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Characterization of lymphocyte subsets in ascitic fluid and peripheral blood of decompensated cirrhotic patients with chronic hepatitis C and alcoholic liver disease: A pivotal study

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Abstract

Hepatitis C virus and alcoholic liver disease are major causes of chronic liver diseases worldwide. Little is known about differences between chronic hepatitis C and alcoholic liver disease in terms of lymphocytes' sub-population. Aim of the present study was to compare the sub-populations of lymphocytes in both ascitic compartment and peripheral blood in patients with decompensated liver cirrhosis due to chronic hepatitis C and alcoholic liver disease. Patients with decompensated liver cirrhosis due to hepatitis C virus or alcoholic liver disease evaluated from April 2014 to October 2016 were enrolled. Whole blood and ascitic fluid samples were stained with monoclonal antibodies specific for human TCRαβ, TCRγδ, CD3, CD4, CD8, CD19, CCR6, CD16, CD56, CD25, HLA-DR, Vα24. Sixteen patients with decompensated liver cirrhosis were recruited (9 with hepatitis C virus and 7 with alcoholic liver disease). In ascitic fluid, the percentage of both $CD3^+CD56^-$ and $CD3^+CD56^+$iNKT cells resulted higher in hepatitis C virus patients than in alcoholic liver disease patients ($1.82 \pm 0.35\%$ vs $0.70 \pm 0.42\%$ ($p < 0.001$) and $1.42 \pm 0.35\%$ vs $0.50 \pm 0.30\%$ ($p < 0.001$), respectively). Conversely, in peripheral blood samples, both $CD3^+CD56^-$ and $CD3^+CD56^+$iNKT cells resulted significantly higher in alcoholic liver disease than in hepatitis C virus patients ($4.70 \pm 2.69\%$ vs $1.50 \pm 1.21\%$ ($p < 0.01$) and $3.10 \pm 1.76\%$ vs $1.00 \pm 0.70\%$ ($p < 0.01$), respectively). Both elevation of iNKT cells in ascitic fluid and reduction in peripheral blood registered in hepatitis C virus but not in alcoholic liver disease patients might be considered indirect signals of tissutal translocation. In conclusion, we described relevant differences between the two groups. Alcoholic liver disease patients displayed lower number of $CD3^+CD4^+$ cells and a higher percentage of $CD3^-CD16^+$, $V\alpha24^+CD3^+CD56^-$ and $V\alpha24^+CD3^+CD56^+$iNKT cells in ascitic fluid than hepatitis C virus positive subjects. Further studies might analyze the role of immune cells in the vulnerability toward infections and detect potential targets for new treatments especially for alcoholic liver disease patients.

Keywords

ascites, decompensated cirrhosis, inflammatory response, lymphocyte immunophenotyping, natural killer T cells

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Introduction

Hepatitis C virus (HCV) infection and alcoholic liver disease (ALD) are the two major causes of chronic liver diseases in developed countries.[1] The onset of ascites in a cirrhotic patient generally runs in parallel with deterioration of clinical status and poor prognosis.[2] While the overall cirrhosis mortality rate has falling in the last three decades,[3] HCV mortality rate have been increasing since 1990s.[4] Little is known regarding the differences and/or similarities between HCV and ALD at the cellular level[5] and, to the best of our knowledge, few data on human peritoneal cells and lymphocyte immunophenotyping are available. Notably, susceptibility to bacterial infections due to cirrhosis-associated immune dysfunction and bacterial translocation through gut mucosa, represent common findings in patients with decompensated cirrhosis.[6] In particular, infections play a chief role in the onset of acute-on-chronic liver failure.[7]

The epidemiological relevance of both chronic hepatitis C (CHC) and ALD and the well-known differences between these two disorders in terms of relationship between pathogenic *noxae* and immune system, explains the scientific interest of the study. The main role of infections (especially bacterial ones) in the natural history of advanced liver disease and the consequent weight of immune system represent other reasons of attention. Furthermore, to analyze the cellular subpopulations in two different sites (blood and ascites) seems to be interesting to characterize the cells translocation.

The aim of this pivotal study was to analyze and compare the sub-population of lymphocytes in the ascitic compartment and in the peripheral blood in patients with decompensated liver cirrhosis due to CHC and ALD.

