

## VIRAL HEPATITIS

**Protease inhibitors-based therapy induces acquired spherocytic-like anaemia and ineffective erythropoiesis in chronic hepatitis C virus patients**

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Liver Int. 2016; 36: 49–58. DOI: 10.1111/liv.12900

**Abstract**

**Background & Aims:** The addition of protease inhibitors, boceprevir (BOC) or telaprevir (TRV), to peg-interferon and ribavirin (PR) increases the incidence of anaemia in patients with chronic hepatitis C virus (HCV) infection. Although genetic variants in inosine triphosphatase (ITPA) gene have been linked to the haemolytic anaemia induced by PR, the mechanism sustaining severe anaemia during triple therapy is still unknown. This study aims to elucidate the molecular mechanisms underlying anaemia in chronic HCV patients with combined therapy. **Methods:** We studied 59 patients with chronic HCV genotype-1: 29 treated with TRV/PR and 30 with BOC/PR. We evaluated biochemical and haematological parameters, red cell index at baseline, 4, 12, 16 and 24 weeks of treatment; in a subgroup, we performed functional studies: osmotic fragility, red cell membrane protein separation, mass spectrometry analysis, quantification of erythroid microparticles release. IL28B and ITPA polymorphisms were also evaluated. **Results:** We found early acute normochromic normocytic haemolytic anaemia (4–8 weeks) followed by a late macrocytic hypo-regenerative anaemia with inappropriate low reticulocyte count (12–24 weeks). Studies on red cells revealed: (i) presence of spherocytes; (ii) increased osmotic fragility; (iii) abnormalities in red cell membrane protein composition; (iv) reduced membrane-cytoskeleton stability; (v) increased release of erythroid microparticles. ITPA polymorphisms impacted only the early phase of anaemia. **Conclusions:** The bimodal pattern of anaemia in chronic HCV patients on triple therapy might be because of acquired spherocytic-like anaemia in the early phase, followed by hyporegenerative anaemia, most likely related to the combined effects of PR and TRV or BOC on erythropoiesis.

**Abbreviations**

BOC, boceprevir; Hct, haematocrit; HCV-1, HCV genotype-1; HCV, hepatitis C virus; ITPA, inosine triphosphatase; MAF, minor allele frequency; MCHC, mean cellular haemoglobin concentration; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MP, microparticle; MS, mass spectrometry; peg-IFN $\alpha$ , interferon alpha; PI, protease inhibitor; PR, peg-interferon and ribavirin; RBC, red blood cells; RBV, ribavirin; RDW, red cell distribution width; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; SNP, single nucleotide polymorphism; TRV, telaprevir.

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Handling Editor: Alessio Aghemo

Received 11 April 2015; Accepted 9 June 2015

Additional Supporting Information may be found at [onlinelibrary.wiley.com/doi/10.1111/liv.12900/supinfo](http://onlinelibrary.wiley.com/doi/10.1111/liv.12900/supinfo)

**Keywords**

chronic hepatitis C – first-generation protease inhibitors – microparticles – spherocytic-like anaemia

About 170 million individuals worldwide have chronic hepatitis C virus (HCV) infection, accounting for about one-third of all cases of chronic viral hepatitis (1). More than 350 million people die each year from complications of HCV-related cirrhosis and hepatocellular carcinoma (WHO 2013). Treatments for chronic HCV infection are evolving.

Individuals infected with HCV genotype-1 (HCV-1) have been considered the most difficult to treat and since 2011 the standard of care for HCV-1 is triple therapy with pegylated-interferon-alpha (peg-IFN $\alpha$ ) and ribavirin (RBV) (PR) plus one of the two-first-wave, first-generation protease inhibitors (PIs), telaprevir (TVR) and boceprevir (BOC). Sustained virological response, defined as undetectable HCV-RNA in serum 24 weeks after the end of treatment, is achieved in approximately 70% of cases (2). However, the addition of PIs to PR worsened side effects, with anaemia being one of the most serious one (3, 4), particularly in cirrhotic patients (5).

Previous studies showed that anaemia induced by RBV is mainly related to red blood cells (RBC) membrane oxidation resulting in increased erythrophagocytic extravascular removal (6). This is also associated with a bone marrow suppression component mediated by peg-IFN $\alpha$  and RBV actions (7). The severity of PR-induced anaemia is further increased by BOC or TVR, through a still unknown mechanism(s). A genome wide association study has identified single nucleotide polymorphisms (SNPs) within inosine triphosphatase (ITPA) gene as being significantly associated with RBV-induced anaemia (8, 9), suggesting ITPA-SNPs as useful marker to predict the degree of anaemia in HCV patients receiving triple therapy (10). However, the recent revision of data from two

large cohorts of triple-treated patients has underlined that the higher incidence of severe anaemia is not only associated to ITPA genetic variants, but additional still unknown mechanisms should also contribute to it (11).

Since 2014, new direct-acting antivirals have been licensed in the USA and EU for use as part of combination therapy for HCV infection, with better tolerability (2, 12, 13). However, these molecules are costly and this represents in several countries a major limitation in the access to treatment for many patients, particularly for those with a lower degree of liver fibrosis. For this reasons, the antiviral scheme employing the combination of PR, with or without first- or second-generation PIs, is expected to be used much longer.

The aims of this study are to assess type of anaemia in chronic HCV patients treated with either TVR or BOC to disentangle the mechanisms of anaemia because of anti-HCV present treatment.

