Liver-directed gene therapy of chronic hepadnavirus infection using interferon alpha tethered to apolipoprotein A-I

Pedro Berraondo1,⁎,1, Marianna Di Scala1, Kyle Korolowicz2, Linta M. Thampi2, Itziar Otano1, Lester Suarez1, Jessica Fioravanti1, Fernando Aranda1, Nuria Ardaiz1, Junming Yang2, Bhaskar V. Kallakury1, Robin D. Tucker4, Marcos Vasquez1, Stephan Menne4,⁎, Jesús Prieto1,⁎, Gloria González-Aseguinolaza1,⁎

1Division of Hepatology and Gene Therapy, Center for Applied Medical Research, University of Navarra, Pamplona, Navarra, Spain; 2Department of Microbiology & Immunology, Georgetown University Medical Center, Washington, DC, USA; 3Department of Pathology, Georgetown University Medical Center, Washington, DC, USA; 4Division of Comparative Medicine, Georgetown University Medical Center, Washington, DC, USA

Background & Aims: Current hepatitis B virus (HBV) management is challenging as treatment with nucleos(t)ide analogues needs to be maintained indefinitely and because interferon (IFN)-α therapy is associated with considerable toxicity. Previously, we showed that linking IFNα to apolipoprotein A-I generates a molecule (IA) with distinct antiviral and immunostimulatory activities which lacks the hematological toxicity of IFNα.

Methods: Here, we analyse the antiviral potential of an adeno-associated vector encoding IFNα fused to apolipoprotein A-I (AAV-IA) in comparison to a vector encoding only IFNα (AAV-IFN) in two animal models of chronic hepadnavirus infection.

Results: In HBV transgenic mice, we found that both vectors induced marked reductions in serum and liver HBV DNA and in hepatic HBV RNA but AAV-IFN caused lethal pancytopenia. Woodchucks with chronic hepatitis virus (WHV) infection that were treated by intrahepatic injection of vectors encoding the woodchuck sequences (AAV-wIFN or AAV-wIA), experienced only a slight reduction of viremia which was associated with hematological toxicity and high mortality when using AAV-wIFN, while AAV-wIA was well tolerated. However, when we tested AAV-wIA or a control vector encoding woodchuck apolipoprotein A-I (AAV-wApo) in combination with entecavir, we found that AAV-wApo-treated animals exhibited an immediate rebound of viral load upon entecavir withdrawal while, in AAV-wIA-treated woodchucks, viremia and antigenemia remained at low levels for several weeks following entecavir interruption.

Conclusions: Treatment with AAV-IA is safe and elicits antiviral effects in animal models with difficult to treat chronic hepadnavirus infection. AAV-IA in combination with nucleos(t)ide analogues represents a promising approach for the treatment of HBV infection in highly viremic patients.

© 2015 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Introduction

Chronic hepatitis B (CHB) is a global health problem, with more than 350 million individuals worldwide infected with hepatitis B virus (HBV) [1]. Available treatment options include nucleoside/nucleotide analogues (NAs) that are highly effective in suppressing HBV replication and preventing liver disease progression. However, NAs do not affect HBV RNA transcription from the covalently-closed circular DNA molecule of HBV within infected hepatocytes and, therefore, have only limited impact on HBV surface antigen serum levels. Since NAs do not directly stimulate immune clearance of HBV-infected hepatocytes, in most cases viral replication rebounds upon cessation of therapy. As a consequence, prolonged or even life-long treatment of HBV is needed. This long-lasting therapy is associated with high costs and the risk of emergence of drug resistant HBV variants [2]. Pegylated interferon alpha (PegIFNα) is an alternative therapy for CHB. This cytokine is endowed with immunostimulatory properties and targets both HBV transcription and replication. Antiviral response to PegIFNα treatment is often durable but the use of this drug is limited due to low response rates and...
Research Article

frequent side effects that are sometimes severe [3]. Therefore, new and alternative treatment approaches for the control of CHB are warranted.