Materials and methods

Patients

Patients with decompensated cirrhosis due to CHC or ALD consecutively subjected to paracentesis from April 2014 to October 2016 were prospectively recruited in the present pivotal study.

The following exclusion criteria were considered: ongoing or recent bacterial infection, recent antibiotic consumption (<4 weeks), presence of Transjugular intrahepatic portosystemic shunt, hepatocellular carcinoma and liver transplant. Selection of patients according to the study criteria has been described in the Supplementary Figure 1.

The diagnosis of liver cirrhosis was based on liver biopsy or indirect sign of advanced liver disease (at blood test, Ultrasound and Transient Elastography). Child-Pugh score and Model for end-stage liver disease (MELD) score were evaluated by laboratory findings.

We classified enrolled patients in two groups according to etiology of liver disease. HCV group included patients with diagnosis of cirrhosis, ascites and active HCV infection (positive HCV-RNA level) without other causes of chronic liver damage. An additional specific exclusion criterion for this group was the absence of antiviral therapy over the past 6 months. ALD group comprised patients with diagnosis of cirrhosis, ascites and alcohol use disorder again without other causes of chronic liver damage. An additional specific exclusion criterion for this group was a minimum period of abstinence of 6 months.

Baseline evaluations included clinical history with special focus to alcohol consuming, non-invasive arterial pressure and blood tests. We registered the following serum biochemical parameters: hemoglobin, hematocrit, creatinine, total bilirubin, electrolytes (sodium, potassium and calcium), albumin, INR, alanine aminotransferase, aspartate aminotransferase, gamma glutamyl-transpeptidase, alkaline phosphatase, ferritin and anti-nuclear, anti-smooth muscle and anti-liver kidney microsome type 1 antibodies (data not shown). Screening for hepatitis virus infection comprised anti-HCV antibodies, evaluation of HCV-RNA copies and anti-hepatitis B antibodies.

The study protocol was in conformity with the ethical guidelines of the 1975 Declaration of Helsinki. All recruited patients gave their written informed consent to undergo to all procedures foreseen by the study (including paracentesis).

Immunophenotype of lymphocytes of ascitic fluid and peripheral blood

We collected peripheral blood samples and heparinized ascitic fluid samples by paracentesis from all enrolled patients. Full blood and lymphocyte counts were measured using a routine hematology analyzer (Sysmex XE-2100 (Dasit, Italy)). Samples were centrifuged for 15 min at 400 g at

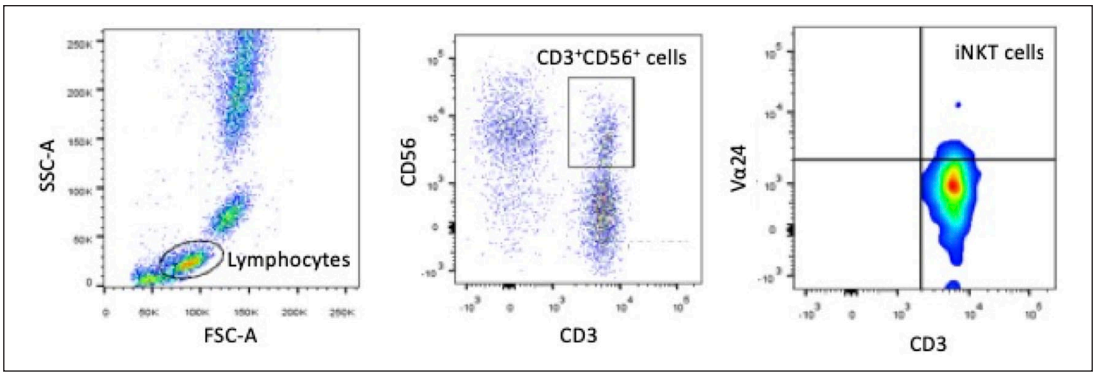

Figure 1. The figure illustrates the gating strategy to discriminate NKT lymphocytes. Lymphocytes are gated according to the side-scattered/plot, and therefore selected for $CD3^{+}CD56^{+}$lymphocytes; among these, lymphocytes expressing the surface Vα24 marker have been sorted.

Table 1. Baseline clinical characteristics of the enrolled patients.