**Patients and methods****Patients and study design**

We enrolled 59 chronic HCV-1 patients treated with TVR ( $n = 29$ ) or BOC ( $n = 30$ ). For inclusion and exclusion criteria see Supplementary material. Patients were examined at baseline, week 4 (end of PR lead-in phase), and 12 (8 weeks of PI + PR). Patients receiving TVR triple therapy were examined at week 16 (end of 12 weeks of PR/TVR), and those receiving BOC triple therapy at week 24 (20 weeks of PR/BOC) (Fig. S1). Informed consent was obtained by all enrolled patients according to Declaration of Helsinki and principles of Good Clinical Practice. This study protocol was approved by the ethics committees of the AOUI-Verona, University of Verona and University of Udine.

**Anaemia definition, haematological parameters and RBC osmotic fragility assay**

Anaemia (Hb < 10 g/dl) and severe anaemia (Hb < 8.5 g/dl) were defined at any time during treatment. Early anaemia corresponded to Hb < 10 g/dl during the first 4 weeks of treatment. Osmotic fragility curves were evaluated after blood sampling (0 h) and at 24 h incubation at room temperature (14–16).

**ITPA genotypes and predicted activity**

ITPA-SNPs rs1127354 and rs7270101 genotyping was performed by direct sequencing, and the predicted ITPA activity was estimated as described (8). See Supplementary material.

**Key points**

- In chronic HCV patients, triple therapy induced a bimodal pattern of anaemia, which is characterized by an early acquired spherocytic-like anaemia, worsened by an inappropriate low reticulocyte count.
- The hyporegenerative anaemia appears in late phase of triple therapy, most likely related to combine effects of PR and TRV of BOC on erythropoiesis.
- ITPA polymorphisms impact only the early phase of anaemia but not its degree.
- Functional studies of red cells from chronic HCV patients during triple therapy revealed changes in membrane protein composition, reduced red cell membrane mechanical stability and increased release of erythroid microparticles.

### Red cell membrane protein separation, immune-blot and mass-spectrometric analyses

A subgroup of 21 patients (TRV,  $n = 9$ ; BOC,  $n = 12$ ) underwent functional studies. RBC membrane proteins were prepared and separated on SDS-PAGE. Gels were either stained with colloidal Coomassie or transferred to nitrocellulose membrane for immunoblot analysis (17–20). Image analysis was performed by Image Quant Las Mini 4000 Digital Imaging System (GE Healthcare Life Sciences, Little Chalfont, UK), and densitometric analysis by ImageQuant TL software (GE Healthcare Life Sciences) (20–22). Bands differently expressed in colloidal Coomassie-stained gels were used for mass spectrometry (MS) (19, 23, 24). MS was performed using a Tofspec SE (Micromass, Manchester, UK). See Supplementary material.

### Erythroid microparticles

Erythroid microparticles (MPs) were isolated from plasma of patients and healthy subjects as reported (25, 26). See Supporting information.

### Statistical analysis

Median, mean, range and SD were used as descriptive statistics. The differences between the two treatment groups and demographical, clinical and pathological features at baseline were tested using non-parameters methods for continuous variables and chi squares and exact test for dichotomous variables, as appropriate.  $P$  value  $<0.05$  was considered significant in two-tailed tests. All the computations were carried out using the STATA program version 12.0 (STATA Statistics/Data Analysis 12.0 – STATA Corporation, College Station, TX, USA). See Supporting information.

## Results

### Patient characteristics

The baseline characteristics of the patients are shown in Table 1. Median age was 55 years and 72.9% were males. Forty-three (72.9%) patients had clinical or histological compensated cirrhosis in Child-Pugh class A. Upper gastrointestinal endoscopy was performed before

**Table 1.** Baseline characteristics of the patients

Characteristics	Telaprevir ( $n = 29$ )	Boceprevir ( $n = 30$ )	Overall ( $n = 59$ )
Age (years)	53.4 (39.8–73.9)	56.8 (41.3–70.6)	55.7 (39.8–73.9)
Male gender	21 (72.4)	22 (73.3)	43 (72.9)
METAVIR F3/F4 ( $n = 46$ )	19 (82.6)	18 (78.3)	37 (80.4)
Cirrhosis*	23 (79.3)	20 (66.7)	43 (72.9)
Oesophageal varices	5 (17.2)	3 (10.0)	8 (13.6)
Splenomegaly†	19 (65.5)	14 (46.7)	33 (55.9)
<i>Treatment history</i>			
Naive	6 (20.7)	5 (16.7)	11 (18.6)
Prior relapse	10 (34.5)	5 (16.7)	15 (25.4)
Prior partial response	3 (10.3)	7 (23.3)	10 (17.0)
Prior null response	6 (20.7)	9 (30.0)	15 (23.4)
Undetermined	4 (13.8)	4 (13.3)	8 (13.6)
ALT (IU/L)	92 (17–289)	75 (22–572)	81 (17–572)
AST/ALT ratio	0.76 (0.42–1.12)	0.71 (0.47–1.33)	0.76 (0.42–1.33)
Albumin (g/L)	41.5 (33.3–47.9)	42.5 (34.5–49.2)	41.8 (33.3–49.2)
MCV (fL)	90.6 (80.6–98.9)	90.5 (80.1–97.7)	90.5 (80.1–98.9)
Hb (g/dl)	15.0 (11.3–16.9)	15.3 (13–17.3)	15.2 (11.3–17.3)
Platelets ( $10^9/L$ )	146 (74–282)	149 (88–290)	149 (74–290)
Creatinine (mg/dl)	0.80 (0.55–1.18)	0.83 (0.62–1.16)	0.81 (0.55–1.18)
IL28B <i>rs12979860</i>	6/22/1	5/19/3	11/41/4
CC/CT/TT			
ITPA <i>rs1127354</i>	21/7/0	26/4/0	47/11/0
CC/CA/AA			
ITPA <i>rs7270101</i>	23/4/1	25/5/0	48/9/1
AA/AC/CC			

Data are expressed as median (range) or number (%).

