ORIGINAL ARTICLE

Phase 1–2 pilot clinical trial in patients with decompensated liver cirrhosis treated with bone marrow–derived endothelial progenitor cells

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The aim of this nonrandomized, open label, phase 1 clinical trial was to evaluate the safety and the feasibility of the treatment with autologous bone marrow–derived endothelial progenitor cells (EPC) in decompensated liver cirrhosis. In addition, the changes in liver function and hepatic venous pressure gradient (HVPG) and their relation with the characteristics of the cellular product were analyzed. Twelve patients with Child-Pugh ≥8 liver cirrhosis underwent bone marrow harvest for *ex vivo* differentiation of EPC. The final product was administered through the hepatic artery in a single administration. Patients underwent clinical and radiologic follow-up for 12 months. The phenotype and the ability to produce cytokines and growth factors of the final cellular suspension were analyzed. Eleven patients were treated (feasibility 91%). No treatment-related severe adverse events were observed as consequence of any study procedure or treatment. Model for end-stage liver disease score improved significantly (*P*0.042) in the first 90 days after cells administration and 5 of the 9 patients alive at 90 days showed a decreased of HVPG. There was a direct correlation between the expression of acetylated-low density lipoprotein and von Willebrand factor in the cellular product and the improvement in liver function and HVPG. The treatment with EPCs in patients with decompensated liver cirrhosis is safe and feasible and might have therapeutic potential. Patients receiving a higher amount of functionally active EPC showed an improvement of liver function and portal hypertension suggesting that the potential usefulness of these cells for the treatment of liver cirrhosis deserves further evaluation. (Translational Research 2016;■:1–12)

Abbreviations: EPC = endothelial progenitor cells; VEGFR = vascular endothelial growth factor receptor; vWF = von Willebrand Factor; acLDL = Acetylated-low density lipoprotein; CXCR4 = C-X-C chemokine receptor type 4; VEGF = vascular endothelial growth factor; EGF =

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epidermal growth factor; HGF = Hepatocyte growth factor; IGF = insulin like growth factor; CT-1 = Cardiotrophin-1; IL-6 = Interleukine 6; SDF = stromal cell-derived factor; HVPG = Hepatic Vein Pressure Gradient; ICG = indocyanine green clearance

AT A GLANCE COMMENTARY

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Background

In the experimental setting, endothelial progenitor cells (EPC) have been regarded as a potential treatment for liver cirrhosis, but no clinical studies have investigated the safety and the feasibility of the treatment with autologous EPC in this setting.

Translational Significance

In this pilot clinical trial, bone marrow–derived EPC were administered through the hepatic artery in patients with decompensated cirrhosis. The treatment was feasible and safe, and the results suggest that EPC administration might be beneficial for liver function and portal hypertension as bridging therapy, whereas waiting for liver transplantation or as supportive therapy for patients not eligible for transplantation.

INTRODUCTION

End-stage liver cirrhosis is the main indication for liver transplantation in Europe and the ultrasound (US).[1,2] Regrettably, many patients awaiting transplantation are not transplanted due to high mortality and dropout rates while in long waiting lists as result of organ shortage.[2] Moreover, a significant amount of patients are not suitable for transplantation for different reasons, mainly comorbidities or advanced age. In absence of liver transplantation 1-year mortality rate among patients with decompensated liver cirrhosis is very high, ranging from 20% to 55% in Child-Pugh score B and C, respectively.[3,4] In the setting of end-stage liver cirrhosis, regenerative therapies could help in bridging patients for transplantation and improving the quality of life of those patients that are not eligible for the intervention. Bone marrow progenitor cells have been advocated as a potential therapeutic tool for different types of liver disease.[5-8] In the last 15 years, transplantation of different bone marrow progenitors has proved beneficial in animal models of acute and chronic liver diseases.[8-10] In patients with chronic liver disease, the administration of bone marrow progenitors by different routes was also shown to provide therapeutic benefit.[10-15] However, studies are very heterogeneous regarding design, characteristics of patients, cells used, and administration route, so no definitive conclusions can be drawn regarding the safety and the efficacy of this treatment. Recently, endothelial progenitor cells (EPC), a subtype of bone marrow progenitors, showed hepatoprotective activity in experimental models of liver injury.[16-19] EPC represent a small percentage of bone marrow progenitors and can be detected in the bloodstream at a very low concentration. EPC are recruited to damaged tissues and seem to contribute to tissue regeneration, especially through their ability to release cytoprotective factors[9,20] and to stimulate vasculogenesis and neoangiogenesis.[21,22] Experimental data suggest that during liver damage these cells produce cytokines and growth factors that promote scar tissue degradation and hepatocyte proliferation.[23] EPC were characterized for the first time in 1997,[21] but we still lack a universal agreement on the phenotype criteria that should be used for their identification.[24] Yet, there is a general agreement that 2 different populations of EPC can be identified, late and early EPC.[22,25,26] These 2 cell subsets exhibit different expression of progenitor cell markers (CD133) and endothelial markers (vascular endothelial growth factor receptor [VEGF-R] 1 and -2); however, they show a similar vasculogenic ability. Compared with early EPC, late EPC show lower expression of progenitor markers (CD133 and CD 34), whereas the expression of vascular endothelial growth factor receptor (VEGFR)-1 and VEGFR-2 is higher. The expression of the common leukocyte antigen (CD45) gradually decreases from mononuclear cells to late EPC.[22] In culture, they are typically recognized by their spindle-shaped aspect and by their ability to form tubes. The capacity for uptaking acLDL and binding Ulex-lectin constitute 2 major functional markers of these cells.[21] Although experimental studies suggest that EPC may revert liver fibrosis and improve the prognosis of liver diseases,[16-18] there is no clinical support to the use of EPC in the treatment of patients with liver cirrhosis. Furthermore, there is no evidence that EPC for therapeutical use can be obtained from the bone marrow of cirrhotic patients.

Therefore, the main aim of this phase 1–2 trial was to evaluate the feasibility and safety of the administration of autologous bone marrow–derived EPC in patients with liver cirrhosis. Their effect on liver function and portal hypertension was also assessed as a secondary aim.

MATERIAL AND METHODS

Study design and inclusion and/or exclusion criteria. This was a nonrandomized, open label, phase 1–2 clinical trial to evaluate the safety (primary end point), feasibility, and efficacy (secondary end points) of treatment with autologous bone marrow–derived EPC in patients with decompensated liver cirrhosis.

