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ORIGINAL ARTICLE

Role of EpCAM+ CD133+ extracellular vesicles in steatosis to steatohepatitis transition in NAFLD

Rocío Muñoz-Hernández[1,2,3] | Sheila Gato[1,2] | Antonio Gil-Gómez[1,2] | Rocío Aller[4,5] | Angela Rojas[1,2] | Laura Morán[6,7] | Javier Gallego[1] | Elena Blázquez-López[2,6,8] | Rocío Gallego-Durán[1,2] | Rocío Montero-Vallejo[1,2] | Vanessa García-Fernández[1,3] | Douglas Maya-Miles[1,2] | María del C Rico[1,2,9] | Francisco J Cubero[2,6,7] | Javier Vaquero[2,6,8] | Javier Ampuero[1,2,3,9] | Rafael Bañares[2,6,8] | Manuel Romero-Gómez[1,2,3,9]

[1]SeLiver Group, Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Sevilla, Spain

[2]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Madrid, Spain

[3]Departamento de Medicina, Facultad de Medicina, Universidad de Sevilla, Seville, Spain

[4]Department of Medicine, Dermatology and Toxicology, Universidad de Valladolid / Gastroenterology Unit, Hospital Clínico Universitario de Valladolid / BioCritic, Group for Biomedical Research in Critical Care Medicine, Valladolid, Spain

[5]Centro de Investigación Biomédica en Red de Enfermedades Infecciosas (CIBERINFEC), Instituto de Salud Carlos III, Madrid, Spain

[6]Instituto de Investigación Sanitaria Gregorio Marañón (IiSGM), Madrid, Spain

[7]Department of Immunology, Ophthalmology and ENT, Complutense University School of Medicine, Madrid, Spain

[8]Servicio de Aparato Digestivo, Hospital General Universitario Gregorio Marañón, Madrid, Spain

[9]UCM Digestive diseases, Hospital Universitario Virgen del Rocío, Sevilla, Spain

Correspondence
Rocío Muñoz-Hernández and Manuel Romero-Gómez, SeLiver Group, Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Sevilla 41013, Spain.
Email: rociomunoz@us.es and mromerogomez@us.es

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Abstract

Background and Aims: Extracellular vesicles (EVs) have emerged as a potential source of circulating biomarkers in liver disease. We evaluated circulating AV+ EpCAM+ CD133+ EVs as a potential biomarker of the transition from simple steatosis to steatohepatitis.

Methods: EpCAM and CD133 liver proteins and EpCAM+ CD133+ EVs levels were analysed in 31 C57BL/6J mice fed with a chow or high fat, high cholesterol and carbohydrates diet (HFHCC) for 52 weeks. The hepatic origin of MVs was addressed using AlbCrexmT/mG mice fed a Western (WD) or Dual diet for 23 weeks. Besides, we assessed plasma MVs in 130 biopsy-proven NAFLD patients.

Results: Hepatic expression of EpCAM and CD133 and EpCAM+ CD133+ EVs increased during disease progression in HFHCC mice. GFP+ MVs were higher in AlbCrexmT/mG mice fed a WD (5.2% vs 12.1%) or a Dual diet (0.5% vs 7.3%). Most GFP+ MVs were also positive for EpCAM and CD133 (98.3% and 92.9% respectively), suggesting their hepatic

Abbreviations: ALT, alanine aminotransferase; ASGPR1, asialoglycoprotein receptor 1; AST, aspartate aminotransferase; BMI, body mass index; CAP, controlled attenuated parameters; CD133, prominin-1; DM, diabetes mellitus; EpCAM, epithelial cell adhesion molecule; EVs, extracellular vesicles; GGT, gamma glutamyl transferase; HDL, high-density lipoprotein; HFHCC, high fat, high carbohydrate and cholesterol; HTA, hypertension; LDL, low-density lipoprotein; MVs, microvesicles; NAFLD, non-alcoholic fatty liver disease; NASH, steatohepatitis; ROC, receiver operating characteristic curve; TC, total cholesterol.

Rocío Muñoz-Hernández and Manuel Romero-Gómez contributed equally as senior authors.

origin. In 71 biopsy-proven NAFLD patients, EpCAM+ CD133+ EVs were significantly higher in those with steatohepatitis compare to those with simple steatosis (286.4±61.9 vs 758.4±82.3; $p<0.001$). Patients with ballooning 367±40.6 vs 532.0±45.1; $p=0.01$ and lobular inflammation (321.1±74.1 vs 721.4±80.1; $p=0.001$), showed higher levels of these EVs. These findings were replicated in an independent cohort.

Conclusions: Circulating levels of EpCAM+ CD133+ MVs in clinical and experimental NAFLD were increased in the presence of steatohepatitis, showing high potential as a non-invasive biomarker for the evaluation and management of these patients.

KEYWORDS
CD133, EpCAM, extracellular vesicles, NAFLD

1 | INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, affecting about a quarter of the world's adult population.[1] The spectrum of liver damage in NAFLD ranges from simple steatosis (SS), an asymptomatic state to more severe forms of the disease, characterized by the appearance of steatohepatitis (NASH) and liver fibrosis. Liver biopsy remains the gold standard diagnostic tool for patients with NAFLD despite its significant disadvantages (low acceptance, invasiveness and heterogeneity). Indeed, the diagnosis of NASH requires a histological examination to confirm the presence of lobular inflammation and hepatocyte ballooning degeneration, with or without fibrosis.[2] Thus, developing novel non-invasive biomarkers of NASH is strongly needed.