\*histological and/or clinical cirrhosis defined by ultrasonography features suggestive of cirrhosis based on a quantitative scoring system derived from the appearance of the liver margins, parenchymal echotexture, portal vein calibre and spleen diameter, supplemented with the presence of oesophageal varices.

†longitudinal diameter  $>10$  cm on abdominal ultrasound.

$P > 0.05$  for each comparison of distributions or proportions between Telaprevir and Boceprevir using Mann–Witney test for unpaired data and chi-squared or exact test.

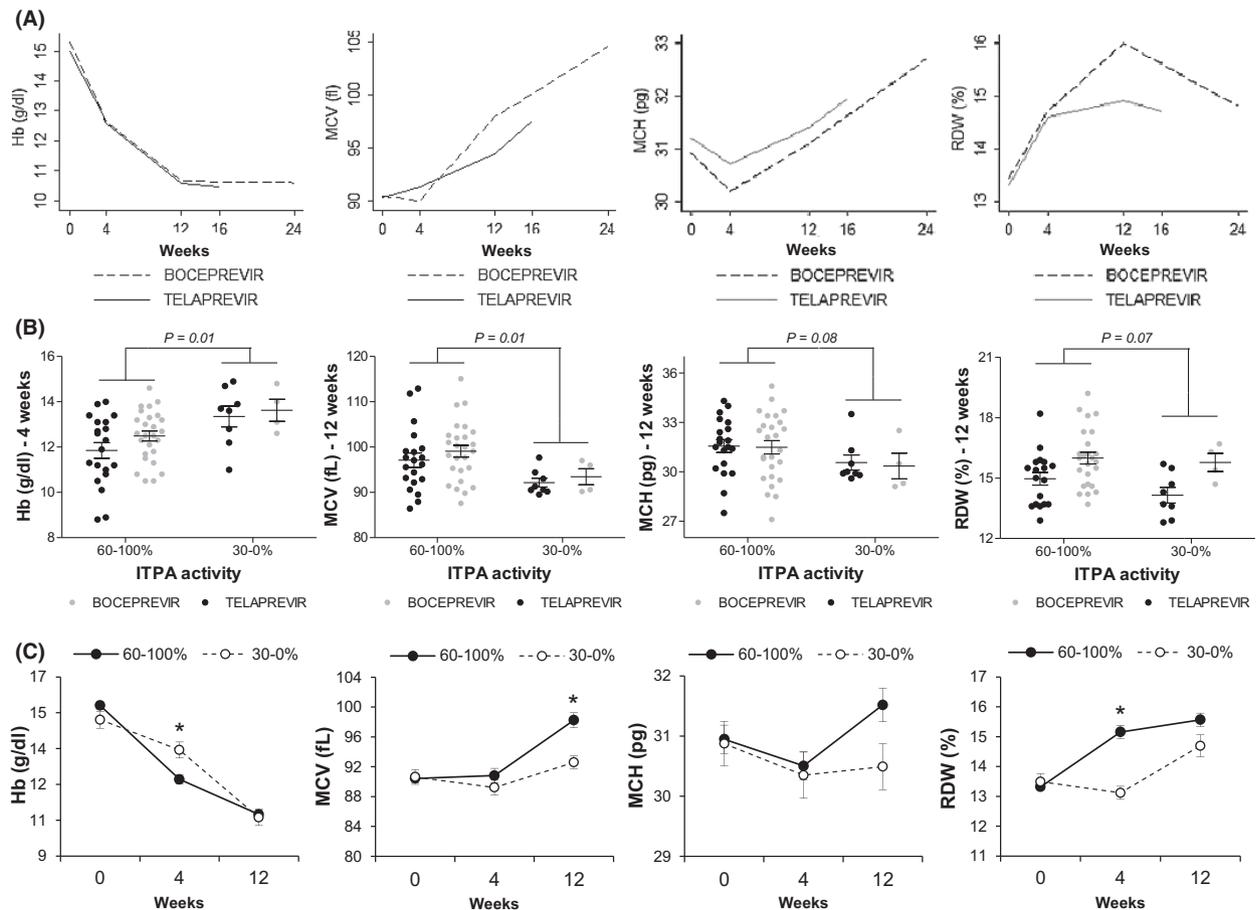
therapy in all cirrhotic patients and eight of them had oesophageal varices. All patients had abdomen ultrasound performed at baseline and splenomegaly was present in 33 of them, including 26 cirrhotics. Twenty-nine patients received triple therapy with TVR and 30 with BOC. The demographical and clinical characteristics were similar between TVR and BOC groups ( $P > 0.05$  for each variable).

