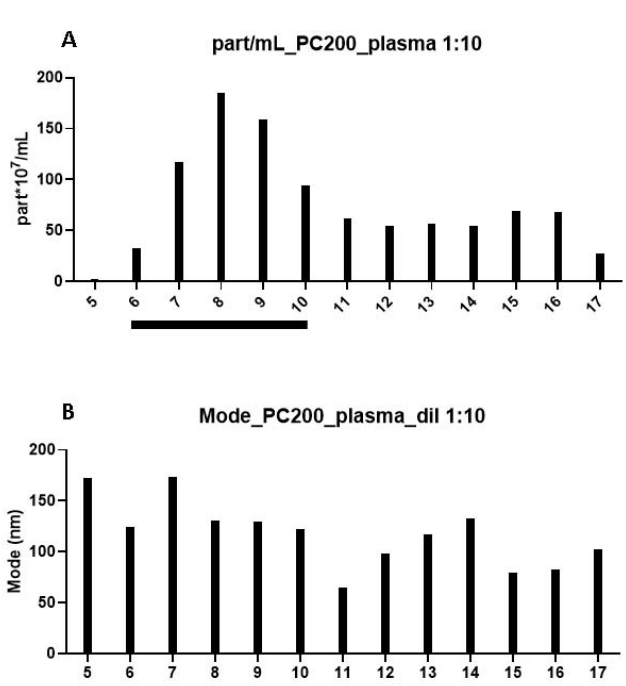
Juan José Gallego, Alessandra Fiorillo, Franc Casanova-Ferrer, Amparo Urios, Mª Pilar Ballester, Lucia Durbán, Javier Megías, Teresa Rubio, Andrea Cabrera-Pastor, Desamparados Escudero-García, Vicente Felipo, Carmina Montoliu*

Contents:

Supplementary methods

RNA extraction from cell culture pellet

The RNA of cell culture pellet was extracted using TRIzol RNA Isolation Reagent (Sigma Aldrich). Cells were homogenized with 300 µL of TRIzol RNA Isolation Reagent, mixture with chloroform and centrifuged 15 minutes at 13.000 g at 4ºC. Aqueous phase containing RNA was transferred to new tube and mixture with isopropyl alcohol to precipitate RNA, then centrifuged 15 minutes at 13.000 g at 4ºC and was washed with cold 75% ethanol. Finaly, RNA was resuspended in RNase-free water. Concentration and quality of RNA was checked using NanoDrop™ 2000 (Thermo Fisher).



C

PC200-PLASMA	Protein UG/UL
F1	0,000
F2	-0,002
F3	-0,002
F4	0,000
F5	-0,002
F6	-0,001
F7	0,009
F8	0,020
F9	0,026
F10	0,044
F11	0,088
F12	0,160
F13	0,329
F14	1,089
F15	1,673
F16	2,269
F17	2,594
F18	2,922

D

Fraction	Particles/mL(*10^7)	Protein (mg/mL)	Ratio Particles/Protein (*10^{10})
6	40	-0,001	
7	120	0,009	13,3
8	175	0,02	8,7
9	160	0,026	6,15
10	100	0,044	2,2
11	60	0,088	0,6
12	50	0,16	0,3
13	55	0,329	0,16

Figure S1. Nanosight analysis and protein quantification of F5 to F17 fractions eluted from plasma sample. A) Fractions F6-F10 are enriched in EVs. **B)** Size (nm) of eluted fractions. F6 to F10 fractions contained EVs with 100-150 nm of size. **C)** Protein quantification in fractions. **D)** Particles/protein ratio in fractions F7-F13

