Circulating CD8 lymphocytes predict response to atezolizumab-bevacizumab in hepatocellular carcinoma

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Table of contents

Supplementary Description of Human Cohorts and study protocol

The study cohort is a prospective series of 37 patients referred to the Division of Hepatobiliary and Immunoallergic Diseases, IRCCS S.Orsola-Malpighi Hospital of Bologna and to the Division of Gastroenterology and Hepatology, Fondazione IRCCS Ca' Granda of Milan between May 2022 and November 2022, for the treatment of intermediate/advanced HCC not amenable of potentially curative treatments or TACE. HCC was staged according to the BCLC staging system [1]. Forty patients were screened and three of them did not receive the indication for atezolizumab-bevacizumab treatment due to previous liver transplantation (one case), severe cardiovascular risk (one case) and venous leg ulcers (one case). Treatment was performed with atezolizumab (1200 mg) associated with bevacizumab (15 mg/kg), according to a 3-weeks intravenous infusion schedule.

A second prospective series of 15 patients with advanced HCC treated by TKIs (sorafenib in 6 cases and lenvatinib in 9 cases) was tested as controls. Patient's characteristics are reported in **Table S1**. A further prospective cohort of 18 patients was prospectically enrolled followed by the two enrolling centers was added, from December 2022 to May 2023, aiming to validate the putative predictive role of baseline CD8+ and CD8+PD-L1+ peripheral lymphocytes and the dynamic changes of CD8+PD1+ peripheral lymphocytes. Among these novel 18 patients assessed with the same criteria of the previous cohort, the first imaging evaluation after treatment start, showed that 13 patients were responders (7 stable disease and 6 partial response) and 5 patients displayed a progression at the first TC evaluation and

subsequently confirmed 4-5 weeks later (Table S1). The study was conducted in accordance with the Declaration of Helsinki. All patients were informed about the finality and procedures of the study, which was previously approved by the local ethics committee (Comitato Etico Area Vasta Emilia Centro – AVEC, on June, 6th, 2021 - approval number 528/2021/Sper/AOUBo) and signed the informed consent. Physical examination and laboratory tests were performed at baseline and before each treatment cycle. The baseline imaging (CT or MR) was performed in close proximity of treatment start while the first imaging assessment (CT or MR) was performed 6-8 weeks after the first drug infusion and every 8-10 weeks thereafter. Treatment efficacy was evaluated by contrast-enhanced CT or MR according to RECIST 1.1 criteria [2]. For the purposes of this study the analysis was focused to the first two follow-up assessment. The study protocol consisted in repeated study blood tests before and during treatments. PBMC were tested before each drug infusion in the atezolizumab-bevacizumab arm, and in patients treated by TKIs at 3-4 weeks after TKIs start and then with a bimonthly schedule. The baseline and early on-treatment variation of the percentage of CD8+, PD1+, PD-L1+, CD8+PD1+ and CD8+PD-L1+ were assayed in peripheral lymphocytes. Data were prospectively collected and subsequently compared in patients experiencing response to treatments, stable disease, or progression. The only patient experiencing a pseudoprogressive disease was classified as a responder.

Supplemental Materials and Methods

Analysis of lymphocytes phenotype by flow cytometry

In the present study, the attention was focused on lymphocytes identified by using side scatter (SSC) versus CD45 flow cytometric plot as a validated method for distinguishing white blood cells [3-4]. Recently, this approach was also used for the detection of leukocyte subtypes in patients with COVID-19 [5]. To characterize the immunophenotype, peripheral blood was collected on a Ficoll gradient vacutainers (Becton, Dickinson and Company, Franklin Lakes NJ, USA) for PBMC separation. Once separated 500.000 PBMC were diluted in PBS and incubated for 20 min at room temperature (RT) with four fluorochrome-conjugated anti-human antibodies supplied by Miltenyi Biotec: CD45 (clone REA747, Miltenyi Biotec GmbH), CD8 (clone REA734, Miltenyi Biotec GmbH), PD1 (Clone REA1165, Miltenyi Biotec GmbH), PD-L1/CD274 (clone REA1197, Miltenyi Biotec GmbH). The use of the cellular antigen CD45 for gating purposes assures to eliminate debris and possible residues of other cell populations included in PBMC from the recorded events and therefore from the analyses. Antibodies dilution was chosen after appropriate titration and isotypic controls were used to set negative gates. Before immunophenotypic analyses red cells were removed by using a lysis buffer (BecKman Coulter). Flow cytometry analyses were performed by using Cytoflex S (BecKman Coulter) daily checked with S calibration beads to keep the setting and histogram uniform during time.