The role of extracellular vesicles (EVs) in cellular communication within tissues and organs has gained significant attention during the last few years, particularly in acute and chronic diseases.[3,4] EVs are small membrane vesicles released by different cell types in resting or under activating conditions, into several biological fluids such as blood, urine, or saliva. EVs contain proteins, lipids and coding/non-coding RNAs of the cell of origin.[5] Microvesicles (MVs) are membrane-derived EVs in the size range of 100–1000 nm that are generated by blebbing from the plasma membrane of nearly all cells, also called large EVs.[6,7] Large EVs express cell surface markers from their parent cell, which enable the characterization of their cellular origin through flow cytometry approaches.[8,9] Previous studies have shown that parenchymal and non-parenchymal liver cells release EVs in biological fluids indicating liver disease progression.[10,11] In this context, several studies demonstrated that changes in hepatocyte-derived EVs under lipotoxic conditions promote inflammation and fibrosis in the liver.[12] A twofold increase of circulating ASGPR1+ EVs in cirrhotic patients compared to healthy controls has been previously reported,[13] and Haertel et al. showed that the combination of Annexin V, ASGPR1, EpCAM and CD133 MVs could help to identify patients with liver tumours.[14]

Hepatocyte-driven liver regeneration involves the proliferation of pre-existing hepatocytes which is the primary generation mode,[15] when the regenerative capacity of hepatocytes and/or cholangiocytes is exhausted hepatic progenitor cells (HPCs) are thought to play an important role in this process.[16] HPC may exist in or near the canal of Hering,[17] and current studies have demonstrated HPCs activation and proliferation in patients with severe and chronic liver disease such as non-alcoholic fatty liver disease[18,19] and have been identified and isolated from liver tissue from all age donors.[20,21] Previous studies have shown that these cell populations are positive for endodermal progenitor markers such as epithelial cell adhesion molecule (EpCAM) and Prominin-1 (CD133),[22–24] and they can express high levels of albumin.[25–27] Despite EpCAM+ CD133+ cells are associated with tumours, in an early stage of the disease, where the liver is submitted to chronic injury, EpCAM+ CD133+ cells are activated and are thought to play a role in liver regeneration without giving rise to hepatocellular carcinoma[28] and could also be releasing specific EVs into circulation. Given the asymptomatic nature of the early stages of NAFLD, the development of non-invasive biomarkers capable of detecting the transition from steatosis to NASH is strongly needed. This study aimed to evaluate the potential of EpCAM+ CD133+ EVs as a novel biomarker to detect the transition to more severe forms of the disease.

Key points

Hepatic expression of EpCAM and CD133 increases with NAFLD progression and EpCAM+ CD133+ extracellular vesicles are produced by the liver after chronic liver injury. Our study showed that AV+ EpCAM+ CD133+ EVs represent a useful biomarker for the difficult task of separating patients with steatohepatitis from those with simple steatosis.

2 | METHODS

2.1 | Animal procedures and ethics statements

2.1.1 | High fat, high carbohydrate and cholesterol animal model

Thirty-one C57BL/6J mice were purchased from Charles River Laboratories (Cedex, France) at 5 weeks of age. Mice were raised in

a temperature-controlled room of 23°C with constant humidity and maintained on a 12/12-h light/dark cycle with ad libitum access to diet and water at the Institute of Biomedicine of Seville (IBiS). After 1 week of acclimatization, the mice were assigned to the following study groups: (a) Control diet ($n=12$), Teklad ENVIGO++++; (b) High fat, high carbohydrates and cholesterol (HFHCC) diet composed by 39.8% kcal from fat, 15.7% kcal from protein and 45.4% kcal from carbohydrates (5TJT TestDiet®) plus 42 g/L (55% fructose and 45% glucose) carbohydrates in drinking water for up to 52 weeks. All animal care and experimental protocols followed the guidelines for the care and use of laboratory animals of our Institution and the European Community Policy for Experimental Animal Studies. (19/02/2016/023).

2.1.2 | AlbCrexmT/mG mice—Liver damage mouse model

The mT/mG mice mouse strain (Stock 07676, Jackson) was cross-bred with mice expressing Cre-recombinase under the control of the *Alb* promoter. In the litters, the EGFP protein is expressed in the plasma membrane (mG) in the cells expressing Cre-recombinase, whereas the dTomato protein is expressed in the absence of Cre (mT) (*AlbCrexmT/mG mice*, EGFP in hepatocytes, dTomato in the rest of the cells), as described.[29] Male and female 10-week-old C57BL6/J mice were treated with WD (D09100301; Research Diets, Inc., New Brunswick, NJ) for 23 weeks. Controls were fed with a chow diet (Altomin, Lage, Germany). In addition, male and 10-week-old female C57BL6/J mice were treated with a Dual diet consisting of Western diet and 10% vol/vol EtOH absolute in sweetened drinking water containing 6.75% D-glucose for 23 weeks, as previously described.[30] All procedures were carried out according to Spanish legal requirements and animal protection law approved by Comunidad de Madrid (PROEX125.1/20). Animals were maintained in the Animal Facility in the Faculty of Biology, Complutense University of Madrid under a 2-h light/dark ad libitum according to the guidelines of the Federation for Laboratory Animal Science Associations (FELASA).

2.1.3 | Collection of samples and determination of EpCAM and CD133 MVs

Retro-orbital blood was extracted and centrifuged at 2000*g* for 20 min at 4°C in heparin-coated microtubes. Samples were collected and pooled for a total of 100 μL of plasma from five mice per group to determine EpCAM and CD133 large EVs levels. In brief, 30 μL of sample were incubated with 5 μL of GFP antibody Alexa Fluor 488 (ThermoFisher scientific, MA, USA,), 5 μL of anti-EpCAM APC (Invitrogen, MA, USA) and 5 μL of CD133 PeCY7 (Invitrogen, MA, USA) for 15 min at RT. Next, we distinguished phosphatidylserine positive MVs using 5 μL of Annexin V-Brilliant Violet with 95 μL of Annexin buffer incubating for 20 min at RT (BD, Bioscience). MVs were analysed using a LSRFortessa (BD Biosciences, San Diego, CA). MVs size was determined using calibration beads Megamix-plus side angle light scatters (SSC) (0.16, 0.20, 0.24 and 0.5 μm) (Biocytex, Marseille, France). Data represent the mean of two independent experiments. MVs levels of HFHCC-fed mice were determined in 5 μL of heparin-plasma sample following the protocol above described with EpCAM FITC (Invitrogen, MA, USA), CD133 (PeCy7) and Annexin V- PE (BD, Bioscience, NJ, USA).

2.2 | Histological evaluation and immunohistochemistry

Liver tissues from HFHCC-fed mice and Dual diet mice were fixed in 4% buffered formalin solution and embedded in paraffin. Successive 4-μm-sections were obtained and prepared for immunohistochemistry. Liver tissue sections were deparaffinized with citrate buffer and rehydrated with decreasing concentrations of ethanol. EDTA-antigen retrieval was subsequently performed, and 3% hydrogen peroxide was used to quench peroxidase activity for 20 min. Sections were then incubated with CD133 (Cell signalling, 1:1000) and EpCAM (Abcam, 1:300) overnight at 4°C and the nuclei were counterstained with haematoxylin. For HFHCC-fed mice, EpCAM and CD133 expression in oval cells in liver tissue, were evaluated by an experienced pathologist from the pathology department of Virgen del Rocio University Hospital. In dual mice liver tissue, Images were captured by Olympus X61 at 20× magnification. Photomicrographs of EpCAM and CD133-positive areas for WD and Dual mice were taken at 20× magnification and analysed using the open-source software ImageJ.