Parameter	All patients	ALD patients	HCV patients	p
Number of patients, n (%)	16	7 (43.7%)	9 (56.3%)	
Age, years	71.81 ± 11.12	70.9 ± 9.73	72.6 ± 12.9	NS
Male sex, n (%)	10 (62.5%)	5 (71.4%)	5 (55.5%)	NS
Child-Turcotte-Pugh mean score	9.27 ± 1.22	9.57 ± 1.62	9.00 ± 0.76	NS
MELD mean score	16.6 ± 4.76	17.29 ± 3.55	16.00 ± 5.81	NS

ALD: alcoholic liver disease; HCV: hepatitis C virus; NS: non-significant; MELD: model for end-stage liver disease.

room temperature within 60 min from collection, supernatants were discarded, and pelleted cells were resuspended in 1 ml of phosphate buffered saline (PBS), counted in a Bürker chamber and finally adjusted to a concentration of 1.0 x 10^6/ml. A hundred microliters of this suspension or 100 µL of heparinized peripheral blood were incubated for 30 min at room temperature by the addition of 50 µL of rabbit IgG (concentration 1 mg/mL) to saturate Fc gamma binding sites. Cells were then stained at room temperature in five different tubes (Becton Dickinson, USA) for 20 min in the dark. They were divided as follows: (1) TCRαβ, TCRɣδ, CD3, CD19; (2) CD16, CD3, CD8, CD56; (3) CD25, CD4, CD8, HLA-DR; (4) Vα24 (invariant NKT (iNKT) cells), CD3, CD56; and (5) CD4, CD8, CCR6 (Figure 1 explains the gating strategy). One milliliter of FACSlyse (Becton Dickinson, USA) was then added to the tubes and incubated for 20 min in the dark. Cells were then washed and after centrifugation resuspended in 1 ml of PBS. Eventually, cells from ascitic fluid samples or peripheral blood samples were assessed by 6-color acquisition system on a FACSCantoII (Becton Dickinson, USA) acquiring at least 50,000 events and analyzed using a FACSDiva software (Becton Dickinson, USA).

Statistical analysis

A physician trained in statistics encoded all samples into a dedicated database in anonymous form. We expressed data as mean (± standard deviation) or median (with range) as applicable. Confidence interval (CI) is presented where appropriate. We performed full descriptive analysis of all analyzed variables. Comparisons between groups were made by Chi-square-test and Fisher's exact test for qualitative variables, and Mann–Whitney U-test for continuous ordinal data. A significance level of 0.05 was considered for all tests. We analyzed data using SPSS 25.0 (IBM, Milan, Italy).

Results

Patients' characteristics

The baseline clinical patterns of patients are shown in Table 1. A total of 16 patients were recruited, almost equally distributed between ALD (7, 43.7%)

and HCV (9, 56.3%) patients. The population was homogeneous in terms of both age and gender.

No differences were detected in terms of Child-Pugh and MELD score between ALD and HCV patients (9.57 ± 1.62 vs 9.00 ± 0.76, p = NS and 17.29 ± 3.55 vs 16.00 ± 5.81, p = NS, respectively).

Immunological analysis

In ascitic fluid (Table 2), the percentage of $CD3^+CD4^+$was statistically lower in the ALD group than in HCV group (40.50 ± 5.30% vs 56.50 ± 6.89% ($p < 0.01$)), whereas the percentage of $CD3^-CD16^+$ cells resulted lower in HCV patients (21.7 ± 6.48% vs 11.12 ± 6.17% ($p < 0.05$)).

Both $V\alpha24^+CD3^+CD56^-$ and $V\alpha24^+CD3^+CD56^+$cells of the ascitic fluid resulted higher in HCV patients than in ALD ones (1.82 ± 0.35% vs 0.70 ± 0.42% ($p < 0.001$) and 1.42 ± 0.35% vs 0.50 ± 0.30% ($p < 0.001$), respectively) (Table 2). Conversely, in peripheral blood samples, the same cells resulted more elevated in ALD patients than in HCV group (4.70 ± 2.69% vs 1.50 ± 1.21% ($p < 0.01$) and 3.10 ± 1.76% vs 1.00 ± 0.70% ($p < 0.01$), respectively) (Table 3).

No other statistically significant differences were detected in ascitic fluid and in peripheral blood samples among the other cell populations (Tables 2 and 3).