### BOC or TVR triple therapy induced a bimodal anaemia

Anaemia and severe anaemia were observed in 9 (30.0%) and 1 (3.3%) patients in the BOC group and in 14 (48.3%) and 1 (3.4%) patients in the TVR group. In the BOC group, 12 (40.0%) reduced RBV dose, 5 (16.7%) reduced RBV dose and received either EPO or transfusion; in the TVR group, 12 (41.4%) reduced RBV dose, 1 (3.4%) received EPO, and 8 (34.5%) required combined measures. Hb levels decreased rapidly from baseline to 4 and 12 weeks of treatment, and then remained stable at 16 or 24 weeks of therapy with TVR or BOC respectively (Table S1, Fig. 1A). The decrease in

Hb levels (TVR group, 15.0–10.5 g/dl; BOC group, 15.3–10.6 g/dl) was similar in both groups ( $P > 0.05$ ). The mean cell volume (MCV) was stable in both groups between 0 and 4 weeks, and increased afterwards. Consequently, the mean cell haemoglobin (MCH) significantly decreased from the baseline to 4 week ( $P < 0.001$ ) and then increased at 12, 16, and 24 weeks of treatment in both groups ( $P < 0.001$ ). The RBC distribution width (RDW) significantly increased up to 12 weeks, followed by a significant decrease afterwards in both groups ( $P < 0.05$  for each comparison).

The time trend of these haematological parameters from the baseline to 4, 12, 16 and 24 weeks was statistically significant using mixed linear regression models with a spline term for time as independent variable ( $P < 0.001$  for each parameter) without differences between the two groups. The differences between baseline and at 16 and 24 weeks values were statistically significant for all parameters when also including in the model group, presence of cirrhosis and splenomegaly as independent variables. A similar time effect of TVR/BOC-PR combination therapy on RBC indices



**Fig. 1.** Haematological parameters in triple-treated HCV patients. (A) Haematological parameters during triple therapy. (B–C) Distribution of RBC indices according to predicted ITPA activity during triple therapy. Data are presented as mean  $\pm$  SD. \* $P \leq 0.01$ .

was observed in the subgroup of 21 patients analysed for functional studies (Table S2). Reticulocytes response was significantly different between the two groups at 4 weeks (no baseline measure was available) and it showed a significant decrease after BOC therapy from 4 to 24 weeks and no change in TVR group. It is of interest to note a lacking in reticulocyte response to the degree of anaemia in both TRV and BOC (Table S2).

#### ITPA polymorphism and pattern of anaemia in chronic HCV patients undergoing triple therapy

We evaluated protective ITPA-SNPs rs1127354 and rs7270101 for anaemia. Both polymorphisms were in Hardy–Weinberg equilibrium ( $P > 0.05$ ) (Table 1). No differences in the minor allele frequencies of the two SNPs between control Italian population TSI ( $n = 98$ ) and HCV patients ( $n = 58$ ) were observed (A: 0.11/0.09, rs1127354; T: 0.10/0.09, rs7270101;  $P > 0.05$  for each comparison). No significant differences were found comparing the two treatment groups.

We divided our cohort in two subgroups, according to the predicted ITPA activity on the basis of ITPA-SNPs: (i) patients with normal/intermediate enzymatic activity (60–100%,  $n = 46$ ) and (ii) patients with low/very low activity (0–30%,  $n = 12$ ). Patients with 0–30%-ITPA activity showed a higher Hb level after 4 weeks of treatment with either TRV ( $13.4 \pm 0.5$  g/dl) or BOC ( $13.6 \pm 0.5$  g/dl) compared to patients with 60–100%-ITPA activity (TRV,  $11.9 \pm 0.3$  g/dl; BOC,  $12.5 \pm 0.2$  g/dl) ( $P = 0.01$ ) (Fig. 1B,C). However, both groups reached the same Hb level after 12 weeks of treatment (Fig. 1C).

At 12 weeks, patients with 60–100%-ITPA activity exhibited a markedly increased MCV (TRV,  $97.1 \pm 1.6$  fl; BOC,  $99.1 \pm 1.3$  fl) compared to those with 0–30%-ITPA activity (TRV,  $92.2 \pm 1.0$  fl; BOC,  $93.5 \pm 1.8$  fl) ( $P = 0.01$ ) (Fig. 1B,C). Similarly, a tendency to increase MCH and RDW in 60–100% patients (MCH: TRV,  $31.6 \pm 0.4$  pg; BOC,  $31.5 \pm 0.4$  pg – RDW: TRV,  $15.0 \pm 0.3\%$ ; BOC,  $16.0 \pm 0.3\%$ ) compared to 0–30% patients (MCH: TRV,  $30.6 \pm 0.5$  pg; BOC,  $30.4 \pm 0.8$  pg – RDW: TRV,  $14.2 \pm 0.4\%$ ; BOC,  $15.8 \pm 0.4\%$ ) was observed after 12 weeks of treatment, although not significant (Fig. 1B,C).

#### Spherocytosis-like anaemia in the early phase of treatment

Functional studies on RBC showed the presence of micro and macrospherocytes at 4, 8, 12 and 16 weeks of treatment with either BOC or TRV (Fig. 2A). Both direct and indirect agglutination tests for immune-mediated haemolytic anaemia were negative. At baseline, the osmotic fragility curves at 0 and 24 h were similar to those observed in healthy controls (data not shown). After 4, 8, 12 and 16 weeks of treatment, an increased osmotic fragility at 24 h after blood sampling was

observed, as indicated by the appearance of haemolysis at 156 mOsm point. This was more marked in TVR-treated patients at 8 weeks of therapy compared to BOC ones (Fig. 2B).