Patients with liver cirrhosis and a Child-Pugh score >7 were evaluated. The main exclusion criteria were upper gastrointestinal bleeding or any other severe disease (including infections) within 30 days before inclusion and hepatocellular carcinoma or any other cancer within 5 years before inclusion. The full list of inclusion and exclusion criteria is provided in Supplementary table I.

Recruitment and treatment were carried out in the Liver Unit at Clinica Universidad de Navarra.

Ethics. All patients provided a written informed consent. The study protocol conforms to the ethical guidelines for human research, as reflected in a priori approval by the local human research committee, the institutional review board, and by the Spanish Agency for Medicinal Products (Agencia Española de Medicamentos y Productos Sanitarios).

Study procedures and methods. ***Bone marrow harvest, cell isolation, and differentiation.*** Between 50 and 100 mL of bone marrow were drawn in heparin-coated syringes from the iliac crest under sedation. The procedure was performed according to standard clinical practice by a specialized hematologist and took approximately 20 minutes. After collection, bone marrow blood was immediately transported in the same syringe used for the collection to the Cell Therapy Area Laboratory for subsequent processing. Bone marrow derived mononuclear cells were isolated by Ficoll gradient and counted in a Neubauer Chamber using Trypan blue staining. Cells were cultured on fibronectin-coated plates (10^6 cells/cm^2) in the presence of endothelial complete medium (ECM) for 4 days for differentiation into EPC. The ECM consisted of endothelial basal medium (Good Manufacturing Practice [GMP]-grade, LiStarfish; Italy) enriched with 5% autologous plasma, recombinant human VEGF (hVEGF; 10 ng/mL; R&D system; USA), recombinant human insulin like growth factor-1 (hIGF-1; 10 ng/mL; GMP-grade Novozymes; Denmark), basic fibroblast growth factor (1 ng/mL; GMP-grade Cellgenix; Germany), recombinant human EGF (hEGF; 10 ng/mL; Promokine; Germany), and hydrocortisone (1 μg/mL; Actocortina, Takeda Farmaceutica; Spain). At day 4 of differentiation, nonadherent cells were discarded and adherent cells were cultured until day 7 in ECM. At day 7, adherent cells were collected by using recombinant cell-dissociation enzymes (TrypLE Select, Gibco) and counted. EPCs were identified by their spindle-shaped aspect and by the expression of markers analyzed by flow cytometry (Fig 1B and D). The following markers were analyzed: CD31, CD34, CD14, VEGFR-2, VEGFR-1, CD133, CD90, CD117, von Willebrand factor (vWF), CXCR4, ID1, and CD45. EPC functionality was estimated by assessing 3 typical characteristics of EPC population: (1) uptake of DiL-acetylated-LDL cholesterol (Biomedical technologies, MA, USA); (2) Ulex europaeus Lectin-FITC Binding (Sigma-Aldrich, MO, USA); (3) tube-structure formation over Matrigel matrix (BD Bioscience, Belgium). Tube structures were observed by microscopy[9,21] (Fig 1C).

In parallel to the clinical manufacturing process, the ability of the cells to produce cytokines and growth factors was assessed in a small aliquot of cells. For this purpose, at day 4 of differentiation, the medium was removed in one well of the culture plate and endothelial basal medium without growth factors, was added to adherent cells until day 7. Supernatant was collected at day 7 and stored at −80°C for measurement of VEGF, EGF, HGF, IGF, CT-1, IL-6, and SDF by ELISA kits (R&D; USA). To capture the ability of the cells to produce cytokines and growth factors independently of the number of cells retrieved, the concentration of these substances was then calculated per million of cells counted in the well.

As quality control, before the administration, a series of surface markers and microbiological test were performed in a small aliquot of the cellular suspension within 4 hours prior the treatment. The cellular product needed to fulfill the following release criteria: negative Gram staining, mycoplasma test negative, viability >60%, VEGFR-1, vWF, and Ulex-binding >40% (the last 3 were tested by flow cytometry). If the release criteria were fulfilled, the cells were suspended in a final volume of 50 mL with ringer lactate added with hAlbumin 1%, packed in a 50 mL syringe, and stored at 4°C until its administration. The remaining surface markers were analyzed by flow cytometry after the administration. A detailed scheme of manufacturing process, images of cells and phenotype profile are shown in Fig 1. For safety reasons, the maximum amount of cells to be infused was established at 100×10^6. The entire manufacturing process was performed at the Cell Therapy Laboratory of Clínica Universidad de Navarra under GMP conditions.

Cells administration. Once the patient was ready for angiography, the syringe was transported in a sterile container at room temperature to the angiography room where the cell product was administered through the proper hepatic artery through a femoral approach