Immune cell numbers were quantified as the frequency of positive cells (%) referred to 5000 gated lymphocytes. In detail, the percentage of PD1+, CD8+, PD-L1+, CD8+PD1+, CD8+PD-L1+ on 5000 peripheral lymphocytes was assessed and their dynamic variations after the first drug infusion were calculated as the ratio between the time points at three weeks, hereafter referred to as T1, and baseline hereafter referred to as T0. Analyses were also performed on absolute numbers of immune cells combining the information provided by the flow cytometer and the blood cell count.

Table S1. Comparison of flow cytometric readings in different blood sample

	Blood sample	**CD8+** $T_{0/-5}$	**PD1+** $T_{0/-5}$	**CD8+PD1+** $T_{0/-5}$	**CD8+** T_1	**PD1+** T_1	**CD8+PD1+** T_1	**FC**
Patient 1- T_0	A	94.16	32.4	30.5	97.56	22.7	24.16	0.79
Patient 1- T_0	B	94.22	32.0	30.42	97.41	22.37	24.42	0.80
Patient 2- T_0	A	34.76	16.36	11.18	38.52	53.65	83.2	7.44
Patient 2- T_0	B	34.30	16.60	11.32	38.04	54.02	83.35	7.36
Patient 3- T_0	A	83.25	15.36	3.29	80.74	25.78	10.6	3.22
Patient 3- T_0	B	83.62	15.51	3.43	80.52	25.06	10.78	3.14
Patient 4- T_0	A	89.38	29.26	28.66	93.52	61.18	61.46	2.14
Patient 4- T_{-5}	B	89.96	28.72	28.30	93.52	61.18	61.46	2.15
Patient 5- T_0	A	82.30	17.30	14.70	89.84	37.34	37.4	2.54
Patient 5- T_{-5}	B	82.30	16.90	15.30	89.84	37.34	37.4	2.47

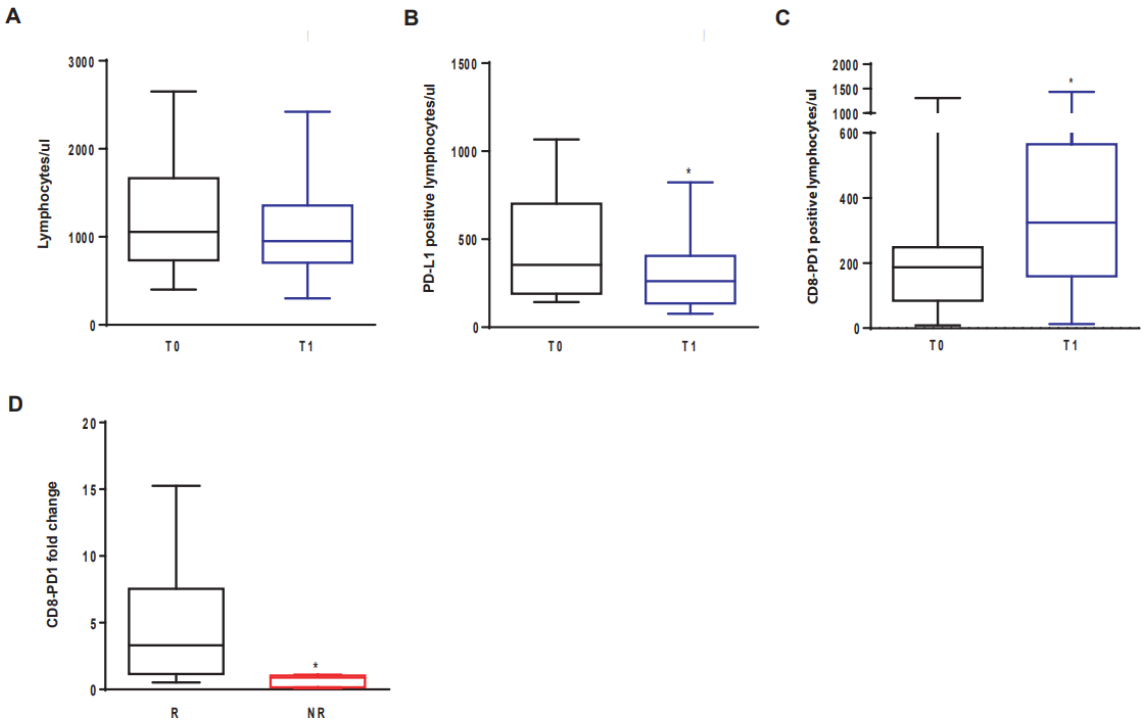
T_0: Baseline (day of first drug infusion).
T_1: 20 days after the first drug infusion.
T_{-5}: Five days before baseline (5 days before the first drug infusion).
FC: CD8+PD1+ fold change (FC): in the first three patients the FC was calculated by using two different blood samples (A and B) obtained in the same day. In the fourth and fifth patients the FC was calculated by using two different blood samples (A and B) obtained in different days (T_0, day of first drugs infusion and T_{-5}, 5 days before the first drugs infusion).

