Keyhole-limpet-hemocyanin induces myeloid dendritic cells in chronic hepatits C and compensated cirrhosis – results from a pilot study

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ABSTRACT:

Background: Keyhole-limpet-hemocyanin (KLH) is a high molecular weight protein used as vaccine component and therapeutic agent in superficial bladder cancer. Immune-stimulatory properties through enhanced activation and maturation of dendritic cells have been proposed. Dendritic cell dysfunction plays a major role in chronification of hepatitis C virus infection, thus suggesting KLH as a possible therapeutic agent. This study aimed to investigate the safety and efficacy of KLH in patients with chronic hepatitis C (CHC) and compensated cirrhosis.

Material and methods: Fourteen therapy naïve cirrhotics ineligible for interferon treatment were given five subcutaneous injections of KLH 1 mg each throughout eight weeks of therapy. FACS analysis from peripheral blood was performed to monitor immunological effects. CHC clearance was chosen as primary endpoint.

Results: Study therapy was tolerated well; there were fifteen adverse events in nine patients, three of them serious but unrelated to KLH. Treatment with KLH induced peripheral blood myeloid dendritic cells 9.4 fold but failed to clear CHC in the investigated population.

Conclusions: KLH administration is safe in compensated cirrhotics with CHC, is immunologically active but unable to clear CHC infection. Due to its beneficial safety profile and the low treatment costs, treatment with KLH warrants further investigation in hepatic diseases.

Keywords:

Chronic hepatitis C, Keyhole-limpet-hemocyanin, Antiviral treatment

Introduction

Chronic hepatitis C (CHC) is a worldwide healthcare problem affecting 130 to 170 million patients.[1] Antiviral therapies used during the last decade were either combinations of pegylated interferon-alpha (peginterferon-alpha) with the nucleotide-analogue ribavirin alone, with ribavirin plus a protease-inhibitor (boceprevir or telaprevir) or the recently approved interferon-free combinations of direct-acting-antivirals.[2,3,4,5,6,7] However, costs, side effects, and contraindications may limit patient access to this treatment.[8]

Keyhole-limpet-hemocyanin (KLH) is the oxygen transport protein of the Californian keyhole limpet, *megathura crenulata.* This low molecular weight protein (40 kDa) is used as a vaccine adjuvant and in bladder cancer. [9,10] Recently, we observed a 52-year-old female patient with CHC and liver cirrhosis who received treatment for superficial bladder cancer with KLH. Standard sensitization with two times 1mg KLH was performed. [11] The hepatitis C virus (HCV) RNA concentration was estimated prior to and after the injections, with a decline in HCV RNA four months after the injections (5,040,000 IU/ml to 682 IU/ml) and subsequently disappeared without relapse.

Chronification of HCV infection is suspected to be an insufficient T-helper and cytotoxic T-lymphocyte response to and subsequent clearance of circulating viral antigens. [12,13] Studies in non-cirrhotic CHC patients revealed an impaired number and functionality of circulating plasmacytoid and myeloid PBDCs. [14,15] HCV core protein and nonstructural protein 3 (NS3) shape the cytokine profile of PBDC in favor of a predominantly regulatory IL-10 secretion, making those cells poor stimulators of T cell proliferation. [16,17] Additionally, HCV NS4 is able to promote secretion of IL-10 by monocytes thus inhibiting IL-12 production by PBMCs in response to LPS and IFN-γ. NS4 inhibited LPS-induced DC maturation and hindered their capacity to stimulate

proliferation and IFN-γ secretion by allospecific T cells. [18] This phenomenon is also displayed by failure of PBDCs from HCV patients to initate Th1 cells due to improper TLR4 signalling, decreased interferon alpha production and their induction of regulatory T cells.[19] These characteristics are thought to promote chronic infection and viral persistence.[20] Owing the in-vitro findings, DCs have repeatedly been stated to be a drug target in the therapy of CHC infection.[21,22] PBDCs exert their allostimulatory capacity through the expression of surface co-stimulatory molecules, e.g. CD86,40,80,83. The ability of KLH to induce these molecules and to elevate the production of the prototypic inflammatory cytokine IL-12 reflects the role of KLH in cancer immunology and its potential in driving Th1 dominant cellular immune responses.[23,24] Accordingly, it has been shown in CHC that KLH loaded PBDCs are able to induce T cells in general as well as cytotoxic T-lymphocytes (CTL) in vitro.[25,26] Since CTL induction is known to play a pivotal role in HCV clearance.[27,28] These properties of KLH may be of antiviral potency.