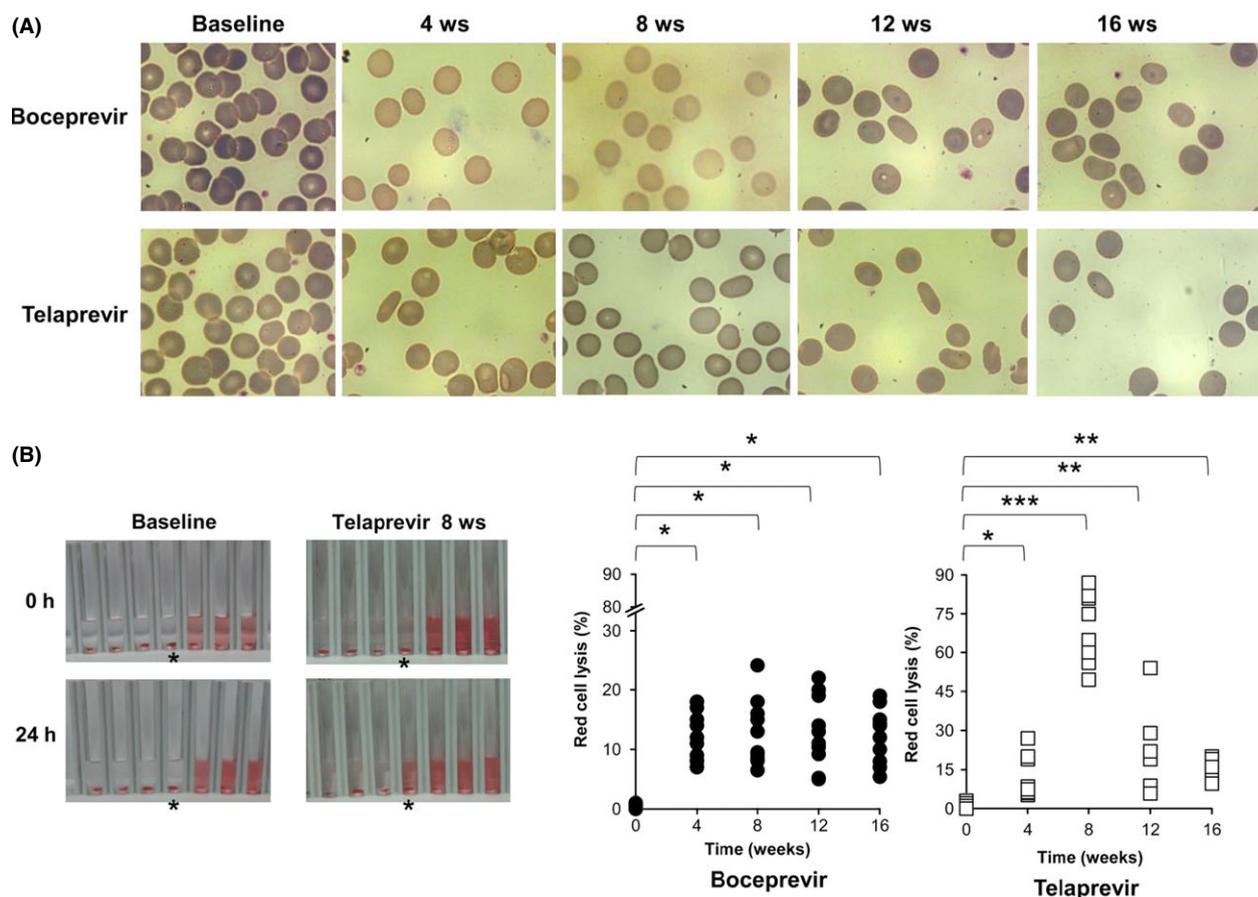
The analysis of RBC membrane proteins showed no major protein defects in patients at baseline (Fig. 3A). In BOC group a significant reduction compared to baseline in  $\alpha$ -spectrin (8 weeks:  $66.6 \pm 2.5\%$ , 12 weeks:  $65 \pm 4.3\%$ ,  $n = 12$ ;  $P < 0.05$ ),  $\beta$ -spectrin (8 weeks:  $67 \pm 1.1\%$ , 12 weeks:  $71 \pm 2.2\%$ ,  $n = 12$ ;  $P < 0.05$ ) and in band 4.2 (8 weeks:  $55.2 \pm 3.9\%$ , 12 weeks:  $58 \pm 4.1\%$ ,  $n = 12$ ;  $P < 0.05$ ) was observed. Similarly, in TRV group we found a significant reduction compared to baseline in  $\alpha$ -spectrin (8 weeks:  $78 \pm 3\%$ , 12 weeks:  $69 \pm 2.8\%$ ,  $n = 9$ ;  $P < 0.05$ ),  $\beta$ -spectrin (8 weeks:  $73 \pm 3.4\%$ , 12 weeks:  $66 \pm 4.7\%$ ,  $n = 9$ ;  $P < 0.05$ ) and in ankyrin (12 weeks:  $52 \pm 1.2\%$ , 16 weeks:  $61 \pm 1.7\%$ ,  $n = 9$ ;  $P < 0.05$ ) (Fig. 3A). The identification of these proteins was then validated by MS (data not shown). The image analysis of the gels revealed also the appearance of addition bands in the molecular range between 135 kDa and 245 kDa (Fig. 3A). These bands were numbered from 1 to 7 and 1 to 6 in RBC from, respectively, BOC and TRV groups and identified by MS. We found fragments of ankyrin (band 1, 2, 4, 7 in BOC and band 2, 3, 5 in TRV) and membrane associated small GTPase (band 3, 5, 6 in BOC and band 1, 4 in TRV) (Table S3). The level of  $\beta$ -adducin Ser-726 phosphorylation was significantly increased in a time dependent fashion in both groups compared to baseline levels (Fig. 3B). The levels of  $\beta$ -adducin phosphorylation in TRV group were higher compared to BOC group, suggesting a more severe cytoskeleton instability of RBC from TRV-treated patients than BOC ones (Fig. 3B).