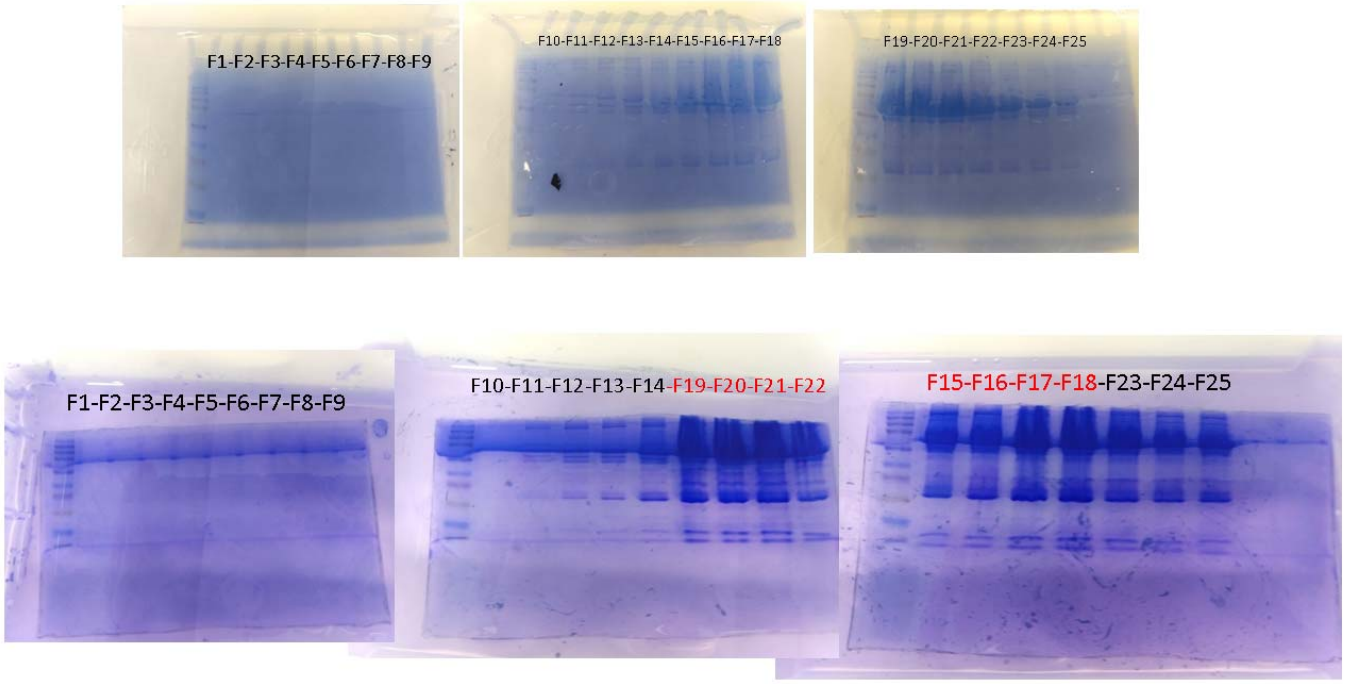


Figure S2. Protein detection by Coomassie blue staining in fractions eluted (F1-F25 fractions) from two plasma samples.