The clinical observation in the above reported patient led - in conjunction with this immunological background – to the rationale that the coincident KLH injections could have been the causative factor for HCV clearance.

This study aimed to examine the safety and efficacy of subcutaneous KLH in patients with untreated CHC of any genotype, compensated liver cirrhosis, and contraindications to peginterferon / ribavirin / boceprevir or telaprevir treatment (since combinations of direct-acting-antivirals were not approved at the time of study design and conduction).

Material and methods

Setting

This prospective, single arm, single center, and interventional pilot study evaluates the safety and efficacy of KLH in compensated cirrhotics with CHC. A double-blinded study design was not considered ethically justifiable in this group of patients at this early stage. The study was performed and all data were collected between January and October 2012. Data were monitored thrice during the study period by an approved Clinical Trial Monitor.

Patients

CHC infection was confirmed by HCV RNA positivity for at least six months. All patients were ineligible for interferon CHC therapy in conformity with the respective guidelines.[29,30] Interferon-free regimens were not available or approved at the time of study design and conduction. Inclusion and exclusion criteria are shown in Table 1.

Liver cirrhosis was confirmed by histology, sonography or Fibroscan® (Echosens S.A.S.U., Paris, France; performed by WS, BS or RES).[31] Only measurements with success rates >80% and interquartile range <30% were used for fibrosis judgment.

For safety reasons, possible hepatic decompensation was monitored clinically (including clinical judgment of hepatic encephalopathy), by laboratory parameters of liver synthesis and the routine evaluation of ultrasound signs of portal hypertension (spleen size, ascites, portal vein perfusion).

Study schedule and drug studied

Patients were scheduled for a structured interview, clinical and laboratory examinations at baseline, weeks one, two, four, six, eight, 12, 18, 24, and 32. They

received five injections of 1 mg KLH (Immucothel®, Biosyn Arzneimittel GmbH, Fellbach, Germany) subcutaneously at baseline and weeks one, two, four and eight. The chosen schedule was based on the maximum sensitization dose approved for KLH in its urological indication.

Laboratory studies

In addition to standard laboratory parameters, the baseline exam also included hepatitis B serology and an HIV antibody test.

The serum HCV RNA level determined upon every study visit was measured at a ISO 9001:2008-certified laboratory with the COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, version 2.0 (Roche Molecular Systems, Inc., Branchburg, NJ). The limits of detection and quantification were identical with 15 HCV RNA IU/ml. Runs were performed in concordance to the manufacturer's instructions. HCV genotypes were determined with the TRUGENE HCV Genotyping Kit (Siemens Healthcare Diagnostics Inc., Tarrytown, NY) according to the manufacturer's instructions.

Immunological measurement (FACS analysis)

Samples for peripheral blood FACS analysis were obtained at study baseline (prior to the first injection), after the study treatment (five KLH injections, i.e. week 10) and 24 weeks after the last KLH dose (i.e. week 32). Freshly drawn and anti-coagulated (EDTA) whole blood (100 µl) was processed for surface staining with the indicated antibodies for 20 minutes at room temperature (all antibodies used in this study were purchased from BD Biosciences, Franklin Lakes, New Jersey, USA).
After staining, the mixture was lysed in 2 ml of lysis buffer (FACS lysing solution; BD Biosciences, Franklin Lakes, New Jersey, USA) washed once with PBS, and after resuspension in PBS, analysed immediately on a LSR II flow cytometer using

FACSDiva software (BD Biosciences, Franklin Lakes, New Jersey, USA). Instrument calibration was done daily using CS&T calibration beads. Compensation settings for single-color controls were established with antibody-capture beads (BD CompBeads), and boundaries for gating were adjusted using fluorescence minus one (FMO) controls, especially for staining of CD80, CD83, CD40 and CD86 markers.