#### Release of erythroid microparticles after 8 weeks of triple therapy

We evaluated the release of erythroid MPs in plasma samples from triple-treated patients at baseline and at the different time points. We did not find significant differences in erythroid MPs from chronic HCV patients compared to healthy controls at baseline (data not shown). Nevertheless, at 8 weeks of treatment we found significant increased amount of erythroid MPs in both BOC and TRV groups compared to healthy controls (Fig. 3C), indicating a disruption in membrane-skeleton network related to triple therapy.

#### Discussion

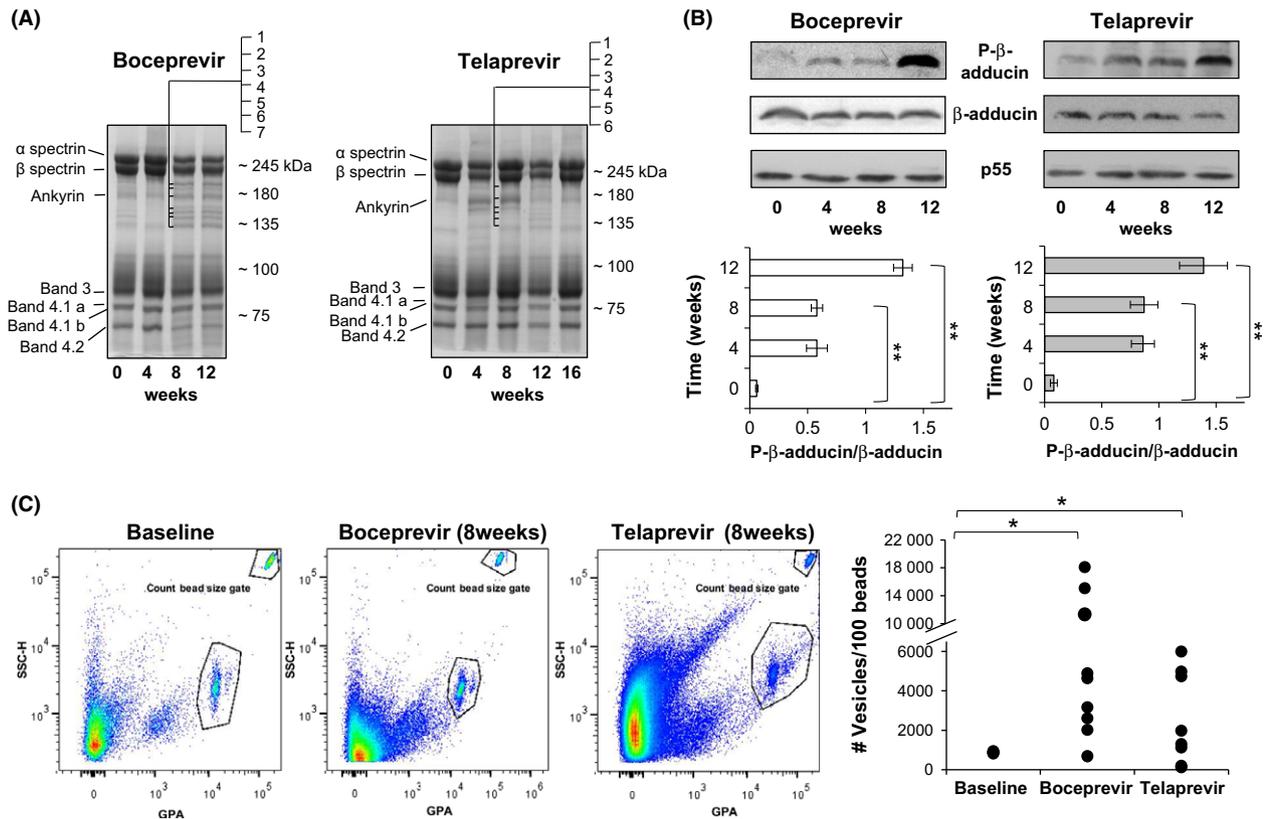
Here, we found a bimodal profile of anaemia in HCV patients undergoing triple therapy, characterized by an early phase with acute haemolytic spherocytosis-like anaemia, corresponding to the first 8 weeks of treatment, followed by the appearance of a hyporegenerative component at 12 weeks of treatment, which is



**Fig. 2.** Morphological and biochemical parameters in triple-treated HCV patients. (A) RBC morphology from patients at baseline and after BOC/TRV treatment. Blood smears were stained with May-Grunwald-Giemsa. One representative image from 9 BOC- and 12 TRV-patients. (B) Osmotic fragility curves of RBC at 0 and 24 h after blood withdrawal; \*increased osmotic fragility at 156 mOsm. One representative image from 9 BOC- and 12 TRV-patients for each time points. The charts show the red cell lysis % at 156 mOsm in treated patients. \* $P < 0.05$ ; \*\* $P < 0.05$ ; \*\*\* $P < 0.001$ .