2.3 | Immunofluorescence

Successive 4-μm hepatic sections were obtained and prepared for immunofluorescence. Paraffin sections were deparaffinized in xylol and rehydrated in graded alcohol series. Sections were then washed in distilled water and heated in a pressure cooker for epitope retrieval (in 10 mM citrate buffer [pH 6.0], 5 min). Slides were blocked with 2.5% horse serum and then incubated (overnight at 4°C) with primary antibodies against CD133 (1:100) and EPCAM (1:50). Sections were then washed with PBS. For fluorescence visualization of antibody reactions, primary antibodies were detected using secondary antibodies labelled with the fluorochromes Alexa Fluor (Alexa anti-mouse A488 and Alexa anti-rabbit A568, Invitrogen, MA, USA). To detect cell nuclei, sections tissues were incubated with 4′, 6-diamidino-2-phenylindole (DAPI) and then mounted on Vectashield mounting medium for fluorescence (Vector Laboratories, Peterborough, UK). Negative controls omitting the primary antibody step were run in parallel. Images were obtained using confocal microscopy (Leica Stellaris 8).

2.4 | Study population

Patients with biopsy-proven NAFLD were enrolled at the outpatient clinics of Virgen del Rocío University Hospital (Seville, Spain) or the

Clinical Valladolid University Hospital. Exclusion criteria were as follows: age < 18 years, significant alcohol intake (≥30 g/day in men and ≥20 g/day in women), evidence of viral autoimmune hepatitis, HIV, recreational drugs abuse or drug-induced fatty liver or other metabolic liver diseases (such as haemochromatosis or Wilson's disease). The clinical assessment included structured questionnaires to determine risk factors (hypertension, T2DM, dyslipidaemia, alcohol consumption and smoking status), monitor physical activity and control current medication along with other anthropometric measures (age, sex) and routine and liver function biochemical panels.

Percutaneous liver biopsies were obtained in all patients under local anaesthesia and ultrasound guidance. Biopsy samples were fixed in formalin and embedded in paraffin blocks. Specimens were stained with haematoxylin–eosin and Masson's trichrome. An experienced pathologist assessed the samples to determine the grade of steatosis, ballooning and inflammation, in order to classify patients by SAF score.[31] Blood samples were collected at inclusion. Fasting (overnight) blood samples were drawn from participants into tubes containing clot activator and separator gel (SST), K3-EDTA and heparin-Vacutainer TM. The index of insulin resistance (Homeostatic Model Assessment for Insulin Resistance, HOMA-IR) was calculated using the following formula: glucose (mg/dL) × insulin (mU/mL)/405. Finally, biochemical parameters were determined in routine laboratory at both hospitals.

The study was conducted in accordance with the ethical guidelines described in the Declaration of Helsinki and the international conference on harmonization guidelines for good clinical practice. Both centres received the approval of its Ethics Committee and ensured that participants understood the study and provided written informed consent.

2.5 | Determination of EPCAM CD133 MVs in NAFLD patients

Samples of peripheral blood were collected using a 21-gauge needle. Heparin-buffered blood samples were obtained and processed less than 1 h after venipuncture at 3000*g* for 10 min. For flow cytometry, 250 μL of plasma was centrifuged for 20 min at 3500*g* at room temperature to obtain platelet-free plasma sample; after that, samples were centrifuged for 30 min at 17 000*g* at RT twice to obtain large EVs pellet. All samples were frozen and stored at −80°C until assayed. The cellular origin of plasma MVs was determined by immunostaining using selective fluorochrome-labelled antibodies. Fifty microlitre of processed plasma was incubated with 3 μL of a monoclonal antibody anti-human CD326 (EpCAM) coupled to Alexa fluor 488 (Invitrogen, MA, USA) and 3 μL of anti-human CD133 (Prominin-1) coupled to PeCY7 (Invitrogen, MA, USA). After that, MVs were then incubated with 5 μL of Annexin V-PE (BD Biosciences, CA) for 20 min at RT to label phosphatidylserine. The negative control (zero value) was obtained using the isotype antibodies.

Large EVs were identified as events with a 0.1–1 μm diameter on forward light scatter (FSC) and side-angle light scatters (SSC) intensity dot plot representation, by comparison to flow cytometry calibration beads (Megamix-plus side angle light scatters SSC [0.16, 0.20, 0.24 and 0.5 μm], Biocytex, Marseille, France), indicating that our EVs isolation strategy mainly detected large vesicles or microvesicles. Finally, 50 μL of Flow Count Beads (Beckman Coulter, Marseille, France) were added to obtain MVs/μL. Data represent the mean of two independent experiments. Microvesicles were analysed by flow cytometry (BD LSR Fortessa; BD Biosciences).

2.6 | Statistical analysis

Categorical variables were expressed as absolute frequencies and percentages (*n*, %), and non-categorical variables as the mean ± SEM or SD. The Kolmogorov–Smirnov test was used to assess whether variables followed a normal distribution. Comparisons between groups were made using T-tests to compare variables with normal distribution, Mann–Whitney U tests for those with non-normal distribution, and χ^2 tests for qualitative variables. Bonferroni correction was applied to confirm differences between groups. All variables reaching statistical significance after Bonferroni correction ($p < 0.05$) in the univariate regression model were included in the multivariate regression model along with well-established prognostic indicators (age, sex and BMI). Possible associations were analysed using Spearman rank-order correlation. The predictive cut-off for EpCAM CD133 MVs was identified using the receiver operating characteristic (ROC) curve method and using the Youden index. Comparisons between AUCs were performed using STATA (v. 16, StataCorp LLC, TX). Data were compared and analysed using SPSS software (version 22.0; SPSS, Chicago, IL), and GraphPad Prism (version 7.0; GraphPad Software, La Jolla, CA) was used for graphics. For all analyses, $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Hepatic EpCAM and CD133 expression increase with the progression of NAFLD and co-expresses in human liver tissue

Mice fed with the HFHCC diet showed liver steatosis from week 13 and clear signs of steatohepatitis at 52 weeks of diet, showing: (a) higher body weight, macro–microvesicular steatosis (>90%), inflammatory foci at lobular, portal and periductal areas, hepatocyte degeneration (ballooning), moderate fibrosis and high prevalence of oval cells; (b) higher expression of EpCAM and CD133 proteins in liver tissue compared with mice with simple steatosis (Figure 1A,B). The reactivity of EpCAM and CD133 in oval cells appears from week 39 of diet onwards, 100% of animals had mild expression of CD133 in week 39 and 64% moderate levels of EpCAM at the same timepoint, reaching severe expression of both markers only when liver tumour appears ($p < 0.001$ for both EpCAM and CD133).