The following conjugated mouse monoclonal antibodies were used for the enumeration of cytotoxic T-cells, helper T-cells, NK-cells, and regulatory T cells (Tregs): CD45-APC-H7, CD3-V500, CD4-V450, CD8-FITC, CD25-PE, CD16-PerCP-Cy5.5, CD56-PE-Cy7 and CD127-Alexa Fluor 647. For determination of mDCs and pDCs, the following panel of conjugated antibodies were used: CD45-APC-H7, lineage marker (lin-1)-FITC, HLA-DR-PerCP-Cy5.5, CD123-PE-Cy7, CD11c-APC, including the activation/maturation markers CD80-V450/CD83-PE and CD40-PE/CD86-V450, respectively. A gate was set in the side scatter vs. anti-CD45 plot which included both the lymphocyte and monocyte populations and 50.000-60.000 events in that gate were routinely recorded. Myeloid and plasmocytoid dendritic cells were gated as described previously.[32]

Endpoints

The primary endpoint defined was an unmeasureable HCV viral load upon week 24. Secondary endpoints were defined as HCV RNA concentrations at week 1, 2, 4, 8, 12, 18 and 32 and alanine aminotransferase (ALT) levels at all visits.

Ethical approval

This study was performed in accordance with Good Scientific Practice and the Declaration of Helsinki. The study protocol was approved by the ethics committee of the Medical University of Graz and the national governmental authorities (Austrian

Agency for Health and Food Safety, AGES). Written informed consent was obtained from every patient prior to study interventions. Prior to study start, the protocol was registered in a clinical trials database (clinicaltrials.gov).

Statistical analyses

Data were analyzed using SPSS Statistics 22 software (IBM ® Corporation, USA). The Kolmogorov-Smirnov test was used to test for Gaussian distribution of continuous variables. Descriptives are given as mean ± standard deviation. Student's t-test, one-way-ANOVA, Kruskal Wallis test, Mann Whitney test, Friedman-test, chi squared test or Fisher's exact test were used as appropriate.

Results

Fourteen treatment naïve cirrhotic patients with CHC infection were enrolled in this study (six male, eight female, ages 35-79 years, Figure 1).

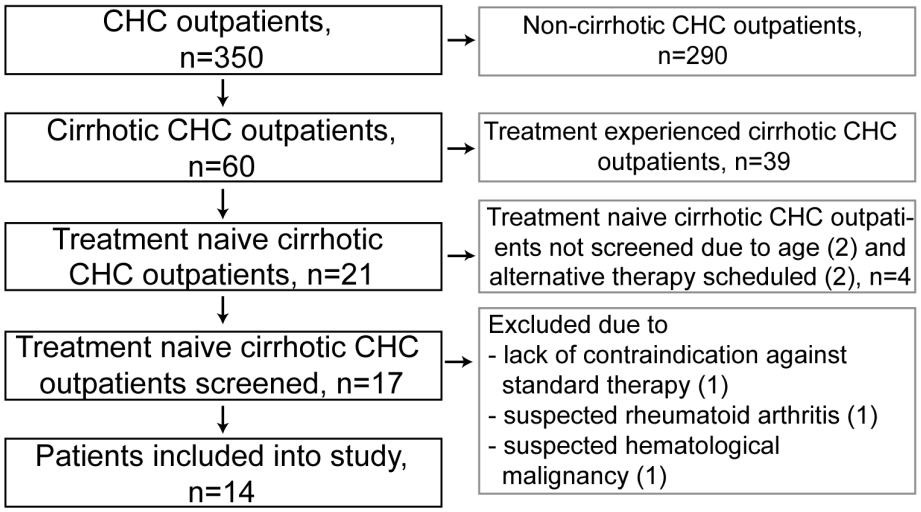

Figure 1

Patients predominantly had hepatitis C genotype 1 (11 of 14; genotype 1a: 2 patients and genotype 1b: 9 patients). Detailed characteristics of patients at study entry are compiled in Table 2.

Patients received the predefined study doses and adhered to treatment and follow up visits. There was neither early treatment discontinuation nor any patient lost to follow up.

Safety

In all patients, therapy was well tolerated and there was neither need to interrupt or abort therapy nor to adjust dosage or therapy interval for medical reasons.

There were fifteen adverse events in nine patients, three of them assessed as serious.

Eight adverse events, none of them serious, were related to the study medication. A detailed summary of adverse events is provided in Table 3. No patient had signs of hepatic decompensation (laboratory or clinical). There were no elevations of gamma-glutamyl transferase or ALT as described in the study medication´s package insert.

Clinical efficacy

Viral load characteristics are shown in Figure 2.