responsible for the lacking bone marrow response. We did not observe differences in the degree of anaemia between BOC- and TRV-treated subjects, in agreement with previous reports (3). The drop in Hb levels was significant at 4 weeks of treatment and was higher in patients with normal ITPA activity compared to subjects with reduced ITPA activity. The RBC indices showed the bimodal change characterized by normochromic normocytic anaemia in the early phase of treatment (0–4–8 weeks) followed by macrocytic anaemia with a significant increase in MCV and MCH values at 12 weeks of treatment, through the end of the therapy. Of note, patients carrying ITPA-SNPs genotype associated to a reduced enzymatic activity exhibited lower MCV and MCH values compared to subjects with normal activity. Nevertheless, all patients result in a comparable degree of anaemia after 12 weeks of treatment, regardless of ITPA activity. Thus, ITPA-SNPs could contribute to explain at least partly the inter-individual variability in early phase of anaemia in the response to triple therapy (27). Our data suggest that anaemia in triple-treated

patients results by a combination of different factors synergizing on anaemia. In fact, during PIs combine therapy, we observe the appearance of micro and macrospherocytes, which was associated with increased osmotic fragility, with negative direct/indirect agglutination tests. Of note, the increased osmotic fragility was more severe in TVR-treated patients than in BOC ones. This might be related to the differences in the ability to cross the cell membranes (28–30). Since erythrocytes survive in the peripheral circulation 120 days, controlled proteolysis of RBC membrane-cytoskeletal proteins is required in membrane-cytoskeletal remodelling (31). Thus, in RBC from triple-treated patients some changes in protease function combined with oxidative stress may affect the machinery involved in membrane-cytoskeletal stability, in agreement with previous reports (32). This is supported by the reduction in  $\alpha$ - and  $\beta$ -spectrins from 4–8 weeks to 12–16 weeks of treatment, indicating a spherocytic-like morphological change. Indeed, reductions of these proteins are linked to hereditary spherocytosis (33). In PIs treated patients, the



**Fig. 3.** Alterations in RBC membrane protein composition and in membrane-cytoskeleton stability. (A) Colloidal Coomassie stained gels of RBC membranes from patients during therapy. One representative image from 9 BOC- and 12 TRV-patients. Bands differently expressed at 8–12–16 weeks of treatment were cut and analysed by MS (Table S2). (B) Immunoblot analysis with specific anti-phospho-β-adducin (P-β-adducin) and β-adducin of RBC membrane proteins from treated patients at the different time points. P55 was used as loading control protein. Relative quantification is shown as P-β-adducin/β-adducin ratio and expressed as mean ± SD. \*\**P* < 0.01. (C) Representative flow cytometric density plot of MPs in plasma from patients at baseline and at 8 weeks of therapy. Erythroid MPs were identified as glycophorin A positive MPs, using phycoerythrin conjugated anti-human CD253a, glycophorin A. MPs were quantified using the known density of fluorescent CytoCount™ beads (circle). \**P* < 0.05.

detection of additional bands between 135 and 240 kDa, identified as ankyrin, might result from either alternative splicing or inhibition of controlled proteolytic cleavage of ankyrin, which is involved in maturation of erythrocyte in the peripheral circulation (34). MS also revealed the presence of small GTPase proteins associated to the RBC membrane from 8 weeks-treated patients. In red cells, small GTPases have been related to events involved in the dynamic association of the membrane-skeleton network, which parallel changes in phosphorylation state of β-adducin (35, 36). Changes in phospho-β-adducin results in disruption of actin-spectrin association, affecting cytoskeleton remodelling (37–39). A time dependent increased in red cell β-adducin phosphorylation was observed, supporting the perturbation in dynamic regulation of cytoskeletal network induced by the combined therapy. Overall, these data indicate that both BOC and TRV promote acute acquired spherocytic-like anaemia in the early phase of the treatment as supported by (i) the presence of spherocytes; (ii) the increase osmotic fragility at 24 h;

(iii) the changes in red cell membrane composition; and (iv) the increased in phosphorylation of β-adducin.

Previous reports on hereditary spherocytosis have shown that the instability between membrane and cytoskeleton network promotes erythroid vesiculation slugging in microcirculation with release of MPs. This results in abnormal RBC volume/surface ratio and generation of spherocytes (40). Accordingly, we found a significant increase in erythroid MPs in both BOC- and TRV-treated subjects at 8 weeks of treatment.

One of the major effects of the presence of spherocytes owing to reduced survival is the increased reticulocyte count in response to anaemia through EPO stimulation. However, in these patients, we did not find the expected increase in reticulocyte count, following the drop in Hb levels, suggesting a failure in erythropoiesis during triple therapy at 8–12 weeks of treatment. This is also supported by the increase in MCV values observed in other disorders characterized by dyserythropoiesis such as Congenital Dyserythropoietic Anaemias (41). The lack of erythropoietic response might be