Discussion

To our knowledge, this represents the first study that evaluated the differences in terms of lymphocyte subpopulation immunophenotyping between HCV and ALD patients. In particular, we analyzed two districts, peripheral blood and ascitic fluid.

The ascitic fluid of ALD patients resulted poorer in term of $CD3^+CD4^+$ cells and Vα24 $CD3^+CD56^-$ and Vα24 $CD3^+CD56^+$ cells, whereas the percentage of $CD3^-CD16^+$ and $CD3^+CD8^+CD16^+$ resulted lower in the ascitic fluid of HCV patients.

$CD3^+CD4^+$ cells represent the typical T helper (T_H) cells, a subgroup of the adaptive immunity that mediate phagocyte activation and favor the production of antibodies by B lymphocytes.[8] In our study, the percentage of these cells together with the total T lymphocytes ($CD3^+$) tended to be lower in ALD patients (without reaching the statistical significance). These results are in keeping with a previous report[9] and may be related to the alcohol-related impairment of immune response.[10]

Vα24 is the best marker to identify a subset of $CD3^+$ lymphocytes that are restricted for the CD1d

Table 2. Immunophenotype of ascitic fluid.

Antibodies	ALD (%)	HCV (%)	p value
TCR αβ	59.0 ± 9.81	70.67 ± 7.03	NS
TCR γδ	7.0 ± 4.34	3.97 ± 2.38	NS
$CD3^+$	69.1 ± 10.61	78.83 ± 8.32	NS
$CD3^+CD4^+$	40.5 ± 5.30	56.50 ± 6.89	<0.001
$CD3^+CD8^+$	29.1 ± 10.55	22.58 ± 8.61	NS
$CD19^+$	2.4 ± 1.17	4.08 ± 3.44	NS
$CCR6^+CD4^+$	5.9 ± 6.38	7.55 ± 6.04	NS
$CCR6^+CD8^+$	2.1 ± 1.18	3.25 ± 3.60	NS
Vα24 $CD3^+CD56^-$	0.7 ± 0.42	1.82 ± 0.35	<0.001
Vα24 $CD3^+CD56^+$	0.5 ± 0.30	1.42 ± 0.35	<0.001
$CD3^-CD16^+$	21.7 ± 6.48	11.12 ± 6.17	<0.05
$CD3^-CD56^+$	21.9 ± 7.59	11.87 ± 5.81	NS
$CD3^+CD16^+$	2.6 ± 3.06	1.15 ± 0.51	NS
$CD3^+CD56^+$	6.9 ± 5.61	6.05 ± 5.34	NS
$CD3^+CD8^+CD16^+$	1.8 ± 0.97	0.60 ± 0.43	NS
$CD3^+CD8^+CD56^+$	7.3 ± 5.99	4.00 ± 3.40	NS
$CD4^+CD25^+$	2.3 ± 2.17	3.63 ± 2.83	NS
$CD8^+CD25^+$	0.2 ± 0.08	0.20 ± 0,15	NS
$CD4^+DR$	6.4 ± 2.13	5.65 ± 4.09	NS
$CD8^+DR$	10.5 ± 7.77	5.55 ± 3.59	NS

ALD: alcoholic liver disease; HCV: hepatitis C virus; NS: non-significant; TCR: T-cell receptor.

Table 3. Immunophenotype of peripheral blood.