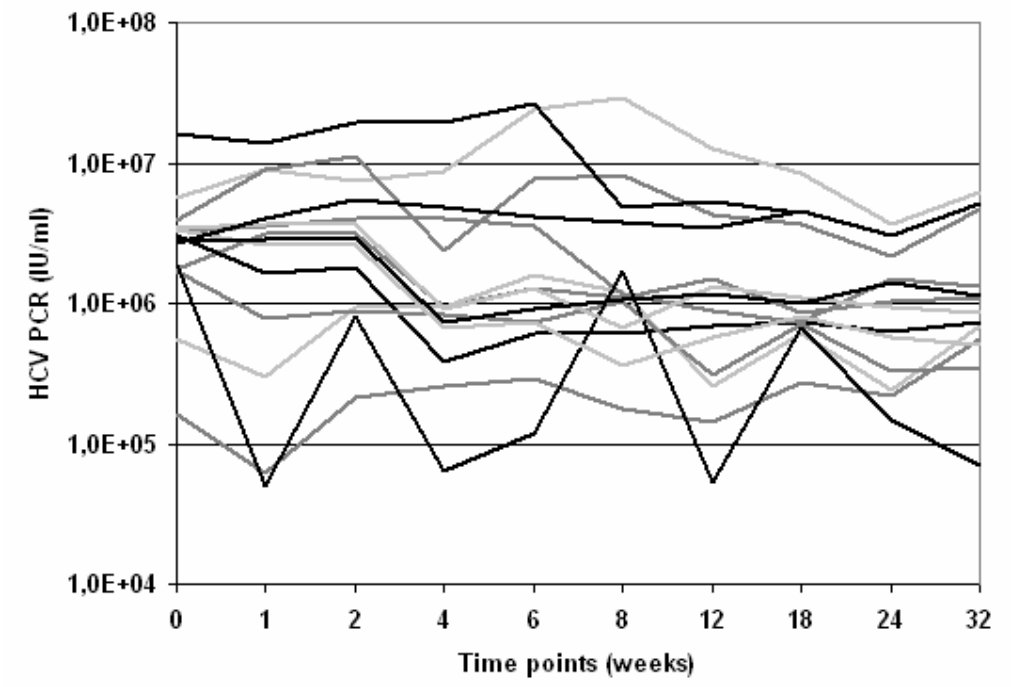

Figure 2

At week 24, HCV RNA was detectable in all patients and the HCV RNA concentration had not changed significantly, meaning that none of the patients had reached viral

clearance. The secondary endpoints (HCV RNA concentration at week 1, 2, 4, 8, 12, 18, and 32) also were not reached and the ALT level did not decline.