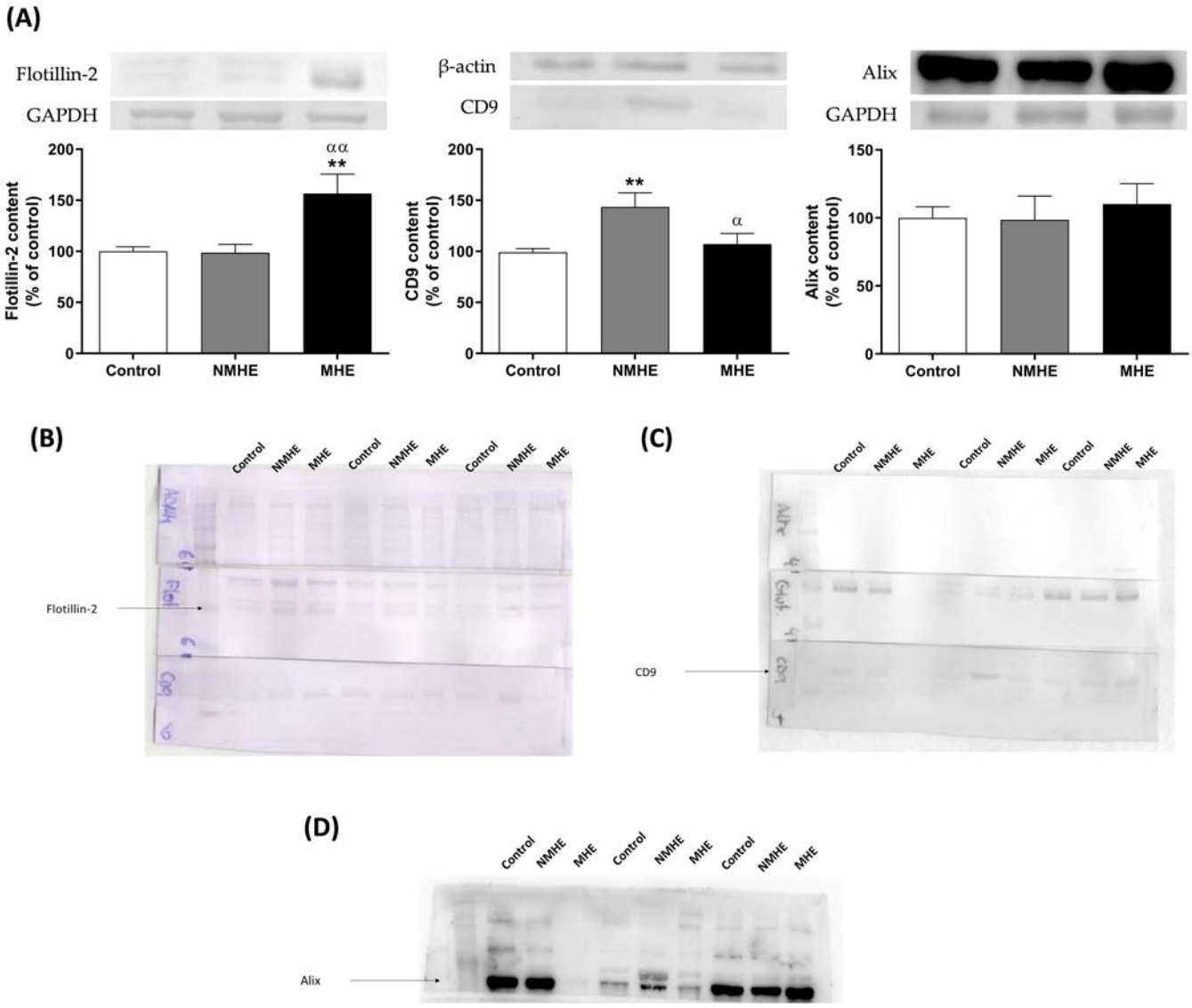


Figure S3. Representative bands, relative cargo and whole blot of extracellular vesicles markers. **(A)** Content of extracellular vesicle markers. (**B-D**) Whole blots of **(B)** flotillin-2, **(C)** CD9 and **(D)** Alix. Depending of molecular weight, β-actin or GAPDH were used as loading control. Values are expressed as percentage of control and are the mean ± SEM. Data were analyzed by univariate analysis of covariance (ANCOVA) with age included as a covariate, followed by Tukey post hoc test. NMHE, MHE, patients without and with minimal hepatic encephalopathy, respectively. Values significantly different from control are indicated by asterisk (*) and from NMHE patients by α. (α: $p < 0.05$; **/αα: $p < 0.01$).