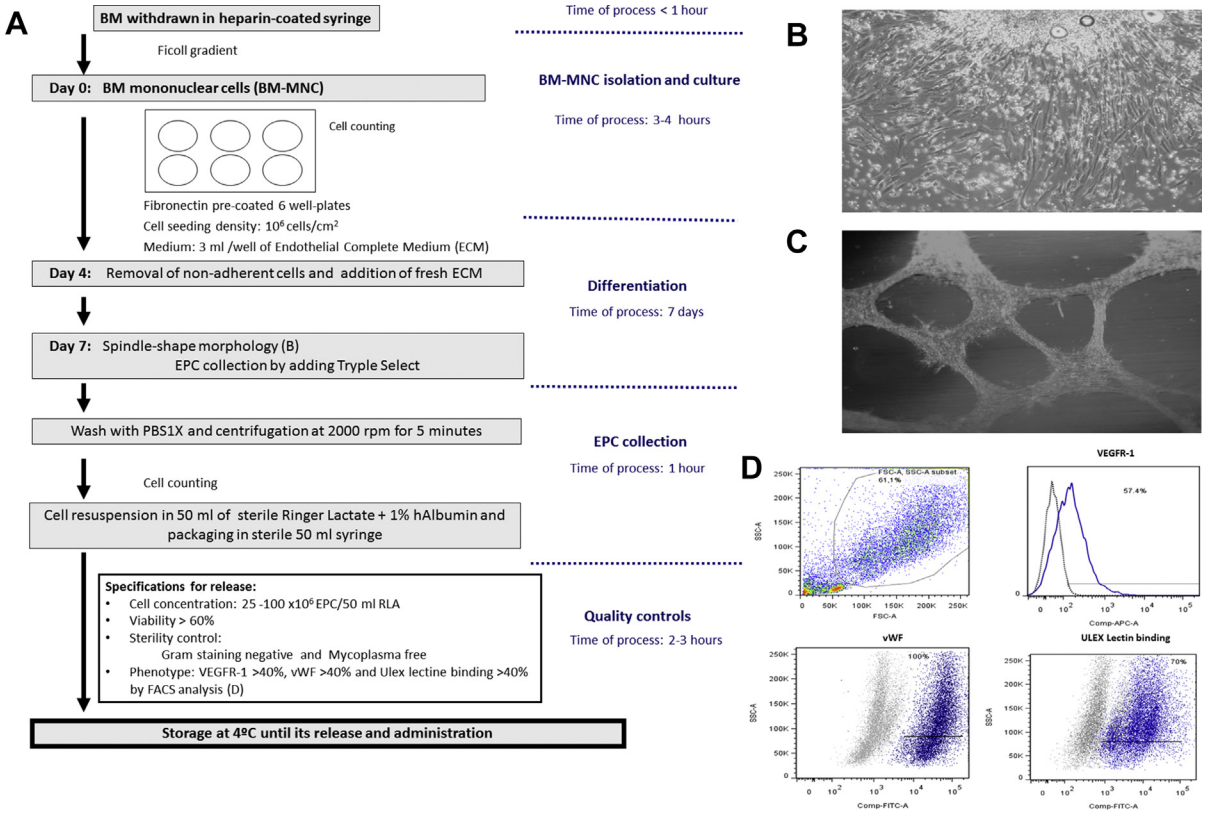

Fig 1. Schematic representation of the manufacturing process (A). Exemplificative images of EPC showing a spindle-shape morphology at 7 day of differentiation (B) and the ability to form tube structures when seeded over matrigel matrix (C). Marker expression analyzed by FACS (D; positive cells are shown in blue).

using a 2.7 F microcathether (Progreat, Terumo, Japan). Cells were injected very slowly over 20 minutes to minimize hemodynamic changes. In patients with anatomic variants of the hepatic artery, cell suspension was divided in 2 equal aliquots and administered via the right and left branches of the hepatic artery. Patients were hospitalized overnight for observation.

Evaluation. At screening, patients underwent a complete clinical, biochemical, and radiologic evaluation to assess their eligibility for the study. After treatment, patients were followed to monitor safety and efficacy. Adverse events, paracentesis, number of episodes of encephalopathy, and any change in the dose of diuretics were recorded at each visit. Physical examination and blood analysis including complete blood count, liver, and renal function tests, albumin, ammonia, and prothrombin time were performed at screening and days 7, 14, 30, 45, 60, 90, 180, and 360 after treatment. Alpha-fetoprotein and protein electrophoresis were obtained at inclusion and days 30, 90, 180, and 360. Liver ultrasound and transient elastography were performed at screening and days 30, 90, 180, and 360. Noninvasive indocyanine green clearance was estimated using a Liver Function Monitor (LiMon-Pulsion Medical Systems AG, Germany) at inclusion and at days 30, 90 and 180. Hepatic vein pressure gradient (HVPG), defined as the difference between the wedged and the free hepatic venous pressures, was measured at the inclusion and day 90 after treatment by catheterization of femoral or right jugular vein. At each visit Child-Pugh, model for end-stage liver disease (MELD) and noninvasive fibrosis scores (AST to platelet ratio index and Fibrosis-4 score) were calculated according to the published formulas.[27,28]

Statistical analysis. Data in tables are shown as median and range or percentage according to the type of variable. To assess the safety and the efficacy of the treatment, changes from baseline in all clinical, biochemical, and radiologic variables, including scores of liver function and fibrosis, have been analyzed. Since each patient served as his own control, the changes of these variables from baseline to different time points during follow-up have been studied with nonparametric tests for repeated measures (chi-squared test or Friedman test). As the most exhaustive clinical evaluation was performed 90 days after treatment administration, differences between days 0 and 90 were expressed as absolute values and change from

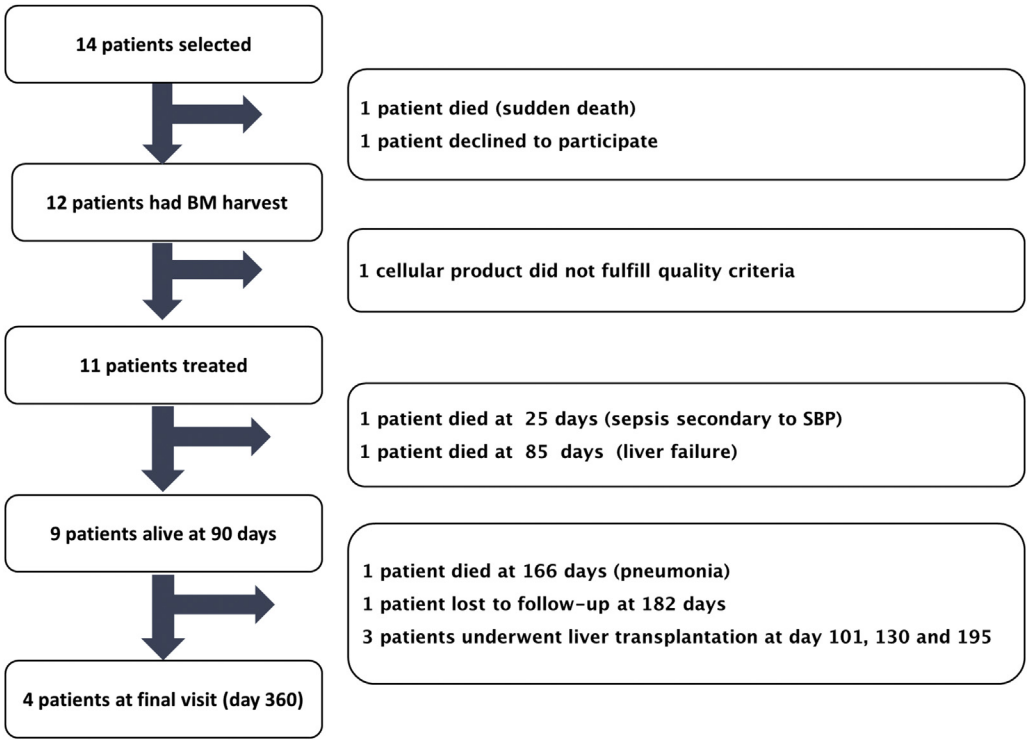

Fig 2. Patients flow chart.

baseline. Safety analysis covered the entire study period. To detect associations between the characteristics of infused cells and changes observed in study variables, differences in phenotype and cytokine production for patients who improved their liver function and portal pressure and patients who did not were compared using *U* Mann–Whitney test. Correlations between cell characteristics and clinical parameters were analyzed using Pearson and Spearman test according to the characteristics of the variables. A P value < 0.05 was considered significant. SPSS software version 20 was used for all statistical analysis.