Table S2. Baseline demographics and clinical characteristics of patients treated with atezolizumab/bevacizumab in the validation cohort.

Patient's characteristics		atezolizumab bevacizumab 18 pts validation	Response to atezolizumab-bevacizumab PR/SD/PD
Age (years old)	<65 ≥65	9 (50%) 9 (50%)	**3/3/2** **3/4/3**
Gender	M F	16 (88.9%) 2 (11.1%)	**5/6/5** **1/1/0**
ECOG PS	0 1	14 (77.8%) 4 (22.2%)	**5/5/4** **1/2/1**
Child-Pugh class	A B C	17 (94.4%) 1 (5.6%) 0	**6/6/3** **0/1/2** **0**
ALBI grade	1 2 3	13 (72.2%) 5 (27.8%) 0	**4/6/3** **2/1/2** **0**
BCLC stage	A B C	0 7 (38.9%) 11 (61.1%)	**0** **4/3/2** **2/4/3**
CLD etiology	HBV active HCV cured HCV NASH/NAFLD alcohol	1 (5.6%) 3 (16.7%) 2 (11.1%) 8 (44.4%) 4 (22.2%)	**0/1/0** **1/1/1** **1/0/1** **2/4/2** **2/1/1**
Nodularity (diameter-cm)	Uninodular<5 uninodular >5 multinodular ≤3 multinodular >3 infiltrating	1 (5.6%) 5 (27.8%) 6 (33.3%) 3 (16.7%) 3 (16.7%)	**1/0/0** **3/1/1** **2/3/1** **0/2/1** **0/1/2**
Size (main lesion in multinodular)	≤3 cm 3-5 cm 5-10 cm >10 cm poorly defined	0 5 (27.8%) 6 (33.3%) 4 (22.2%) 3 (16.7%)	**0/0/0** **2/2/1** **1/4/1** **3/0/1** **0/1/2**
Portal vein invasion	yes no	7 (38.9%) 11 (61.1%)	**2/2/3** **4/5/2**
AFP (ng/mL)	≤20 21-400 ≥401	5 (27.8%) 6 (33.3%) 7 (38.9%)	**2/1/2** **3/1/2** **1/5/1**
Extrahepatic spread	yes no	6 (33.3%) 12 (66.7%)	**1/2/3** **5/5/2**
Response to treatment 1st imaging	partial response (R) stable disease (SD) progression (NR)	6 (33.3%) 7 (38.9%) 5 (27.8%)	

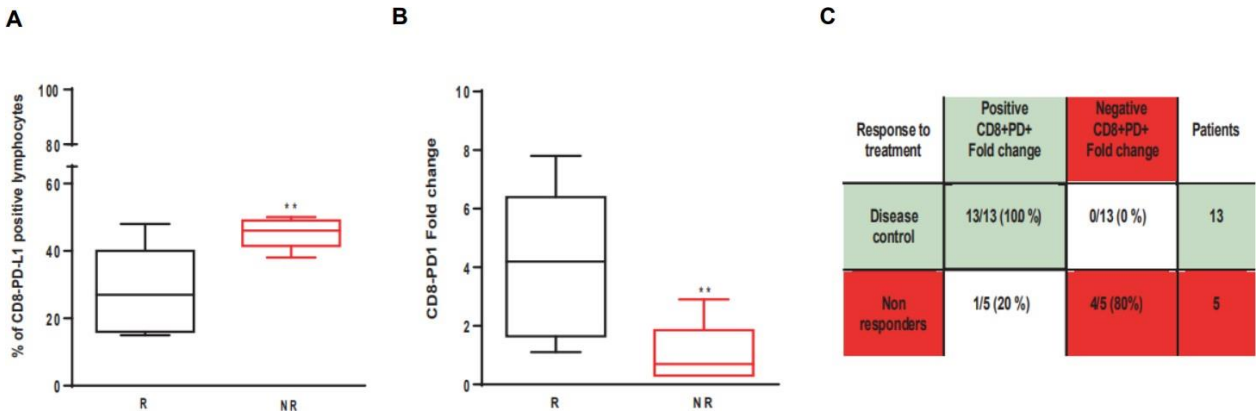
M: male; **F:** female
ECOG PS: Eastern Cooperative Oncology Group Performance Status (0-5). **ALBI:** Albumin-Bilirubin grade for HCC.
PR/SD/PD: Partial Responder/Stable Disease/Progressive Disease according to RECIST 1.1 criteria.
BCLC: Barcelona Clinic Liver Cancer staging system.
CLD etiology: etiology of the underlying Chronic Liver Disease (CLD). In cases of more etiologies were recognized in the same patient, the most relevant was considered. HBV: Hepatitis B virus; HCV: Hepatitis C virus (active infection or previously cured infection); NASH/NAFLD: Non-Alcoholic Steato-Hepatitis/Non Alcoholic Fatty Liver Disease;
AFP: Alfa-Feto-Protein in ng/ml.