Antibody	ALD (%)	HCV (%)	p value
TCR αβ	65.7 ± 9.88	62.8 ± 8.42	NS
TCR γδ	3.9 ± 2.24	4.5 ± 3.85	NS
$CD3^+$	71.1 ± 8.26	70.0 ± 8.53	NS
$CD3^+CD4^+$	46.1 ± 13.50	49.8 ± 10.58	NS
$CD3^+CD8^+$	24.1 ± 11.20	19.2 ± 8.82	NS
$CD19^+$	5.7 ± 6.46	10.0 ± 7.35	NS
$CCR6^+CD4^+$	15.3 ± 9.50	8.7 ± 7.52	NS
$CCR6^+CD8^+$	1.4 ± 0.64	1.2 ± 0.96	NS
Vα24 $CD3^+CD56^-$	4.7 ± 2.69	1.5 ± 1.21	<0.01
Vα24 $CD3^+CD56^+$	3.1 ± 1.76	1.0 ± 0.70	<0.01
$CD3^-CD16^+$	15.2 ± 6.52	10.8 ± 6.24	NS
$CD3^-CD56^+$	14.4 ± 6.49	10.9 ± 5.86	NS
$CD3^+CD16^+$	1.3 ± 1.65	0.3 ± 0.42	NS
$CD3^+CD56^+$	7.2 ± 6.34	8.1 ± 6.81	NS
$CD3^+CD8^+CD16^+$	1.7 ± 1.37	0.3 ± 0.21	NS
$CD3^+CD8^+CD56^+$	7.6 ± 6.60	3.6 ± 3.15	NS
$CD4^+CD25^+$	2.9 ± 1.26	2.4 ± 1.23	NS
$CD8^+CD25^+$	0.3 ± 0.40	0.4 ± 0.59	NS
$CD4^+DR$	5.1 ± 3.03	4.9 ± 2.41	NS
$CD8^+DR$	9.2 ± 7.98	4.8 ± 3.98	NS

ALD: alcoholic liver disease; HCV: hepatitis C virus; NS: non significant; TCR: T-cell receptor.

and are called invariant natural killer T (iNKT) cells.[11,12] Albeit their name recall the natural killer (NK) cells, the classical CDs associated with this subset (CD16 and CD56) are expressed only by a portion of iNKT cells and their appearance is likely to be linked to a late stage of cell differentiation.[13] These cells have been extensively studied in mice, whereas investigations in humans are still lacking.[12] Overall, iNKT cells seem to play a pivotal role in the early phases of antitumor, autoimmune, allergic, inflammatory and antimicrobial responses.[14] In our study, the percentage of iNKT in the ascitic fluid of patients with ALD was significantly lower compared to the HCV group, either when we consider the CD56 non-expressing and expressing cells.

Mathews et al.[15] developed a chronic-plus-binge ethanol feeding murine models demonstrating that iNKT cells are pivotal in the development of liver injury exclusively in the first stages of liver disease.[15] More in depth, only the Vα24 subset of NKT cells (also called type I NKT) seems to be accountable for liver damage, whereas the so-called type II NKT cells (not-invariant) are not involved in the disease process.[16] Our study analyses cirrhotic patients with a long disease course and with an alcohol-free period of at least 6 months. Arguably, these two features may explain the lower percentage of iNKT cells in the ALD group if compared to HCV patients.

Regarding HCV group, iNKT cells seem to be protective against HCV infection.[17] Some studies showed few amount of iNKT cells in both liver and peripheral blood of HCV positive patients, whereas other authors did not confirm this assumption.[18–20] An elegant study demonstrated that while NKT cells from patients without cirrhosis have a regulatory role by balancing anti-fibrotic (interferon (IFN)) and pro-fibrotic (interleukin (IL)-4 and IL-13) cytokines, in HCV cirrhotic patients these cells would acquire a pure pro-fibrotic phenotype.[21] Moreover, the amount of liver-infiltrating NKT cells seems to correlate with the severity of inflammation and fibrosis.[22] We found higher percentage of iNKT cells in ascitic fluid of HCV patients when compared to the ALD group. In addition, we reported a significant reduction of the same cells in peripheral blood samples of HCV patients. These data might indicate a leukocyte migration toward the peritoneal cavity and therefore an indirect sign of tissutal activation and proliferation. It is well known that iNKT cells may contribute to liver injury and fibrosis through lysis, induction of hepatocytes' apoptosis and production of pro-inflammatory mediators.[23] In addition, the reduction of iNKT cells in peripheral blood samples is in keeping with literature reports that correlate this depletion to the persistence of HCV infection.[23] Further studies might investigate the role of these cells at different stages of liver disease.