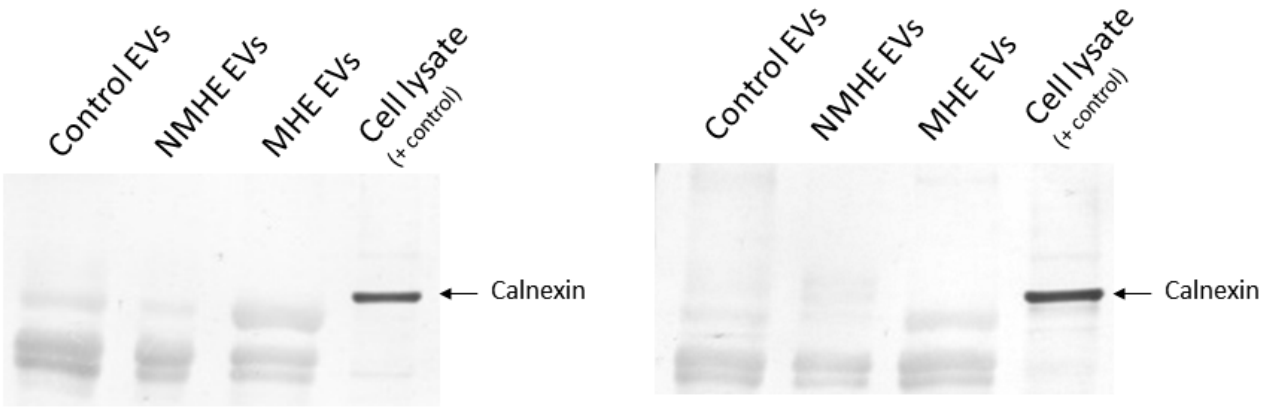
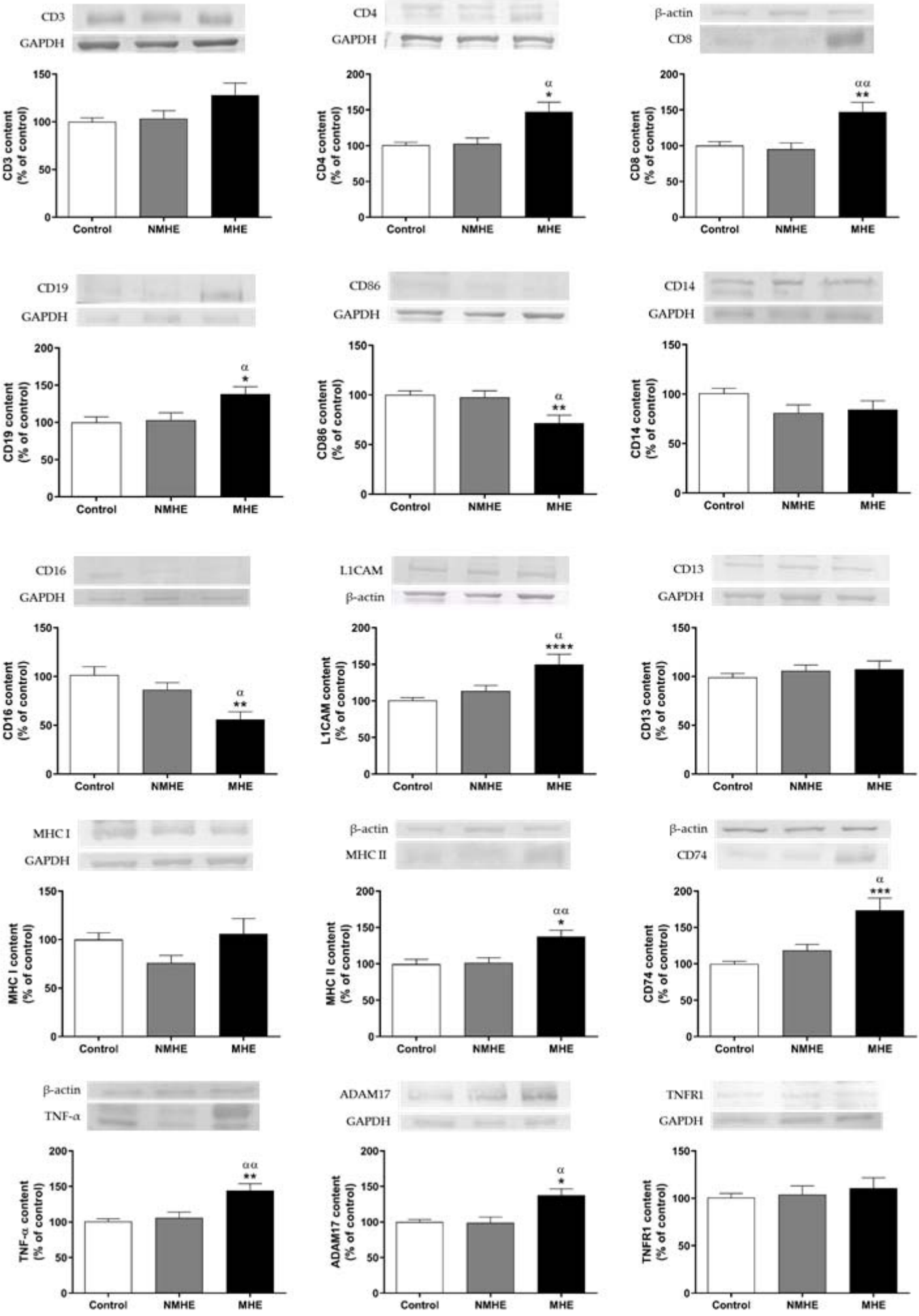