RESULTS

Patients. Fourteen patients with a severely decompensated cirrhosis were screened, half of them in Child-Pugh C stage. Eight patients had hepatitis C-related liver cirrhosis and 6 patients alcoholic cirrhosis. All alcoholic patients had at least 6 months of abstinence before inclusion. Regarding patients with hepatitis C-related cirrhosis, all of them where viremic and did not received any antiviral treatment in the year prior the inclusion in the study or after study inclusion. Five patients were under evaluation for liver transplantation at the time of inclusion. Five patients have refractory ascites and underwent evacuative paracentesis whenever clinically indicated. These patients received albumin infusions after large volume paracentesis. All had a preserved renal function. One patient suddenly died during the screening phase before bone marrow was harvested and another one declined to participate before any study procedure. Twelve patients had finally their bone marrow harvested (Fig 2). Detailed demographic and clinical characteristics of the 12 patients are summarized in Table I.

Feasibility. Bone marrow harvest was feasible in all patients. In one case, the cells obtained after culture did not fulfill the phenotypic criteria established in the protocol to qualify for EPC, and the patient was finally excluded. In another case, a bacterial contamination of the cell product was suspected on Gram staining, the product was discarded and a second bone marrow harvest yielded a cell product suitable for administration. Therefore, 11 patients received the experimental treatment (Fig 2). Treatment was administered in all cases according to the protocol previously described. Six of the 11 treated patients received the treatment directly through the main hepatic artery, whereas in the remaining 5 patients, the treatment was administered in its branches because of variants in the hepatic arterial anatomy. The global feasibility was 91%. There was no need of plasma or platelets transfusion before or after any study procedure.

Characteristics of cellular product. Cellular viability was always higher than 90%. The total amount of cells finally available for administration was very

Table I. Baseline characteristics of patients undergoing bone marrow harvest (n = 12)

Variable	Value
Age, years	58 (45–75)
Sex, males	77%
Etiology (%)	
Alcohol	54%
HCV	46%
Bilirubin, mg/dL	3.89 (0.84–23.3)
Creatinine (mg/dL)	0.9 (0.4)
International normalized ratio	1.45 (1.1–2.02)
Previous episodes of encephalopathy (%)	44%
Ascites (%)	100%
Refractory	41%
Child-Pugh (points)	10 (8–12)
MELD score	16 (11–22)
MELD-Na score	(16–25)

MELD, model for end-stage liver disease.
Data are expressed as median (min-max), unless specified.

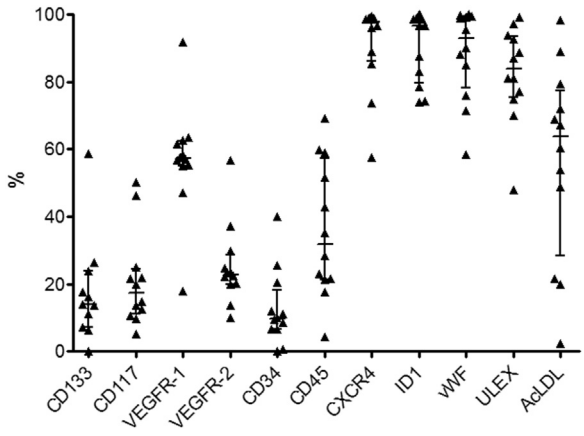

Fig 3. Phenotype characteristics of final cellular product (n = 12).

heterogeneous ranging from 8.45×10^6 to 450×10^6 cells. This cell number was independent of the volume of bone marrow blood withdrawn and of the degree of cytopenia in peripheral blood (although platelet count tended to inversely correlated with the number of cells obtained; Supplementary Fig 1) but directly correlated with the degree of liver insufficiency at baseline expressed as MELD score (R: 0.75, $P = 0.004$; Supplementary Fig 2). The number of cells obtained tended to decrease as the age of patients increased (R 0.5; P 0.059; Supplementary Fig 1). The phenotype of the final cell suspension and its ability to produce cytokines and growth factors varied among patients. There was a high and homogeneous expression of endothelial markers among the cells obtained from different patients, such as vWF and acLDL (acetylated-low density lipoprotein). Similarly, the expression of markers of functionality of EPC, such as Ulex binding ability, was very high in almost all cellular products. The expression of progenitor markers was more heterogeneous among different patients. No relationship was found between age, liver function at baseline, and the etiology of liver disease and phenotypic cell features. A complete characterization of the final cell product and the ability to produce cytokines *in vitro* are shown in Fig 3 and Table II, respectively.

The number of cells administered varied from 8.9×10^6 to 100×10^6 (median 46.6×10^6) as a result of the large variability in the number of cells obtained.

Safety and survival. Treatment was very well tolerated, and no adverse events were related to the cell product. Regarding the short-term safety and tolerability, a single case of mild abdominal pain during arteriography responsive to intravenous paracetamol was the only adverse event related to study procedures. All the patients were hospitalized for overnight observation after the treatment. No significant alterations of blood pressure, heart rate, body temperature, peripheral oxygen saturation, and urinary output, were observed in the 24 hours after the treatment. All of patients were discharged after overnight observation. Regarding the long-term safety and tolerability, along the 12 months of follow-up, as expected in patients with fairly advanced cirrhosis, the number of adverse events including severe adverse events not related to treatment or study procedures was high. The majority were complications of cirrhosis, such as ascites requiring evacuative paracentesis, encephalopathy grade II, variceal bleeding, and hepatorenal syndrome. A detailed list of severe adverse events observed during the study is provided in Table III.