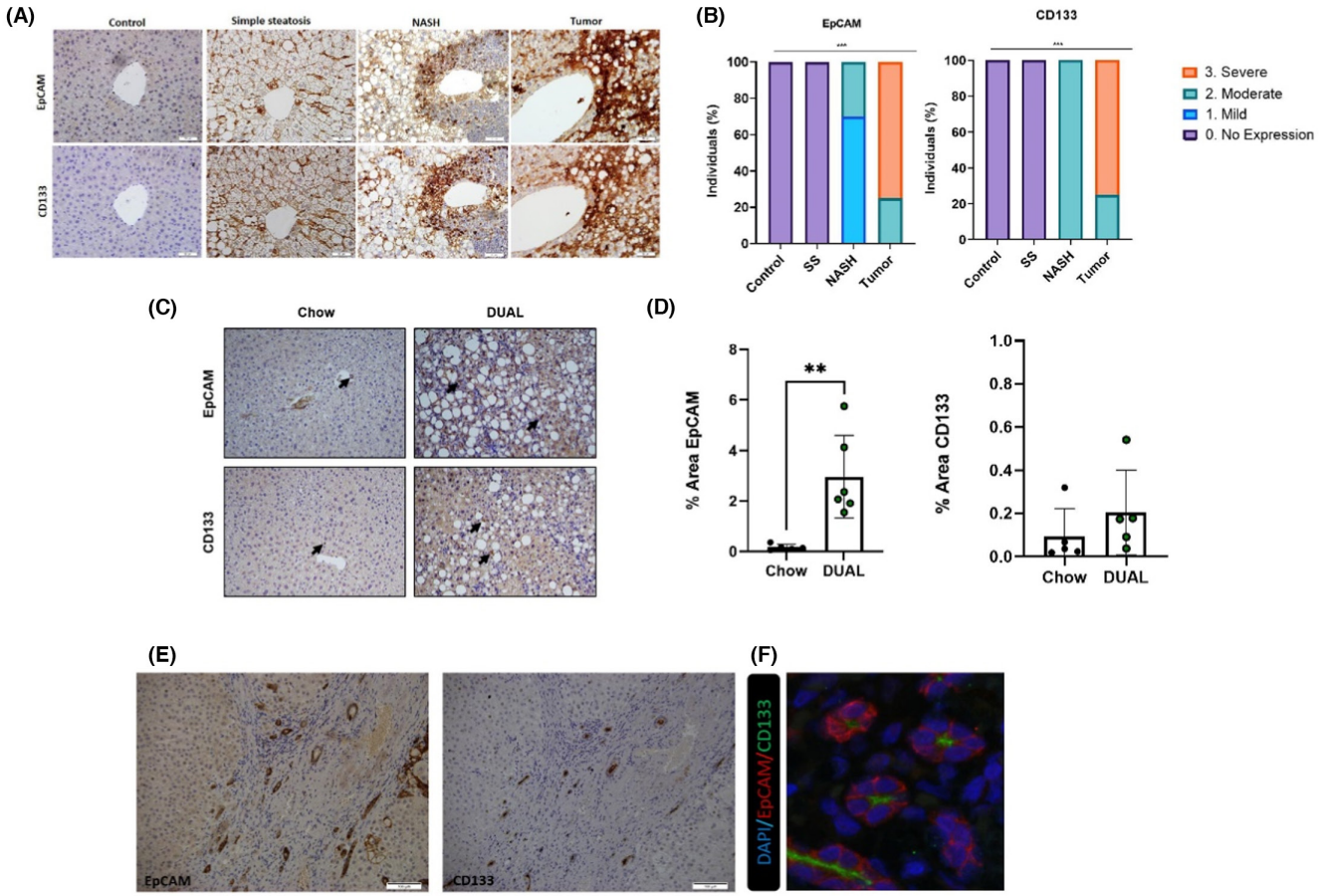

FIGURE 1 Expression of EpCAM CD133 liver tissue. (A) Representative pictures of EpCAM and CD133 oval cell staining according to different stages of the disease in HFHCC-fed mice. (B) Percentage of animals with several degrees of EpCAM and CD133 liver tissue expression. (C) EpCAM and CD133 IHC staining in AlbCrexmT/mG mice with Dual diet after 23 weeks of Dual feeding. (D) Quantification of % positive area of EpCAM and CD133, respectively, using ImageJ software ($n = 5–6$). (E) EpCAM and CD133 IHC in human liver biopsies (10× magnification). (F) Immunofluorescence double staining of hepatic tissue (40× magnification). Data represent the mean ± SEM. $^{*}p < 0.05$ $^{***}p < 0.001$.

Next, we analysed the expression of EpCAM and CD133 in liver tissue of AlbCrexmT/mG mice fed a Dual diet (Figure 1C). Compared with control mice, the liver parenchyma of mice fed a Dual diet showed a significantly increased percentage of positive EpCAM area per view field and a clear tendency towards increased CD133 staining (Figure 1D). As shown in Figure 1, importantly, the expression of EpCAM and CD133 was also confirmed in liver tissue from patients with NAFLD (Figure 1E). As shown in Figure 1F, these cells frequently coexpressed both EpCAM and CD133, thus explaining the double positivity observed in the cell-derived microvesicles.

3.2 | The liver is able to produce AV+ EpCAM+ CD133+ circulating MVs

Mice fed a HFHCC diet for 52 weeks showed significantly higher circulating concentrations of EpCAM CD133 EVs compared to control mice fed with a chow diet (Figure 2A, $p = 0.02$). Besides, we found a positive and significative correlation between levels of this kind of EVs compare to EpCAM ($r = 0.47$; $p = 0.005$) and CD133 ($r = 0.30$; $p = 0.036$) liver tissue expression.