Figure S4. Calnexin representative SDS-PAGE western blots. Calnexin, marker of endoplasmic reticulum, was absent in EVs samples. Positive control used for calnexin was cell lysate of $CD4^+$ T cells.


CD3
GAPDH
CD3 content (% of control)
Control
NMHE
MHE
CD4
GAPDH
CD4 content (% of control)
α
*
β-actin
CD8
CD8 content (% of control)
αα
**
CD19
GAPDH
CD19 content (% of control)
CD86
GAPDH
CD86 content (% of control)
CD14
GAPDH
CD14 content (% of control)
CD16
GAPDH
CD16 content (% of control)
L1CAM
β-actin
L1CAM content (% of control)
****
CD13
GAPDH
CD13 content (% of control)
MHC I
GAPDH
MHC I content (% of control)
β-actin
MHC II
MHC II content (% of control)
αα
CD74
CD74 content (% of control)
***
TNF-α
TNF-α content (% of control)
ADAM17
GAPDH
ADAM17 content (% of control)
TNFR1
GAPDH
TNFR1 content (% of control)

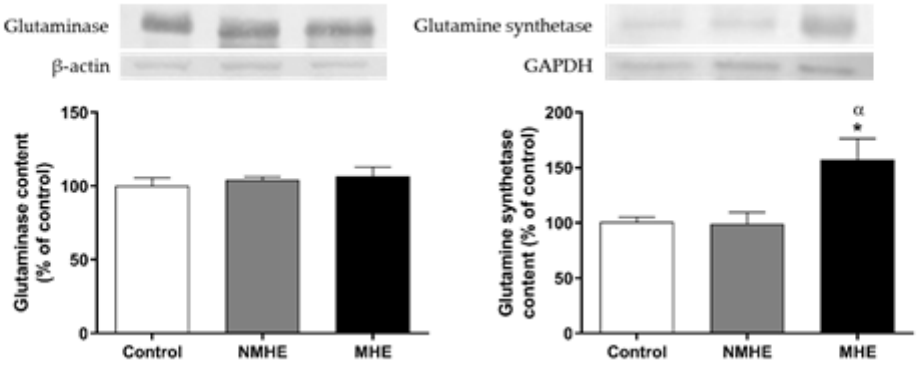


Figure S5. Representative bands and relative extracellular vesicles protein cargo. Content of markers related to immunity cells CD3, CD4, CD8, CD19, CD86, CD14 and CD16; markers related to CNS cells L1CAM and CD13; molecules implicated in activation of immune system and presenting antigens to T cells MHC I, MHC II and CD74; molecules related to inflammation environment TGF-β, TNF-α, ADAM17 and TNFR1; and enzymes related to ammonia metabolism, glutaminase and glutamine synthetase. Depending of molecular weight, β-actin or GAPDH were used as loading control. Values are expressed as percentage of control and are the mean ± SEM. Data were analyzed by univariate analysis of covariance (ANCOVA) with age included as a covariate, followed by Tukey post hoc test. NMHE, MHE, patients without and with minimal hepatic encephalopathy, respectively. Values significantly different from control are indicated by asterisk (*) and from NMHE patients by α. (*/α: $p< 0.05$; **/αα: $p< 0.01$; ***: $p< 0.001$; ****: $p< 0.0001$).