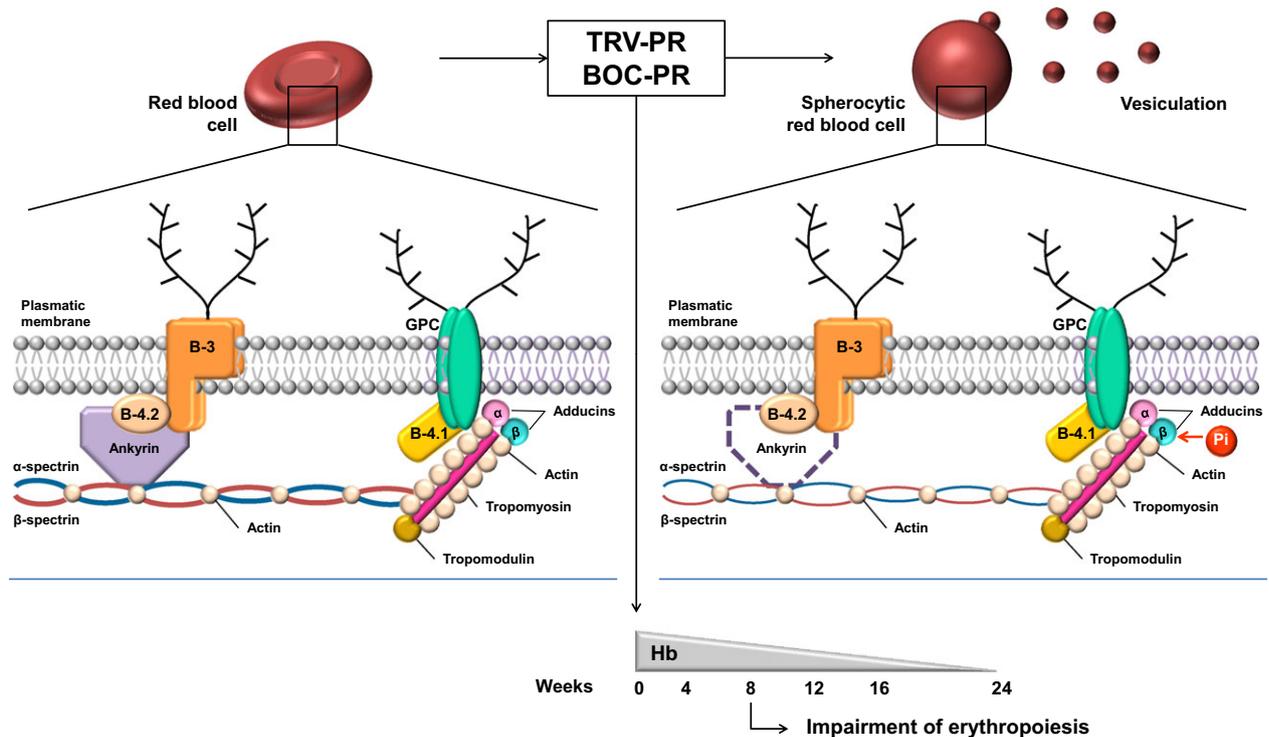
related to the combination of PR effects on erythroid precursors (7), synergizing with BOC and TRV.

This is the first functional study, which aims to unravel the characteristics of anaemia induced by triple therapy for chronic HCV patients. The main weakness of this study is the small number of patients recruited for functional studies, because of requirement of fresh blood to be immediately processed within few hours after sampling. However, the patients were enrolled consecutively among those undergoing triple therapy in Hepatology Centres according to the current guidelines for treatment of chronic HCV patients in routine activity and therefore they can be considered representative of all those treated according to present anti-HCV schedule.

We believe that our results may still be of interest in the era of new antiviral drugs. The treatment of HCV chronic hepatitis has evolved rapidly, starting with the availability of first generation PIs (2). These drugs are allowed to treat HCV-1-infected patients who had failed PR dual therapy. Subsequently the scheme based on the use of triple therapy with sofosbuvir and PR made possible to shorten the treatment, improving its tolerability. Currently, several new drugs besides sofosbuvir are available for the

treatment of chronic HCV hepatitis, including second-generation PIs (13, 42). Despite the advantage in the efficacy and tolerability, the access to treatment with new antivirals is limited by their high cost (43). In several industrialized countries, the access to treatment with new antivirals is reserved only to patients with cirrhosis or severe liver fibrosis while in less advanced stages of liver disease antiviral therapy with PR and first- or second-generation PIs is still currently adopted. In countries where the access to new antivirals will remain to be available only for patients with more advanced liver disease, seem justifiable to consider antiviral schemes based on IFN combinations to eradicate HCV infection in patients with less severe liver disease before the progression to cirrhosis. In addition, the possibility that new generation anti-HCV PIs might induce abnormalities in erythrocyte structure similar to those here described, even when used in IFN-free regimens with RBV, cannot be excluded and will deserve to be evaluated in future studies.

In conclusion, we propose a bimodal anaemia in chronic HCV patients treated with combined therapy: an early acute haemolytic acquired spherocytic-like anaemia (4–8 weeks), which subsequently is sustained



**Fig. 4.** Bimodal anaemia in triple-treated HCV patients. Protein components of erythrocyte membrane and cytoskeleton in physiological conditions. The combined therapy results in acquired spherocytic-like anaemia in the early phase: spherocytic RBC with increased vesiculation; reduction in  $\alpha$ - and  $\beta$ -spectrins (thin lines); increased  $\beta$ -adducin phosphorylation and proteolytic cleavage of ankyrin (dotted line). An impairment of erythropoiesis in the late phase was also observed. B-3, band 3; B-4.2, band 4.2; B-4.1, band 4.1; GPC, glycophorin C; Pi, phosphorylation.

by an impairment of erythropoiesis (8–12 weeks to the end of the treatment) (Fig. 4).

Future studies should be carried out to better define the effects of first- or second-generation PIs combined with PR on erythropoiesis to improve the haematological outcome of chronic HCV patients undergoing triple therapy.

### Acknowledgements

We thank T. Kalfa for helpful discussion.

**Financial support:** This work was supported by PRIN (LDF and AI: 201228PNX83) and FUR\_UNIVR (LDF) (Government-based financial support).

**Conflict of interest:** The authors do not have any disclosures to report.

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