To confirm if EpCAM CD133 EVs were released from hepatocytes, we used *AlbCrexmT/mG* mice fed a WD or a Dual diet (WD + alcohol). In *AlbCrexmT/mG* mice, albumin-expressing cells (hepatocytes and oval cells) present GFP in their plasma membrane (mG), whereas all other cells express the Tomato-dye protein instead (mT) (Figure 2B). Using flow cytometry and amplification of signal with a fluorochrome-conjugated anti-GFP antibody, we showed that *AlbCrexmT/mG* mice fed a WD for 23 weeks presented increased proportions of GFP-positive Annexin V EVs compared with controls (12.1% vs 5.2%), supporting hepatocytes as their cell of origin (Figure 2C). The proportion of AV+ dTomato + EVs was not increased by the WD (58.9% in control vs 44.5% after WD). Importantly, 92.8% and 98.3% of the AV+ GFP+ EVs population in the control and WD groups, respectively, were also EpCAM+ CD133+, indicating that most hepatocyte-derived (GFP+) EVs were also positive for these proteins, while within the dtomato+ population, 19.1% and 19.3% were also positive for EpCAM and CD133 in the control and the WD group respectively.

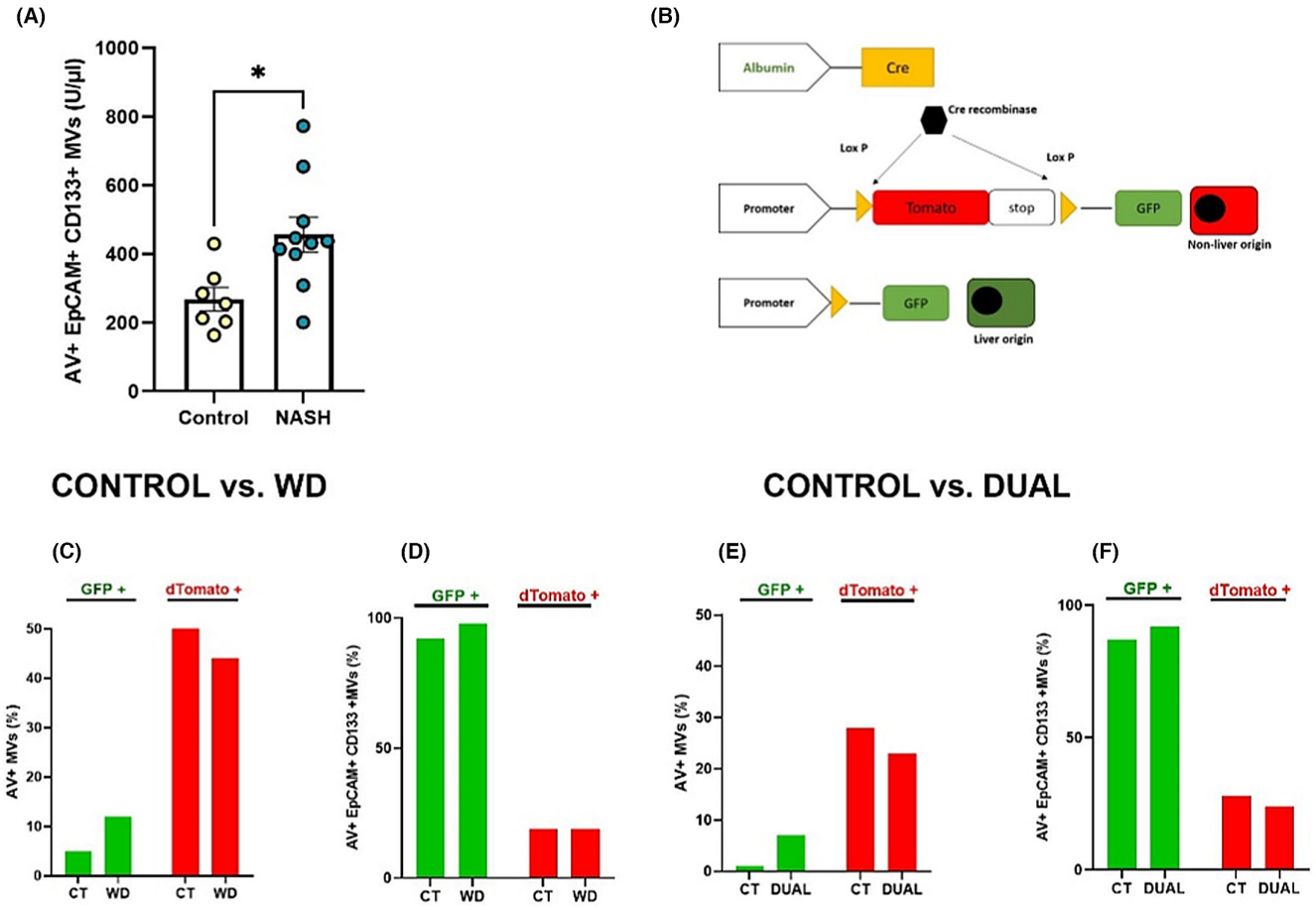

FIGURE 2 (A) Circulating concentration of EpCAM+ CD133+ EVs in mice fed an HFHCC diet (MASH) or a chow diet (Control) for 31 weeks. (B) AlbCrexmT/mG mice. (C, D) GFP+/ −dTomato−+EpCAM+ CD133+ EVs levels in AlbCrexmT/mG mice after 23 weeks with WD or Dual treatment (E, F). Samples were collected and pooled for a total of 100 µL of plasma from five mice per group in AlbCrexmT/mG mice. Data are shown as mean ± SD in the HFHCC-mouse model. $^*p < 0.05$.

Similar results were obtained in *AlbCrexmT/mG* mice fed a Dual diet for 23 weeks (Figure 2E,F). Compared with control mice, mice fed the Dual diet showed a significative elevation of circulating AV+ GFP+ EVs (0.5% vs 7.3%) while levels of dTomato+ EVs remain stable (27.8% vs 23.9%). Again, the proportion of MVs positive for EpCAM and CD133 among the total AV + GFP+ EVs was 87.9% in control mice and 92.9% in Dual mice. In summary, we observed an increase in GFP+ EVs in both NALFD models, and approximately 90% of these GFP+ EVs were EpCAM+ and CD133 + .

3.3 | Circulating levels of AV+ EPCAM+ CD133 EVs were associated with steatohepatitis in patients with NAFLD

Among 130 patients with biopsy-proven NAFLD included in this study, 71 of them composed the discovery cohort. Patients were classified by SAF score as simple steatosis ($n = 20$) or steatohepatitis ($n = 51$). Among their baseline demographic and clinical characteristics (Table 1), patients with steatohepatitis had more fibrosis ($p = 0.046$), higher levels of AST ($p = 0.001$) and ALT ($p = 0.005$), and a higher prevalence of arterial hypertension ($p = 0.002$) than patients with simple steatosis. Patients with steatohepatitis showed significantly higher plasma levels of EpCAM+ CD133+ EVs than patients with simple steatosis (Table 1, and Figure 3A).