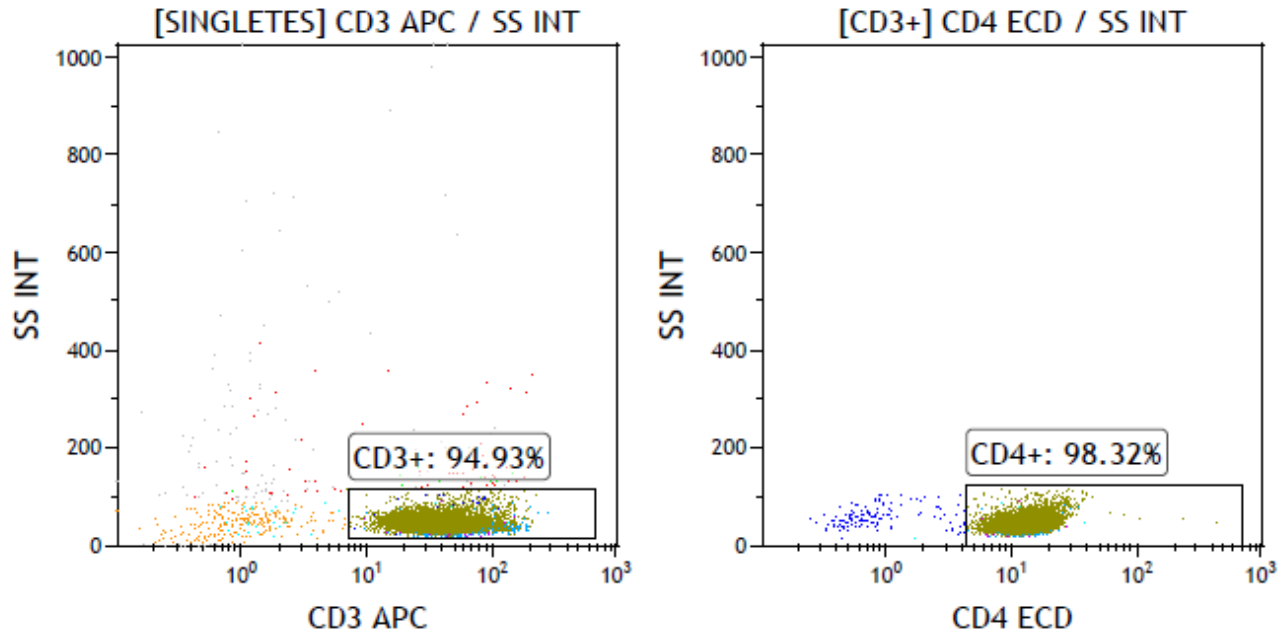


Figure S6. Purity of the CD4$^+$ T cell sample isolated using the EasySep™ Human CD4$^+$ T Cell Isolation Kit. After thawing the PBMC, the analysis of specific markers for CD4$^+$ T cells was performed to determine the purity of the CD4$^+$ sample following the protocol as in the reference [5]. The markers used were CD3 (total T cells) and CD4 (CD4$^+$ T cells). CD4$^+$ T cells were identified as positive for both markers.

Table S1. Primary antibody and dilution used in analysis of EVs protein cargo by immunoblotting.

Protein	Primary antibody		
	Species	Dilution	Company
CD9	Rabbit (rb)	1:500	Sigma-Aldrich
Alix[a]	Rabbit (rb)	1:1000	Proteintech
Flotillin-2	Rabbit (rb)	1:500	Invitrogen
TNF-α	Rabbit (rb)	1:500	Abcam
TNFR1	Rabbit (rb)	1:1000	Abcam
ADAM17	Rabbit (rb)	1:250	Abcam
Glutamine synthetase	Mouse (ms)	1:5000	Thermo Fisher
Glutaminase	Rabbit (rb)	1:1000	Novus Biological
MHC I	Rabbit (rb)	1:1000	Invitrogen
MHC II	Rabbit (rb)	1:1000	Invitrogen
CD74	Mouse (ms)	1:1000	Abcam
L1CAM	Rabbit (rb)	1:1000	Bioss
CD13	Rabbit (rb)	1:500	Bioss
CD3	Rat (rat)	1:500	Abcam
CD4	Rabbit (rb)	1:1000	Novus Biological
CD8	Rabbit (rb)	1:2000	Thermo Fisher
CD19	Rabbit (rb)	1:1000	Abcam
CD14	Rabbit (rb)	1:1000	Abcam
CD16	Rabbit (rb)	1:100	Santa Cruz
CD86	Mouse (ms)	1:200	Abcam
Calnexin	Rabbit (rb)	1:1000	Novus Biological
β-actin	Mouse (ms)	1:5000	Abcam
GAPDH	Mouse (ms)	1:15000	Sigma-Aldrich

Secondary antibodies used were Anti-rabbit, anti-mouse or anti-rat IgG conjugated with alkaline phosphatase (Sigma-Aldrich) at a dilution of 1:4000.