Extrahepatic spread: extrahepatic HCC localization include lung in 3 cases, lymph nodes in 2 cases (associated with peritoneal metastasis in one case), bones in one case.



Supplementary Figure 1. Dynamic changes in immune cell composition evaluated on absolute number of lymphocytes in the early treatment phase.

Box-plot graphic representation of dynamic variations of: (A) Total Lymphocytes in all patients. (B) PD-L1+ lymphocytes in all patients. (C) CD8-PD1 positive lymphocytes in all patients (D) CD8-PD1 fold change (T1/T0) in responders (R) and non-responders (NR) to atezolizumab-bevacizumab. T0: baseline assessment. T1: 3-weeks assessment, before the second drug infusion. * *p<0.05;* by unpaired t-test. Dynamic changes for the number of CD8+PDL1+ lymphocytes at T0 and T1 was 387.48 ± 244.4 vs 265.5±166.33 T-test, p=0.038 and for the number of CD8+PD1+ lymphocytes at T0 and T1 was 222.64 ± 292.2 ± vs 337.92 ± 286.16 T-test, p=0.046.

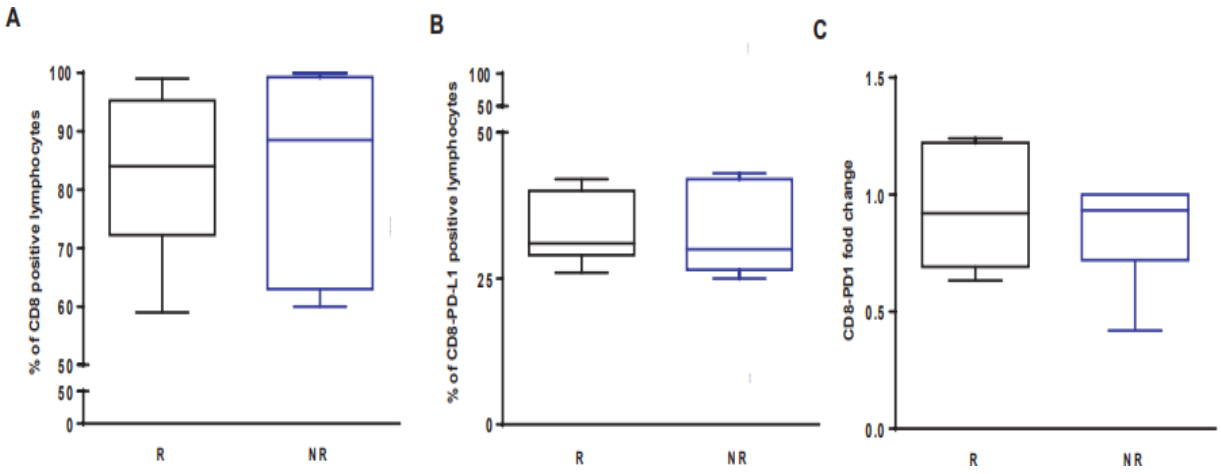


Supplementary Figure 2. Dynamic changes in peripheral lymphocyte populations in the early treatment phase of validation cohort.

Box-plot graphic representation of dynamic variations of: (A) CD8-PD-L1+ lymphocytes in in responders (R) and non-responders (NR); (B) CD8+PD1+ lymphocyte FC at three weeks after treatment start in responders and non-responders. (C) Schematic view of CD8+PD1+ lymphocyte FC (T1/T0) in HCC patients showing disease control or non-response to atezolizumab-bevacizumab.

T0: baseline assessment. T1: 3-weeks assessment, before the second drug infusion.

**p<0.01 by unpaired t-test.



Supplementary Figure 3. TKIs treatments and circulating CD8+ lymphocyte analyses. (A-B) Box-plot graphic representation of CD8+, CD8+PD-L1+ baseline lymphocytes in responder (R) and non-responder (NR) patients subsequently treated with TKIs and (C) CD8+PD1+ lymphocyte fold change (T1/T0) in HCC patients showing disease control or non-response to TKIs.

Supplementary references

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