Three patients died during follow-up, and they all had a severe disease with Child-Pugh scores of 11, 11, and 12 at screening. The first patient died at day 25 because of sepsis secondary to spontaneous bacterial peritonitis (SBP) and had a previous episode of SBP before inclusion. According to the follow-up protocol of the clinical trial, this patient was seen at the institutional outpatient clinic 7 and 15 days after the cell administration. Hemogram, liver, and renal function tests did not show any sign of infection or renal dysfunction nor significant changes as compared with previous laboratory results. Twenty-one days after the treatment, she was admitted because of fever, abdominal pain, oliguria, and tense ascites. Blood analysis showed hepatorenal syndrome type 1 and ascitic and blood culture revealed SBP due to *Klebsiella pneumoniae* with bacteremia. The patient died with multiorgan failure 48 hours after admission, despite the prompt antibiotic and vasopressor treatment. The second patient died at day 85 in a distant hospital

Table II. Ability to produce cytokines in vitro (n = 12)

Cytokines	Concentration
VEGF (pg/mL)	37 (0–1420; IQR 234)
HGF (pg/mL)	1076 (0–10315; IQR 1760)
IGF-1 (pg/mL)	8.5 (0–1076; IQR 193)
Cardiotrophin (pg/mL)	18 (0–1000; IQR 490)
EGF (pg/mL)	3.6 (0–168; IQR 9.7)
IL-6 (pg/mL)	28.6 (0–543; IQR 63)
SDF-1 (pg/mL)	11.2 (0–1324; IQR 247)

Data are expressed as median (min-max; interquartile range). The concentration of cytokines/mL is expressed per 1*10E6 cells recovered.

Table III. Severe adverse events list

Type of SAE	Frequency	Outcome
Ascites (paracentesis)	5	Resolved
Encephalopathy, grade 2	3	Resolved
Spontaneous bacterial peritonitis	3	2 resolved, 1 dead*
Respiratory infection	3	2 resolved, 1 dead
Upper gastrointestinal bleeding	3	Resolved†
Hepatorenal syndrome	2	1 resolved, 1 dead*
HCC	1	Resolved‡
Toracoascites	1	Resolved
Sudden death	1	Dead§
Sepsis	1	Dead*
Head trauma	1	Resolved
Liver failure	1	Dead
Umbilical hernia	1	Resolved

*The patient was admitted because of sepsis and hepatorenal syndrome secondary to SBP.
†In one case, the gastrointestinal bleeding was not related to portal hypertension.
‡Liver transplantation.
§Before bone marrow harvest.

because of encephalopathy and suspected respiratory infection. The third patient died at day 166 because of pneumonia and had a prior diagnosis of chronic obstructive pulmonary disease. One patient was diagnosed of hepatocellular carcinoma 5 weeks after treatment. The tumor was not observed in the liver US performed at screening. Portal vein thrombosis was suspected in the liver Doppler US performed by protocol at day 30. An magnetic resonance imaging ruled out thrombosis but detected a 27-mm nodule with noninvasive criteria of hepatocellular carcinoma. His alpha-fetoprotein levels were below 10 ng/mL at the inclusion and did not show significant changes during the follow-up. The patient had been listed for liver transplantation before inclusion was transplanted 7 months after cell therapy and is still alive and free of recurrence 3 years after transplantation. Three episodes of upper gastrointestinal bleeding were observed after treatment. One occurred 4 weeks after treatment and was due to arterial bleeding at the enteric anastomosis of previous bariatric surgery (not related to portal hypertension). Two patients had variceal bleeding 17 and 19 weeks after treatment. One patient was lost to follow-up at day 182.

Six patients were treated while awaiting liver transplantation. Three were transplanted 101, 130, and 195 days after cell therapy. Two abandoned the waiting list at the end of the follow-up when a relevant improvement in liver function was observed. Their MELD scores improved from 12 to 9 and from 17 to 13, respectively, from days 0 to 360. One patient was removed from the waiting list caused of recurrence of alcohol consumption (Fig 2). None of the patients removed from the waiting list developed hepatocellular carcinoma 2 years after the end of the study.

Changes in liver function and portal hypertension, and complications of cirrhosis. Nine patients were alive 3 months after cell therapy and most of them showed stable or slightly decreased liver function scores. Compared with baseline, MELD score decreased in 6 patients at day 90. The changes in MELD score observed along the first 3 months after treatment were statistically significant ($P = 0.047$). The decrease in MELD score was particularly relevant 2 months after cells administration (median change at day 60: −2 points, range 0 to −4; $P = 0.027$) compared with baseline (Supplementary table II). Similarly, Child-Pugh score decreased in 5 patients although change from baseline did not achieve statistical significance at month 3 (median change: −1 point, range −3 to +1 points). Looking at individual liver function parameters, a significant improvement at month 3 was observed in international normalized ratio (prothrombin time) but not in bilirubin, albumin, or ammonia. HVPG decreased in 5 patients, remained stable in 2 and worsened in the other 2 patients. Median change in HVPG was −10% (range: −27 to +40%; ns; Fig 4). In the 5 patients in which HVPG improved, decrease ranged from −12 to −27% (median −16%), whereas in the 2 patients in which HVPG worsened, increase ranged from 6% to 40% (median 23%). Individual changes in MELD score and HVPG are represented in Fig 4. Liver enzymes showed fluctuations after therapy but always remained below 1.5 × upper normal limit (Supplementary table II). No effect was observed on liver fibrosis scores (Fibrosis-4 score and AST to platelet ratio index), indocyanine green clearance, liver stiffness, or severity of ascites, in terms of number and volume of paracentesis (data not shown).

Relation between cell product characteristics and changes in liver function and portal hypertension. The improvement in liver function was not related to the

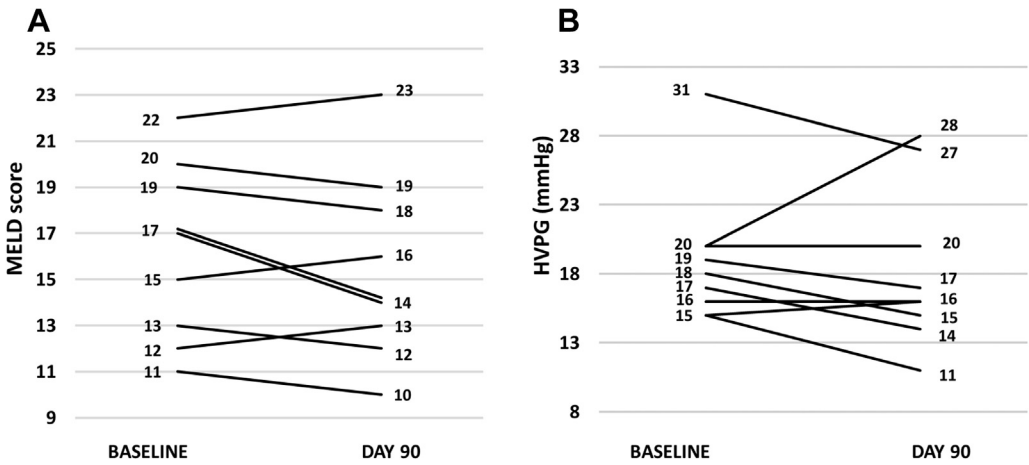

Fig 4. Changes in MELD score (A) and HVPG (B) from baseline to day 90 (n = 9).