Regarding histopathological features of NAFLD, patients with lobular inflammation (mild or severe) had higher levels of EPCAM+ CD133+ EVs (321.1 ± 305.7 vs 721.4 ± 588.3, $p = 0.001$) than those without lobular inflammation (Figure 3B), but no difference was found between patients with mild and severe inflammation (data not shown). Patients with hepatocyte ballooning also showed higher levels of this kind of EVs compared to patients without ballooning (Figure 3C). In contrast, the circulating concentration of EpCAM+ CD133+ EVs was associated neither with the degree of steatosis (S1 ($n = 37$): 633.3 ± 99.1 U/µL vs S2 ($n = 25$): 672.3 ± 117.9 U/µL vs S3 ($n = 9$): 552.7 ± 141.9 U/µL, $p = 0.907$, Figure 3D) nor with the presence of fibrosis (No fibrosis: 611.5 ± 77.5 vs Fibrosis: 649.9 ± 123 U/µL, $p = 0.735$) or its degree (Figure 3E). Multivariate regression showed that age, ALT and circulating EpCAM+ CD133+ EVs were independent predictors of steatohepatitis (rank-ordered by Wald, Table 1).

TABLE 1 Baseline demographic and clinical characteristics of patients in the discovery cohort.

Variables	Simple steatosis ($n=20$)	NASH ($n=51$)	Univariate p-value	Multivariate OR (CI 95%; p-value)
Age, years ± SD	50.3 ± 12.8	57.7 ± 10.6	0.016	1.153 (1.052–1.264); $p=0.003$
Male, sex, *n* (%)	14 (70)	24 (47)	0.081	
BMI (kg/m^2)	31.8 ± 5.2	35.6 ± 7.8	0.047	
Fibrosis (Kpa)	10.8 ± 6.8	14.8 ± 7.7	0.046	
CAP (dB/m)	314.9 ± 64.6	332.8 ± 41.6	0.192	
Bilirubin ± SD (mg/dL)	0.92 ± 1.20	0.56 ± 0.32	0.222	
Platelets ± SD (×10^9/L)	198.8 ± 61.1	222.1 ± 66.6	0.181	
Creatinine ± SD (mg/dL)	0.78 ± 0.19	0.88 ± 0.51	0.561	
AST ± SD (IU/L)	30.80 ± 15.1	53.2 ± 36.7	0.001	
ALT ± SD (IU/L)	36.3 ± 17.8	62.02 ± 45.2	0.005	1.055 (1.006–1.107); $p=0.031$
GGT ± SD (IU/L)	59.1 ± 25.8	101.8 ± 129	0.061	
TC ± SD (mg/dL)	179.2 ± 54.2	181.4 ± 34.9	0.833	
LDL ± SD (mg/dL)	106.1 ± 42.1	104.8 ± 32.9	0.364	
HDL ± SD (mg/dL)	43.7 ± 12.3	46.4 ± 10.8	0.393	
HTA, *n* (%)	8 (5.6)	40 (78.4)	0.002	
DM, *n* (%)	9 (6.3)	11 (21.6)	0.292	
EpCAM+ CD133+ (U/μL) ± SD	286.4 ± 61.9	758.4 ± 82.3	<0.001	1.006 (1.002–1.009); $p=0.009$

Note: Categorical variables are represented by absolute frequencies and percentages (*n*, %). Non-categorical variables are represented by mean ± SEM. *p*-values are from the comparison of SS and NASH groups.

Abbreviations: ALT, alanine aminotransferase, AST, aspartate aminotransferase; BMI, body mass index; CAP, controlled attenuated parameters; CI 95%, 95% confidence interval; DM, diabetes mellitus; GGT, gamma glutamyl transferase; HDL, high-density lipoprotein; HTA, hypertension; LDL, low-density lipoprotein; TC, total cholesterol.

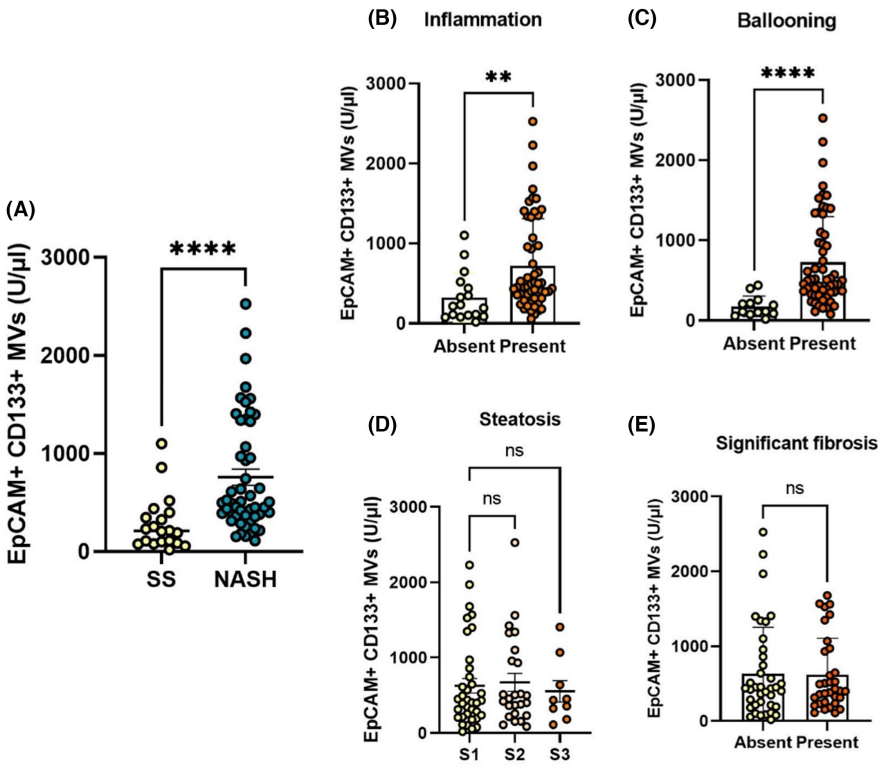

FIGURE 3 EpCAM AND CD133 EVs levels in NAFLD patients. (A) AV+ EpCAM+ CD133+ EVs population in SS versus NASH. (B) EVs levels regarding inflammation, (C) Steatosis grade (D) Fibrosis stage and (E) Ballooning. Data are represented as mean ± SEM ns, no significant, $^*p<0.05$, $^{**}p<0.005$ and $^{***}p<0.001$.