$CD3^{-}CD16^{+}$ and $CD3^{-}CD56^{+}$ are markers for the identification of NK cells, a group of cells that lack antigen-specific cell receptor, do not require antigen presentation to initiate their effector function and that utilize different killing mechanism to exert cytotoxicity.[24] In our study, the level of NK cells not expressing the CD16 (the receptor that mediate the binding of the Fc portion of IgG antibodies activating the antibody-dependent cell-mediated cytotoxicity (ADCC)) resulted to be lower in HCV patients than ALD group. Indeed, the shedding of CD16 with impaired ADCC function is common in HCV-infected patients. This mechanism seems to be the way whereby HCV can escape the immune responses.[25]

Little is known about function of NKT and iNKT in human liver.[26] NKT cells represent 30%–40% of mouse liver lymphocytes, whereas the percentage of $CD3^{+}CD56^{+}$cells among human liver ranges from 3% to 15% and only less than 1% of human liver lymphocytes are considered to be invariant (CD1d-dependent).[27] Studies in mice have demonstrated that NKT cell subsets might be targeted for potential therapeutic intervention, especially in the context of ALD. Indeed, it is known that chronic-plus-binge ethanol feeding in mice activates hepatic iNKT cells, which play a critical role in the development of early ALD.[15] Remarkably, a further study found that only type I NKT (and not type II) become activated after alcohol feeding, and their number decreases after treatment with sulfatide, all-trans retinoic acid or tazarotene.[16]

In our study, the percentage of iNKT in ascitic fluid of patients with ALD was considerably lower than HCV group. However, as stated above, the role of these subsets of cells might be more prevalent in the early stages of the disease (chronic hepatitis) in comparison with cirrhotic one. Further studies might specifically study the lymphocyte subsets in the ascitic fluid of ALD patients at different stages of cirrhosis (cirrhosis at first decompensation *versus* cirrhosis at second or third decompensation episode *versus* end-stage liver disease).

Notably, IL-30 treatment reduces liver fibrosis in mice model through inducing NK group 2, member D-retinoic acid early inducible 1 interaction between NKT and activated hepatic stellate cells.[28] Interestingly, although treatment with αGalCer (a probable activator of iNKT cells) induces liver injury in mice, phase I/II clinical trials have failed to detect any effect in humans.[29,30]

The present study shows some limitations. First of all, the small sample size of our cohort might make statistically weak the reported data. However, the lack of prior research studies in this field has encouraged our Department to conduct a pivotal study with few participants, in order to expand the cohort in the future. Regarding this point, sample size of the study was not *a priori* calculated and consequently the power analysis was not performed.

Another limitation regards the fact that we express our results based on the percentage of the total cell population. In these cases, a correct preservation of the whole cell population is pivotal. However, every step of the cell analysis, from sample collection to laboratory processing, was made in order to limit every condition that would have affected the total cell number (e.g. the loss of cells with adherence capacities from the sample due to container conditions or temperature).

In conclusion, this is the first study describing the differences between lymphocyte subsets in the ascitic fluid of patients either with HCV or ALD related cirrhosis. In particular, ALD patients in comparison with HCV positive subjects presented an ascitic fluid with lower percentage of $CD3^+CD4^+$ cells and higher rate of $CD3\text{-}CD16^+$, $V\alpha24^+CD3^+CD56$- and $V\alpha24^+CD3^+CD56^+$ cells. On the contrary, the same two subsets resulted to be higher in ALD patients when we considered the peripheral blood samples. The findings of the present study might be useful for development of further works that should examine the role of immune cells in in the vulnerability toward infections (especially bacterial ones) and identify potential targets for new treatments. It would be particularly interesting to investigate the role of iNKT cells as potential therapeutic targets for the treatment of ALD.

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Author contributions

Roberto Giulio Romanelli: study concept, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. Gianfranco Vitiello: interpretation of data and critical revision of the manuscript. Stefano Gitto: interpretation of data and critical revision of the manuscript. Maria Grazia Giudizi: acquisition of data. Roberta Biagiotti: acquisition of data. Alessia Carraresi: acquisition of data. Francesco Vizzutti: critical revision of the manuscript. Giacomo Laffi: study concept and design, analysis and interpretation of data, and drafting of the manuscript. Fabio Almerigogna: study concept and design, statistical analysis, interpretation of data, critical revision of the manuscript, and study supervision.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval

Ethical approval was not sought for the present study because all the procedures included in the study are part of normal clinical practice. In particular, the ascitic fluid represents by definition a waste material.

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Informed consent

All recruited patients gave their written informed consent to undergo to all procedures foreseen by the study (including paracentesis).

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Supplemental material

Supplemental material for this article is available online.

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