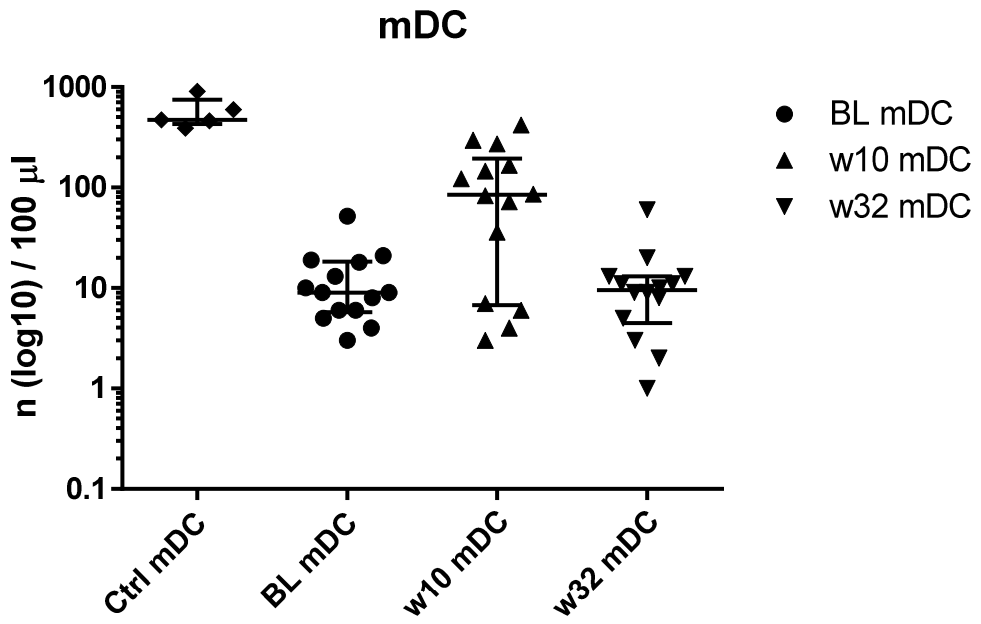

Figure 3

Immunological efficacy

At baseline, peripheral blood mDC numbers were lower in the patient group compared to controls (median 9 vs 475; p<0.001) and increased to 9.4 fold compared to baseline after the five KLH injections at week 10 (median 9 vs 85; p= 0.008, see Fig.3). However, mDC levels after KLH did not reach levels of controls. The effect did not persist after withdrawal of KLH and returned to baseline values upon week 32 (median 9 vs 9; p=0.004 vs week 10, p=0.73 vs baseline). Peripheral blood pDC numbers at baseline were significantly lower in patients than in controls (median 594 vs 1339; p= 0.002). No significant changes were observed in peripheral blood pDCs

during KLH therapy. Furthermore, activation and maturation status of mDCs and pDCs remained unchanged throughout the study. Effector cell numbers (T-helper, cytotoxic T-cells, natural killer cells and Tregs) were significantly lower in patients at baseline compared to controls and remained unaffected by KLH therapy.

Discussion

This pilot study evaluated a potential novel medical treatment for CHC with subcutaneous KLH in fourteen treatment naïve cirrhotic patients ineligible for interferon therapy. The study intervention was tolerated well and there were no serious treatment-related adverse events. Clinically, HCV eradication could not be reached, but hitherto undescribed immunological effects could be observed.

First, a reduction of peripheral blood dendritic cells (PBDCs) in CHC patients is known, but the degree of mDC reduction in compensated CHC cirrhotics demonstrated in this study is surprising.[14] The patients were nearly devoid of mDCs at baseline, could be numerically induced by KLH to the 9-fold number and returned to baseline at study end. Control PBDC numbers resembled the results of earlier studies in healthy individuals and were about 40 times higher than in CHC cirrhotics.[33] Since the impact of CHC on DC number is mild and a possible impact of liver cirrhosis on DC number is not known, a synergistic role of CHC and cirrhosis seems possible and can be speculated on.

Second, we did not observe the anticipated effect of KLH on DC activation and maturation that was shown in vitro.[23] This is of particular interest due to the fact that a in-vitro study recently suggested that monocyte-derived DCs from cirrhotic patients have similar activation and maturation capacities as healthy individuals.[34] These in-vitro observations are not be supported by our in-vivo data.

Impaired PBDC numbers and function may be a major hint to the pathomechanism of viral persistence since impaired DC function correlates with compromised virus-specific T cell responses.[35] Furthermore the virus itself compromises dendritic cell HLA-DR expression, prevents maturation and induces the production of anti-inflammatory cytokines like IL-10.[36]

These direct inhibitions of DCs point out the possible evolutionary survival process of the HCV by stopping the innate immunity of recognizing and defeating the virus. Therefore targeting DCs in the therapy has been proposed recently.[37] The process of the loss of dendritic cell number in patients with CHC is not fully understood; liver disease itself has been proposed and also direct viral influence on DC function.[36,38] Natural killer cell (NKc) crosstalk with DCs could be one possible mechanism of how dendritic cells get reduced in patients with CHC. Immature DCs are at risk of getting killed by activated NKc and it has been shown that activated NKc kill immature but spare mature DCs.[39] The maturation grade is reflected by expression of surface markers and since the presence of HCV induces down regulation of these molecules, induced immaturity of DCs could be a trigger for NKc dependent lysis.[40,41]

For the above reasons, mDC stimulation was targeted and successfully accomplished by KLH treatment, which we herein show to our knowledge for the first time. However, mDC numbers could not be increased to values observed in healthy controls and activation as well as maturation of the numerically increased mDCs remained unaffected, being two major possibilities of hindering effector cell induction and consecutively antiviral activity. Other protocols of KLH administration in terms of more frequent dosing and/or a higher cumulative dose or the application of other immunostimulatory molecules may overcome these limitations in future studies.

In conclusion, the KLH treatment protocol presented led to a desired immunological effect on mDC numbers. To our knowledge, our data reveal for the first time that severely decreased mDCs in cirrhotic CHC patients are temporarily inducible by medical treatment. Nonetheless, the protocol failed to induce effector cells and did not reduce HCV RNA load in our patient cohort of difficult-to-treat patients. No sustained virological response was achieved. The treatment was safe in

a population vulnerable to intervention-related side effects, consistent with long-standing experience with KLH in urological practice.