3.4 | The association between circulating EPCAM+ CD133+ EVs and steatohepatitis and their value as a non-invasive biomarker was confirmed in an independent cohort

The external validation was conducted in a cohort including 27 patients with simple steatosis and 31 patients with steatohepatitis (total $n=51$, 50% males). In this validation cohort, most baseline characteristics were similar in patients with steatohepatitis compared with those with simple steatosis except for higher fibrosis degrees and higher levels of GGT in the former group (Table S1). Compared with the discovery cohort, patients in the validation cohort presented higher platelet count ($p=0.036$), lower AST ($p=0.012$) and higher prevalence of arterial hypertension ($p=0.002$) (Table S2). In this independent cohort, EpCAM+ CD133+ EVs levels were higher in patients with steatohepatitis than in those with simple steatosis (554.1±44.6 vs 353.1±39.3; $p=0.002$), and again levels of EVs were not related to fibrosis or steatosis (Figure 4D,E). In contrast, as shown in Figure 4F,G, levels of EVs were higher in patients with ballooning compared to those without ($p=0.01$), and finally, patients with inflammation showed higher levels of EpCAM+ CD133+ EVs than those without ($p=0.001$).

Finally, to further confirm the role of the EVs as biomarkers, we assessed the added value of EpCAM+ CD133+ EVs to the diagnostic capacity of established clinical/biochemical biomarkers in predicting NASH. In the global cohort ($n=130$), the area under receiver–operator characteristics curve (AUROC [95% CI]) of the clinical parameters (sex, age, BMI, AST, ALT and fibrosis; Table S3) was 0.76 (0.68–0.85); $p<0.001$. After adding the levels of EpCAM+ CD133+ EVs to the regression, the AUROC increased significantly ($p=0.001$) to 0.88 (0.82–0.94); $p<0.001$.

4 | DISCUSSION

Hepatic expression of EpCAM and CD133 and EpCAM+ CD133+ EVs increased during disease progression in HFHCC mice and, interestingly, about 90% of these EVs had liver origin. In NAFLD patients, levels of AV+ EpCAM+CD133+ EVs were higher in those with steatohepatitis than simple steatosis, and levels of EpCAM+ CD133+ EVs were positively correlated with histological features reflecting disease severity such as inflammation and hepatocyte ballooning. Circulating EVs identified in several body fluids such as blood, saliva and urine, have been proposed as markers for liquid biopsies in several diseases.[32–34] EVs are considered attractive biomarkers because they are abundant and stable in fluids[35] and because the lipid bilayer membrane protects the molecular cargo promoting high stability and acting as a reservoir. Furthermore, cell-specific circulating EVs may provide more information on the injured tissue identifying cell type.[36–38] Immune cell-derived circulating EVs using cell surface markers such as CD4+, CD8+ and CD14+ helped to identify patients with NAFLD at risk of suffering from HCC. In addition, circulating CD14+ MVs in NAFLD patients correlated with ALT levels and were correlated with NAS score.[39] Previous studies from our own group have reported a decrease in the number of endothelial and platelet-derived MVs after sustained virological response in Hepatitis C patients, which correlated with improvement of endothelial function.[40] Platelet-derived

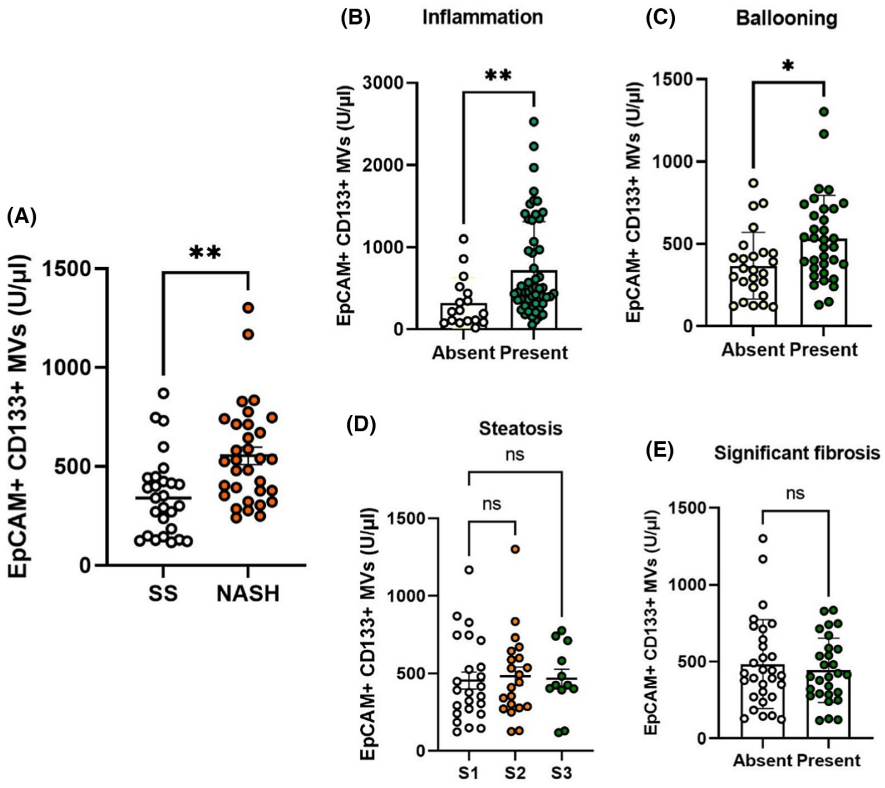

FIGURE 4 EpCAM AND CD133 EVs levels in NAFLD patients (Independent cohort). (A) AV+ EpCAM+ CD133+ EVs population in SS versus NASH. (B) EVs levels regarding inflammation, (C) Steatosis grade (D) Fibrosis stage and (E) Ballooning. Data are represented as mean ± SEM; ns, not significant, $^{*}p<0.05$, $^{**}p<0.005$ and $^{***}p<0.001$.