total number of cells administered (Supplementary Fig 3). However, a direct correlation between liver function scores decrease and the percentage of cells expressing specific EPC markers in the final cell suspension was observed. Patients in whom MELD score decreased at 3 months received a significantly higher amount of cells expressing VEGFR-2 (13.8% vs 22.9%; $P = 0.024$), vWF (76% vs 94.4%; $P = 0.048$), and acLDL (21.7% vs 75.6%; $P = 0.048$; Fig 5A). Similarly, patients in whom Child-Pugh score decreased at 90 days tended to receive a higher amount of cells expressing VEGFR-2 (16.8% vs 24.6%; $P = 0.032$) and wVF (85.7 vs 99.4%; $P = 0.063$; Fig 5B). Contrary, no significant differences in surface markers were detected between patients who improved their HVPG and patient who did not. However, a direct correlation was observed between expression of acLDL and vWF and changes in liver function and HVPG (Fig 6). There was no relationship between baseline MELD and Child-Pugh scores and HVPG and their changes after treatment. Supplementary Table III summarizes individual patient's data, including the characteristics of the cell product, the etiology of the liver disease and the main changes in liver function and portal pressure.

The production of cytokines and growth factors by EPC including VEGF, EGF, HGF, IGF, CT-1, IL-6, and SDF was heterogeneous. A trend for a higher production of HGF, SDF and IGF-1, and lower production of IL-6 was observed in cells administered to patients in whom liver function improved at 3 months compared with patients who did not improved, although statistical significance was not reached. A higher production of VEGF, HGF and IGF, and a lower production of IL-6 was in patients with an improvement in HVPG, although differences were again not statistically significant.

DISCUSSION

The administration of hematopoietic and mesenchymal stem cells derived from bone marrow or from peripheral blood for the treatment of liver cirrhosis has been explored in a number of clinical trials in the last 20 years. Most of these studies used unselected cell populations, whereas others used immunoselected CD34 + or CD133 + fractions. A small number of studies tested the transplant of fetal liver-derived epithelial cell adhesion molecule + cells in different types of chronic liver diseases including congenital biliary atresia and liver cirrhosis. Transplantation of these cells was found to be safe and seems to improve the quality of life and liver function.[29] In this pilot clinical trial, an EPC-enriched cell suspension was generated from autologous bone marrow blood of patients with end-stage liver cirrhosis and administered in the hepatic artery in an attempt to assess the feasibility, the safety and the possible effect of this treatment on liver function and portal hypertension. To our knowledge, this is the first clinical trial in which this specific cell type, derived from autologous bone marrow, is administered in patients with decompensated liver cirrhosis. Bone marrow harvest was safe in all patients and, in fact, there was no need of plasma or platelets transfusion before or after any study procedure. As reported in previous studies,[12,14] our results confirm that the administration of bone-marrow derived cell suspensions through the hepatic artery is a safe procedure in cirrhotic patients. Since more than 25 years, the intrarterial administration of drugs or therapeutic devices is extensively used for the locoregional treatment of liver tumors in cirrhotic patients.[30] Compared with the intraportal injection, this technique carries less bleeding risk, especially considering the coagulation impairment that characterizes this population. Compared with the peripheral

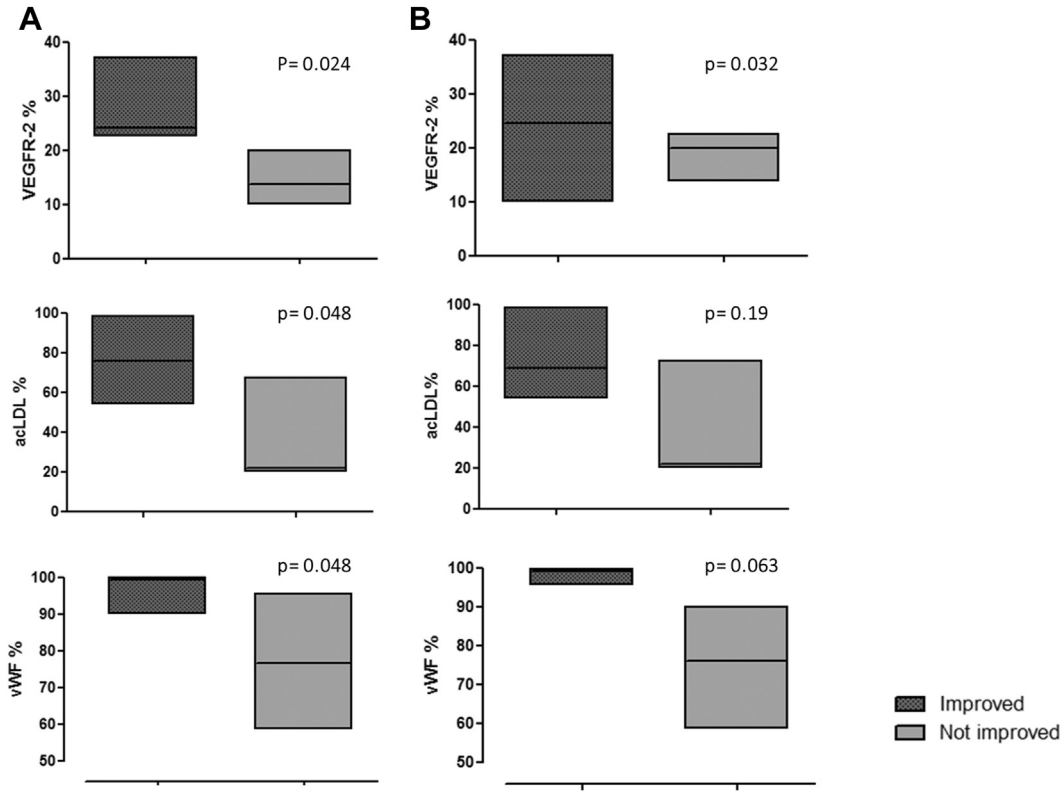

Fig 5. Association between phenotype characteristics and changes in MELD score (A) and Child-Pugh score (B) from baseline to day 90 (n = 9).