[a]For Alix protein, secondary antibody used was anti-rabbit IgG conjugated with horseradish peroxidase (Sigma-Aldrich) at a dilution of 1:4000.

Table S2 (I). Potential biological function of proteins differentially expressed in EVs from MHE assessed using (GO) platform

GO biological process complete
regulation of opsonization
biological regulation
regulation of immune effector process
regulation of immune system process
response to symbiotic bacterium
response to symbiont
response to other organism
biological process involved in interspecies interaction between organisms
response to external biotic stimulus
response to biotic stimulus
response to stimulus
negative regulation of complement activation, classical pathway
negative regulation of humoral immune response mediated by circulating immunoglobulin
regulation of humoral immune response mediated by circulating immunoglobulin
regulation of immune response
regulation of response to stimulus
regulation of humoral immune response
negative regulation of humoral immune response
negative regulation of immunoglobulin mediated immune response
negative regulation of B cell mediated immunity
regulation of complement activation, classical pathway
regulation of complement activation
negative regulation of complement activation
very-low-density lipoprotein particle assembly
fibrinolysis
negative regulation of blood coagulation
negative regulation of coagulation
regulation of coagulation
negative regulation of multicellular organismal process
regulation of blood coagulation
regulation of wound healing
regulation of response to wounding
regulation of hemostasis
regulation of body fluid levels
regulation of biological quality
negative regulation of hemostasis
negative regulation of wound healing
negative regulation of response to wounding
negative regulation of viral entry into host cell
negative regulation of viral life cycle
acute inflammatory response
defense response
response to stress

Table S2 (II)

GO biological process complete
complement activation, classical pathway
humoral immune response mediated by circulating immunoglobulin
humoral immune response
immune response
immune system process
immunoglobulin mediated immune response
B cell mediated immunity
adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains
adaptive immune response
lymphocyte mediated immunity
leukocyte mediated immunity
immune effector process
complement activation
activation of immune response
positive regulation of immune response
positive regulation of response to stimulus
positive regulation of immune system process
renal system process
platelet degranulation
phagocytosis, engulfment
plasma membrane invagination
membrane invagination
negative regulation of endopeptidase activity
regulation of endopeptidase activity
regulation of peptidase activity
regulation of hydrolase activity
regulation of catalytic activity
regulation of proteolysis
regulation of protein metabolic process
negative regulation of peptidase activity
negative regulation of proteolysis
negative regulation of protein metabolic process
negative regulation of hydrolase activity
negative regulation of catalytic activity
negative regulation of molecular function
blood coagulation
coagulation
hemostasis
wound healing
response to wounding
innate immune response
defense response to other organism

Table S3. Correlations between protein content of EVs, PHES score and activation of lymphocytes subpopulations

Protein in EVs		**PHES score[a]**	**Activated $CD3^+$ lymphocytes**	**Activated $CD8^+$ lymphocytes**	**Activated memory $CD4^+$ lymphocytes**	**Activated $CD8^+$ $CD28^-$ lymphocytes**
TNF-α	r	-0.419	0.470	0.447	0.470	0.407
	p	<0.01	0.015	0.02	0.015	0.04
TGF-β	r	0.297		-0.419	-0.462	-0.479
	p	<0.05		0.047	0.03	0.02
MHCII	r	-0.280				
	p	<0.05				

Correlation coeficient (r) and significance (p) for Pearson correlation analysis are shown. [a] For PHES score Spearman correlation analysis was used. Activation of lymphocytes subpopulations was measured as $CD69^+$ cells. EVs, extracelular vesicles; PHES, Psychometric Hepatic Encephalopathy Score.