MVs (CD61+) correlated with fat fraction, ballooning and fibrosis stage in NAFLD patients and have also been proposed as biomarkers for NASH diagnosis.[41] Weil et al. have recently reported that Annexin V+ MVs levels, mainly derived from platelets, inversely correlated with MELD score and were 2.5-fold higher in healthy control than in cirrhotic patients.[42] Patients with lower small AV+ platelet-derived MVs levels had also a higher risk of death or liver transplant during a 6-month follow-up period. Some useful markers have emerged to characterize liver-derived EVs, which included asialoglycoprotein receptor 1 (ASGPR1), bile-acyl-coenzyme A synthetase (SLC275A) and Hepatocyte paraffin 1 (HepPar 1). In a diet-induced mouse model of NASH, the authors reported a significant increase of hepatocyte derived-MVs, staining with ASGPR1 in both, males and females, at 12 and 10 weeks of feeding, respectively, occurring prior to histological evidence of inflammation and correlating with steatosis grade, fibrosis stage, ballooning and lobular inflammation.[31] Previous studies showed a significant increase in circulating calcein fluorescein isothiocyanate (FITC) + EVs in 25 patients with advanced NASH compared to 25 healthy controls.[43] They established a cut-off of 668 EVs/μL with 92% sensitivity and 75% specificity for differentiating patients with or without portal hypertension. SLC27A5 has also been analysed as a hepatocyte-specific marker in MVs, and levels of SLC27A5 were 3–4-fold significantly higher in patients compared to controls, while no differences between with and without fibrosis were reported.[31] HepPar 1 microvesicles were found significantly increased in patients with hepatocarcinoma and may serve as early recurrence biomarker after liver resection.[44] Recently, Povero et al., identified increased levels of SLC27A5+ circulating EVs ranging from 200 to 1000 nm, in patients with primary sclerosing cholangitis compared to healthy subjects, while no differences were found in the level of ASGPR1+ EVs.[45] Besides, adipose-derived EVs have been recently recognized as biomarkers for lipid and glucose metabolism in metabolic liver disease.[46]

When severe insults impede hepatocyte proliferation, the hepatic progenitor cells, identified by specific surface markers such as EPCAM and CD133,[11,47,48] become activated. Here, we described an increment in the hepatic expression of both proteins with the progression of the disease in a HFHCC-fed mice model, reaching higher levels in cancer liver tissue.[18] Previous studies have demonstrated the contribution of hepatocyte-derived EVs to liver mass regeneration.[49] However, studies that investigate levels of large EVs positive for EpCAM and CD133 as potential biomarkers in well-characterized patients with biopsy-proven NAFLD are not yet available. Different EVs subpopulations have been previously associated with NAFLD based on their cellular origin, but our study is the first to combine size discrimination and the double labelling of EVs in several murine models and a large, multicentre well-characterized cohort of biopsy-proven NAFLD patients. In this study, we conclude that higher levels of EpCAM+ CD133+ EVs discriminate between patients with simple steatosis and patients with steatohepatitis.

Our analysis of GFP+ EpCAM+ CD133+ in *AlbCrexmT/mG* mice demonstrated that more than 90% of the total EpCAM+ CD133+ MVs originated in albumin-expressing cells (hepatocytes or oval cells), supporting the utility of this kind of EVs as biomarkers of liver injury in NAFLD patients. Whereas EpCAM+ CD133+ EVs levels were higher in patients with inflammation and ballooning, we found no association between EVs levels and fibrosis or steatosis degree. These results contrast with data recently reported by Nakao et al. showing a correlation between hepatocyte-derived EVs levels and steatosis in 28 morbid obese patients with NAFLD,[50] which could be explained due the variability between cohorts in terms of BMI, age, ALT and fibrosis levels. Payancé A et al. showed that patients with Child–Pugh C had fourfold and 2.2-fold higher levels of hepatocyte-derived MVs measured by ELISA than patients with Child–Pugh A or B respectively.[51] In our study, we found no changes in patients with cirrhosis, which could be explained by the low sample size of cirrhotic patients in the discovery and validation cohorts, or to the different subsets of MVs studied. Previous results analysed these surface markers of MVs in HCC, and concluded that AV+ EpCAM+ CD147+ MVs were elevated in patients with HCC and CCA. Furthermore, the AV+ EpCAM+ ASGPR+ CD133+ could distinguish between tumour and the presence of cirrhosis in tumour-free individuals,[10] while here we report a great discrimination capacity of AV+ EpCAM+ CD133+ EVs in patients with NAFLD without liver tumour. This study acknowledges some limitations. First, the lack of a matched control group due to the ethical impossibility of having a biopsy-proven healthy cohort. Second, our study did not include patients with other aetiologies of liver disease and, therefore, we did not evaluate the capacity of AV+ EpCAM+ CD133+ EVs to identify patients with NAFLD among different aetiologies. However, our study showed that AV+ EpCAM+ CD133+ EVs represent a useful biomarker for the more difficult task of separating patients with NAFLD and steatohepatitis from those with simple steatosis. Further studies may also explore the EpCAM+ CD133+ EVs molecular cargo to determine the mechanisms involved and to use it as a potential therapeutic tool. Finally, the development of new techniques for the isolation and characterization will represent an opportunity to enhance the clinical utility, representing a promising tool for non-invasive diagnosis in the clinical practice. In conclusion, levels of AV+ EpCAM+ CD133+ EVs increased during the transition from simple steatosis to steatohepatitis and were found at greater concentration in patients showing lobular inflammation and ballooning, showing a clinical utility as a potential biomarker for steatohepatitis in patients with NAFLD.

AUTHOR CONTRIBUTIONS

Manuel Romero-Gómez and Rocío Muñoz-Hernández conceived, designed and supervised the study, Javier Ampuero, Rocío Aller and Rafael Bañares selected patients' samples, Sheila Gato, Antonio Gil-Gómez, Angela Rojas, Laura Morán, Javier Gallego, Elena Blázquez-López, Rocío Gallego-Durán, Rocío Montero Vallejo, Vanessa García-Fernández, Douglas Maya-Miles, María del C Rico, Francisco J Cubero and Javier Vaquero generated and analysed data.

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CONFLICT OF INTEREST STATEMENT

The authors do not have any disclosures to report.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

ORCID

Rocío Muñoz-Hernández https://orcid.org/0000-0003-3765-6276
Rocío Gallego-Durán https://orcid.org/0000-0002-9452-1661
Francisco J Cubero https://orcid.org/0000-0003-1658-1041
Javier Vaquero https://orcid.org/0000-0001-8903-7288
Manuel Romero-Gómez https://orcid.org/0000-0001-8494-8947

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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