CHC therapy has evolved rapidly since study commencement and highly effective treatment regimens devoid of significant side effects are available today. However, the observed response on mDC number may be of significant interest in a multitude of liver diseases. For instance, pathogenesis of viral hepatitis - associated hepatocellular carcinoma (HCC) is thought to be influence by hampered PBDC function.[42] KLH-based treatments for HCC, a disease with high mortality and limited therapeutic options, may be a promising approach in the future.

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Author contributions

W.S. and A.M.M. designed and conducted the study, collected samples, analyzed data and wrote the main manuscript text. R.E.S. and B.B. supervised the study and the patient care. W.S., A.M.M., U.K., B.S. were recruiting patients and performed patient care. H.S. planned and supervised the FACS analysis. H.K. performed HCV RNA measurement. R.E.S. and P.F. supervised the analysis of the data and the evolution of the main manuscript text. All authors critically reviewed the manuscript.

Competing interests

The authors declare to have no competing interests regarding the underlying manuscript.

Figure legends

Figure 1 Patients with chronic hepatitis C infection eligible for the study; CHC, chronic hepatitis C

Figure 2 Quantitative hepatitis C viral load during the study period

Figure 3 Myeloid dendritic cell numbers during the study and compared to controls

Tables

Inclusion Criteria:	
1	CHC infection, genotype 1-6
2	Age 18-80 years
3	Women: postmenopausal state
4	Compensated liver cirrhosis (Child Pugh Score 5 or 6)
5	Contraindication against interferon based CHC therapy (dual or triple)
Exclusion Criteria:	
1	Known hypersensitivity to KLH
2	Immunosuppression; treatment with: glucocorticoid, azathioprine, , mycophenolate, TNF alpha blockers, calcineurine inhibitors, interleukin 2 blockers
3	Hepatocellular carcinoma and other malignancies
4	Coinfection with hepatitis B or HIV
5	Pregnancy or lactation
6	Cardiovascular event (stroke, myocardial infarction) during the last six months
7	Uncontrolled diabetes
8	Renal failure (glomerular filtration rate < 50 ml/min) or hemodialysis
9	Severe active autoimmune disorders

Table 1

Inclusion and exclusion criteria for study therapy. CHC, Chronic hepatitis C; KLH, Keyhole-limpet-hemocyanin; TNF, tumor necrosis factor; HIV, human immunodeficiency virus

Age (y)	Sex (m / f)	Height (m)	Weight (kg)	BMI	Viral GT	Viral load (IU/ml)	Cirrhosis (confirmation by)	FS (kPa)	IQR (kPa)	Previous P/R therapy (yes / no)
50	m	1.86	118	34	3a	1,000,000	FS	36.3	4.0	no
52	f	1.52	60	26	1a	180,000	FS	17.4	0.0	no
60	f	1.69	68	24	1b	2,800,000	FS	42.2	7.9	no
60	f	1.63	62	23	1b	3,200,000	FS	30.7	5.3	no
56	f	1.65	79	29	1b	5,700,000	FS	16.2	3.6	no
56	f	1.54	76	32	1a	270,000	FS	40.4	0.0	no
46	m	1.82	83	25	2a	180,000	FS	36.2	2.7	no
63	f	1.70	60	21	1b	5,000,000	FS	12.8	1.2	no
40	m	1.85	98	29	1b	4,100,000	FS	20.9	0.8	no
76	f	1.70	72	25	1b	2,000,000	FS	15.1	2.5	no
35	m	1.93	91	24	1b	460,000	FS	26.3	4.9	no
61	f	1.50	67	30	1b	17,000,000	FS	34.7	6.3	no
60	m	1.73	77	26	1b	2,500,000	FS	26.4	8.7	no
79	m	1.82	74	22	3a	3,600,000	FS	25.1	2.4	no

Table 2

Baseline characteristics of 14 patients enrolled in the study. GT, genotype; FS, fibroscan; IQR, interquartile range

Adverse Events	Number	Related (y/n)
Fatigue, postinjection	1	y
Local erythema, postinjection	4	y
Fever, postinjection	1	y
Maculopapular rash (legs)	1	y
Epididymitis	1	n
Sinusitis	1	n
Ventricular bigeminus	1	n
Diarrhea	1	n
Aggravation of vitiligo (hands)	1	y
Total	12	
Serious Adverse Events		
Norovirus positive gastroenteritis	1	n
Erysipel (legs)	1	n
Banding of esophageal varices	1	n
Total	3	

Table 3

Adverse and serious adverse events that occurred during the study