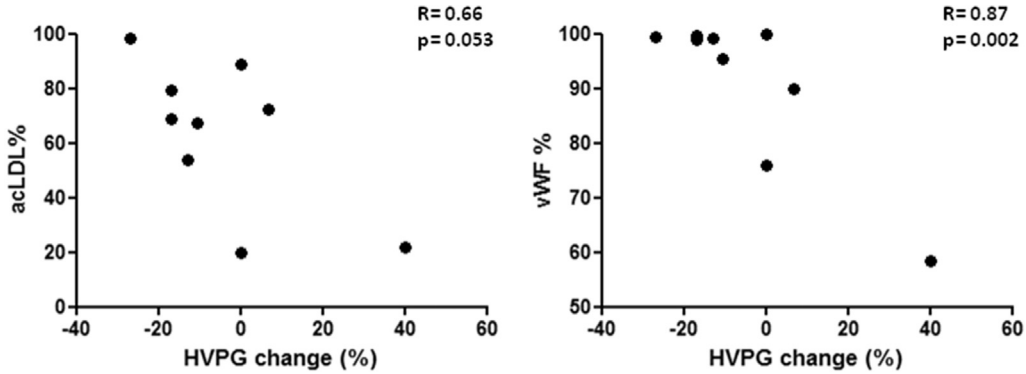

Fig 6. Correlation between acLDL and vWF expression and changes in HVPG (%) from baseline to day 90 (n = 9).

vein injection, we believe that the administration of the cellular product through the hepatic artery allows the highest number of cells reaching the liver parenchyma. Indeed, previous studies using Tc99-marked human fetal liver-derived stem cells injected in the hepatic artery showed that most of the marked cells remained within the liver lobe injected.[31] Unfortunately, in this study, as well as in most of previous clinical trials, since the cellular product is autologous and not labeled with a radioisotope, we were not able to demonstrate the presence of the therapeutic cells in the liver of treated patients. Similarly, due to the study design, it is impossible to assess whether the effect of the treatment is because of the paracrine action of products released by the injected cells on liver-resident cells or it is due to an endocrine effect of cells products released in the bloodstream. However, the relatively late effects observed in liver function and portal hypertension,

between 60 and 90 days after treatment, suggest that this effect is mediated by changes in the resident cells in the liver rather than through an endocrine effect mediated by the release of cells in the peripheral circulation.

No complications related to the procedure were observed in this trial. Despite the potential harm of administering a cellular suspension in the hepatic artery could be an acute increase of the sinusoidal pressure, no bleeding complications were observed in the short term, and a decrease in HVPG was later observed in most patients. The long interval (>3 months) between cell therapy and the 2 episodes of variceal bleeding makes unlikely a causal relation. Moreover, no patients showed a worsening of the severity of their ascites and none of them required an increase of diuretics dosage after the injection. Most adverse events were those expected in a population with end-stage cirrhosis, and none were considered related to the study procedure or the experimental agent.

Regarding feasibility, a suitable therapeutic suspension was obtained in more than 90% of patients. However, the number and the type of cells obtained varied among different individuals and the reason of this variability was not completely understood. In the present study, we observed that the number of cells obtained tended to decrease as the age of patients increased; however, there was no correlation between the phenotypic characteristics of the final cellular suspension and the age of patients, suggesting that bone marrow aging does not determine the ability of EPC generation. In addition, we did not observe any significant relation between the degree of peripheral cytopenia and the phenotype of cells obtained, even the platelets count tended to inversely correlate with the number of cells obtained, suggesting that other factors may drive the differentiation into EPC. Interestingly, it seems that the severity of cirrhosis and not its determining cause, could affect the number of cells obtained from bone marrow, perhaps revealing an attempt of the bone marrow to mobilize different cellular types involved in repair processes during chronic liver injury, as suggested by other investigators.[32] Accordingly, other investigators reported an increased number of circulating EPC in patients with liver cirrhosis, a feature that appears to be in direct correlation with the severity of liver disease.[33]

However, no correlation was found between the characteristics of isolated cells and the severity of liver dysfunction, meaning that the stage of cirrhosis does not determine the characteristics of the final cell product, at least in the conditions used in this study. Other factors should therefore be investigated. Although in animal models mononuclear bone marrow cells cultured for 7 days result in EPC with an "early-like phenotype,"[9] the phenotype of cells in this series resembles more closely "late-EPC" with a lower expression of progenitor markers and higher expression of functionally active EPC.

Considering the poor baseline conditions of the patients included in this study, since all of them suffered severely decompensated cirrhosis, the transient but significant improvement in liver function observed during the 3 months after treatment, reflected by the MELD score, may suggest that these cells could have a potential therapeutic effect on liver dysfunction. The lack of correlation between MELD score and hepatic venous pressure gradient values at baseline and their changes at 3 months suggest that improved liver function was related to treatment and not to the baseline conditions. No correlation between the number of cells administered and the intensity of changes in liver function or HVPG was observed. However, it should be noted that the higher the amount of administered cells expressing endothelial markers, the stronger the clinical benefit. Particularly, patients who received a higher proportion of cells expressing VEGFR-2, acLDL, and vWF showed greater improvement in liver function and portal hypertension and the intensity of acLDL and vWF positively correlated with the intensity of changes in MELD score and hepatic venous pressure gradient. Interestingly, previous studies showed the increased ability of late EPC compared with early EPC, to produce nitric oxide in response to VEGF.[22] It could be hypothesized that this characteristic accounted for the improvement of liver function and portal hypertension observed in the present study among patients who received an higher proportion of cells expressing VEGFR-2, acLDL, and vWF and able to produce higher amounts of VEGF.