We have previously shown that the linkage of IFNα to apolipoprotein A-I (apoA-I) generates a fusion molecule termed InterApo (IA) which maintains the antiviral and immunostimulatory activities of IFNα but is devoid of the hematological and central nervous system toxicities that are mediated by this cytokine [4,5]. In the present study, we aimed at evaluating if liver transduction with an adeno-associated virus (AAV) vector encoding IFNα (AAV-IFN) or IA (AAV-IA) is an effective therapeutic approach for chronic HBV infection. We tested this gene therapy approach in HBV transgenic (HBVTg) mice and in woodchucks with chronic woodchuck hepatitis virus (WHV) infection. The latter is a model which closely reproduces the virological and histopathological features of CHB [6]. An important difference between CHB and chronic WHV infection is that woodchucks frequently exhibit serum viral loads in excess of $10^{10}$ viral genomes (vg)/ml while in humans HBV viremia rarely exceeds $10^7$ vg/ml. The extremely high levels of circulating WHV virions cause exhaustion of immunological effectors thus rendering chronic WHV infection into a condition that is exceedingly difficult to treat. In an attempt to facilitate the response to IA, in one of our treatment protocols, we treated WHV-infected woodchucks with entecavir (ETV) during 4 weeks to see whether a transient decrease of viremia could make viral replication easier to control. Overall, our studies demonstrate that treatment with AAV-IA is safe and exerts antiviral effects both in HBVTg mice and in chronic WHV carrier woodchucks and suggest that combination of IA with NAs is a promising approach to achieve sustained control of HBV replication.

Materials and methods

Animals

Female, 6 weeks old, C57BL/6 mice were purchased from Harlan (Barcelona, Spain). The HBVTg mouse lineage 1.3.32 (inbred C57BL/6, H-2b) was kindly provided by Professor Frank Chisari (The Scripps Research Institute, La Jolla, California, USA). These HBVTg mice encode a 1.3-overlength copy of the HBV genome (serotype ayw), replicate HBV at high levels within the liver and express all of the HBV antigens [7]. HBVTg mice were matched for age (6–10 weeks), sex (male), and serum HBV DNA and HBV surface antigen levels, as determined by real-time quantitative PCR or an enzyme-linked immunosorbent assay (ELISA) (Bioelsa HBSag 3.0, Biokit), respectively. The mice were treated in accordance with the guidelines of the University of Navarra (Pamplona, Spain).

AAV vectors were inoculated in mice via retro-orbital injection in a total volume of 200 μl. For viral challenge, a lethal dose of encephalomyocarditis virus (EMCV) was intraperitoneally injected. Blood samples were drawn by retro-orbital bleeding at the indicated time points. Serum samples were derived from two consecutive centrifugations of blood samples at 9100 g for 5 minutes and stored at −20 °C. Liver samples were stored at −80 °C until assayed.

Captive-born, 12 months old Eastern woodchucks (Marmota monax), neonatally infected with WHV were purchased from Northeastern Wildlife Inc. (Harris, ID). Animal maintenance and handling were performed according to the regulations of the local Animal Care and Use Committee of the University of Navarra. Laparotomy was performed to inject intrahepatically 2.5 × 10¹³ or 1.25 × 10¹² viral genomes of the AAV vectors. The second woodchuck study with additional ETV treatment was performed under an animal protocol approved by the institutional Animal Care and Use Committee of Georgetown University (Washington, DC). ETV (Selleck Chemicals) was mixed with woodchuck liquid diet (Dyets Inc., Bethlehem, PA) and orally administered by dose syringe. Daily, oral ETV dosing at a concentration of 0.5 mg/kg started at day 0 and continued thereafter for 4 weeks. A single dose of 5 × 10¹⁴ viral genomes of the AAV vectors was given at day 0 to woodchucks by intravenous injection. In both studies, blood was collected from the saphena vein or other approved sites using isoflurane or ketamine/xylazine anesthesia for sedation of woodchucks.

AAV vectors

Experiments were performed using AAV serotype 8 vectors encoding luciferase (AAV-Luc), mouse IFNα (AAV-IFN), the IA fusion protein generated by linking mouse IFNα to mouse apoA-I [AAV-IA], woodchuck IFNα [8,9] (AAV-wIFN), woodchuck apoA-I [10] (AAV-wApO) or the IA fusion protein formed by linking wIFNα5 to woodchuck apoA-I (AAV-wIA). All transgenes were placed under the transcriptional control of the elongation factor 1α promoter. The above AAVs were produced by co-transfection of pDPPs.ape and AAV plasmids into 293T cells [11]. Two days later, AAVs were purified from cell lysates by ultracentrifugation in Optiprep Density Gradient Medium-Iodixanol (Sigma-Aldrich, St Louis MO). For AAV titration, viral DNA was isolated using the High Pure Viral Nucleic Acid kit (Roche Applied Science. Mannheim, Germany). The concentration of viral particles was subsequently determined by real-time quantitative PCR using primers specific to the elongation factor 1α promoter (EF-Forward (Fw): 5’-ggtggtgagcaccacacaaaggg-3’ and EF-Reverse (Rv): 5’-cctggagagtccacagtaggaaacag-3’).