In the last years, the role of EPC in liver dysfunction and portal hypertension has been matter of debate.[33,34] The results of this study not only open new therapeutic avenues for liver cirrhosis that is worth exploring but also argue in favor of the hepatoprotective role of these cells, suggesting that their increase in cirrhotic patients may be a bone marrow response to alleviate liver injury. The bone marrow-derived cells used in the present study produced a variety of hepatoprotective growth factors including HGF and IGF-I. This finding may suggest that local production of cytoprotective molecules may underline the potential clinical benefit of autologous EPC. Since, according to the manufacturing process established for this study, EPC were carefully washed before its administration and given the low dose of cytokines and growth factors used in the culture, it is extremely unlikely that the effects observed in liver function and portal hypertension 3 months later are mediated by the use of these growth factors for the culture of EPC. This pilot trial clearly demonstrated the feasibility and safety of

autologous EPC administration via the hepatic artery. The lack of a control arm prohibits definitive conclusions regarding clinical benefit. However, our preliminary data are encouraging and provide useful information for the design of future controlled prospective clinical trials. These studies should take in consideration the phenotypic properties of bone marrow-derived EPC and the possibility of repeated EPC injections. Moreover, since one important insight of this study, is that the higher amount of functional EPC is administered, the stronger clinical effect is observed, the design of next studies should take into account the possibility of sorting EPC before its administration to administer a more EPC-enriched cellular suspension.

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SUPPLEMENTARY DATA

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Appendix

Supplementary Table I. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Child-Pugh score B 8 or higher	Age <18 or >75 years
Ability to sign informed consent	Upper gastrointestinal bleeding or severe infection in the 30 days before inclusion
Ability to follow the study protocol	Chronic encephalopathy that makes impossible the informed consent and the participation in the study
	Hepatocellular carcinoma (including previous)
	Other malignant tumors (except in situ tumors or nonmelanoma skin tumors) in the 5 years before inclusion
	Severe extrahepatic acute disease in the 30 days before inclusion
	Any other chronic decompensated disease
	Any contraindication for the study procedures
	Pregnancy or breast-feeding
	Treatment with experimental agent in the 30 days before the inclusion

Supplementary Table II. Main changes in clinical variables observed during the first 90 days after treatment (n = 9)

Variable	Day 0	Day 30	Day 60	Day 90	Median delta, days 0–90	% Change days 0–90	Patients improved at day 90 (n)
Bilirubin (mg/dL)	3.5 (4.1)	3.9 (3)	3.4 (4)	3 (4.4)	−0.3 (1)	−8 (49)	6
INR	1.5 (0.4)	1.5 (0.3)	1.50 (0.2)	1.4 (0.4)	−0.1 (0.05)	−6 (5)	7
Albumin (g/dL)	2.7 (0.8)	2.9 (0.9)	2.6 (0.8)	2.9 (0.6)	+0.3 (0.7)	+9 (30)	3
AST (X UNL)*	1.02 (0.2)	1.08 (0.2)	1.39(1.0)	1.4 (1.1)	+0.16 (0.4)	+22.2 (42)	1
ALT (X UNL)*	0.4(0.0)	0.4 (0.2)	0.56 (0.5)	0.58(0.5)	+0.15 (0.2)	+36.5(56)	1
γGT (X UNL)*	0.73 (0.2)	0.76 (0.5)	0.92 (0.5)	0.55 (0.5)	0.0 (0.2)	0.0 (41)	3
ALP (X UNL)*	0.87 (0.4)	0.89 (0.0)	1.14 (0.5)	0.98 (0.3)	+0.01 (0.4)	+2.2 (60)	2
Creatinine mg/dL	1 (0.6)	0.8 (0.4)	0.9 (0.2)	0.9 (0.4)	0.0 (0.2)	−7 (75)	NA
Sodium (mEq/L)	135 (4)	134 (2)	135 (5)	134 (6)	−1 (3)	−0.7 (2.6)	5
Ammonia	76 (86)	74 (76)	52 (50)	63 (75)	20 (55)	22 (47)	7
HVPG (mm Hg)	18 (5)	NA	NA	16 (8.5)	−2 (4)	−10.5 (20)	5
MELD	17 (7)	16 (15)	15 (7)	14(6)	−1 (3)	−5.2 (19)	6
MELD-Na	19 (3)	20 (4)	18 (6)	19 (5)	−1 (2)	−6 (17)	6
Child-Pugh	9 (3)	10 (3)	9 (2)	9 (3)	−1 (1)	−9.1 (12.5)	5

Abbreviations: *AST*, aspartate aminotransferase; *ALT*, alanine aminotransferase; *γGT*, gamma-gutamyl tranferase; *ALP*, alcaline phosphatase; *HVPG*, hepatic venous pressure gradient; *UNL*, upper normal limit.
Data are expressed as median (interquartile range).
NA, not applicable since creatinine was lower than 1.4 mg/dL in all patients both, before, and after treatments.
*Liver enzymes are expressed in value × upper normal limit since during the study the laboratory changed their normal value ranges.

Supplementary Table III. Changes in liver function and portal pressure among treated patients (n = 11) in relation to number and phenotype of cells administered and etiology of liver disease

						MELD		Child-Pugh		HVPG (mm Hg)	
Patient ID	**Etiology**	**Number of cells**	**VEGFR-2 %**	**vWF %**	**acLDL %**	**Day 0**	**Day 90**	**Day 0**	**Day 90**	**Day 0**	**Day 90**
Patient 1	HCV	40×10^6	23.2	98.8	79.2	19	18	11	11	18	15
Patient 2	Alcohol	34×10^6	29.9	99.4	98.3	20	19	12	9	15	11
Patient 3	Alcohol	86×10^6	22.6	90.0	72.1	17	14	8	7	15	16
Patient 4	HCV	71×10^6	19.9	58.4	21.7	15	16	9	10	20	28
Patient 5	HCV	8.9×10^6	56.8	85.0	60.4	13	NA	12	NA	24	NA
Patient 6	Alcohol	13×10^6	13.8	76.0	20.0	12	13	9	9	16	16
Patient 7	HCV	107×10^6	10.0	95.5	67.2	22	23	12	12	19	17
Patient 8	Alcohol	16×10^6	37.1	99.6	68.8	11	10	9	8	18	15
Patient 9	Alcohol	116×10^6	20.1	71.5	2.35	27	NA	11	NA	18	NA
Patient 10	Alcohol	46×10^6	24.6	99.1	53.8	17	14	11	10	31	27
Patient 11	HCV	82×10^6	23.6	100	89.0	13	12	8	7	20	20

NA, not applicable since these 2 patients were not alive at day 90.