ELISA for murine IFNα determination

IFNα levels in serum were measured using the VeriKine™ Mouse Interferon Alpha ELISA kit (PBL, NJ, USA) by following the manufacturer’s recommendations.

Hemogram

Blood samples were collected in Microvette 500 Blood Collection tubes with EDTA (Sarstedt, Landskrona, Sweden). The hemogram was analysed using the Drew Scientific HemaVet Hematology Analyser according to the manufacturer’s recommendations.

RNA extraction and cDNA synthesis

Total RNA was extracted from liver tissue using the Maxwell® 16 Total RNA Purification kit (Promega, Madison, Wisconsin, USA). Concentration and purity of RNA was determined by absorbance at 260 and 280 nm in a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). One microgram of mRNA was transcribed to cDNA with Moloney Murine Leukemia Virus reverse transcriptase from Promega by following the manufacturer’s assay instructions. The reaction was incubated for 1 h at 37 °C, denatured for 1 min at 95 °C and cooled at 4 °C. Samples were immediately used for PCR or stored at −20 °C until assayed.

Quantitative PCR analysis of IFN-stimulated genes

Mouse IFN-stimulated-gene 15 (ISG15), ubiquitin specific peptidase 18 (USP18), 2′-5′-oligoadenylate synthetase (2′-5′ OAS) and β-actin expression was determined by quantitative real-time PCR using specific primers for each gene (ISG15-Fw: 5′-gattggccagcaagattg-3′ and ISG15-Rv: 5′-tcctgctgaagaacacta-3′; USP18-Fw: 5′-cccaacagctccatcagg-3′ and USP18-Rv: 5′-atgaccaagctccagg-3′; 2′-5′ OAS-Fw: 5′-acctgctgaagactggc-3′ and 2′-5′ OAS-Rv: 5′-ccagctgctggacccg-3′ and SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). As β-actin transcript levels remain unchanged across different experimental conditions, the expression of this housekeeping gene was used to standardize gene expression (ACT-Fw: 5′-cctggtgctaggcc-3′ and ACT-Rv: 5′-cgctgacccgagc-3′). For determination of HBV DNA or RNA following reverse transcription into cDNA, the following primers were used: HBcAg-Fw: 5′-TTCGACTTCCACCTTAT-3′ and HBcAg-Rv: 5′-GGGAGGGACAGGCTTCTTCTA-3′. Murine GPDH served as a reference and the following primers were used: GPDH-Fw: 5′-TCAC CACAACTGTTCA-3′ and GPDH-Rv: 5′-CAGAAGGCTGGATGCTCC-3′.

The amount of each transcript was expressed by the formula $2^{-Ct}$, where Ct is the point at which gene fluorescence rises significantly above background fluorescence. Real-time PCR reactions were performed using Bio-Rad reagents in accordance with the manufacturer’s recommended protocol.

Virological determinations

Serum WHV and HBV DNA was isolated using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Lewes, United Kingdom) or the QiAmp DNA Mini kit (Qiagen, Valencia, CA), respectively, and were quantified by real-time PCR as described previously [9,12].

Serum WHV surface antigen (WHsAg) was assayed quantitatively against woodchuck serum with a known concentration of WHsAg by using a monoclonal antibody-based ELISA as described (13). Serum HBsAg was analysed using Bioelisa HBsAg 3.0 (Biokit, Barcelona, Spain) following manufacturer’s instructions.

Determination of wIFNα bioactivity

The antiviral activity mediated by wIFNα present in serum was assayed in a cytopathic effect reduction assay using woodchuck WCH17 cells following challenge with EMCV in a 96-well microtiter plate format as described previously (8).

Statistical analysis

Statistical analyses were performed using the Prism 5 computer program (GraphPad Software Inc, San Diego, CA, USA). The survival data were represented in Kaplan-Meier graphs and analysed by the log rank test. The remaining parameters were analysed by one-way ANOVA followed by the Tukey’s multiple comparisons test. The comparisons of WHV viremia and WHsAg relapse after ETV withdrawal were performed by fitting the data to a dose-response stimulation curve followed by the extra sum-of-squares F test. p <0.05 values were considered significant.

Results

IA but not IFNα can be expressed in the liver for a prolonged period of time without significant hematological toxicity

We generated AAV vectors encoding IA or IFNα for evaluating the feasibility and safety of long-term expression of these molecules in rodents. As shown in Fig. 1A, immunoreactive IFNα was detected in serum of mice injected with a single dose of 5 × 10^11 vg of AAV-IFN or AAV-IA, but IFNα levels were substantially higher with the latter vector. However, while mice treated with AAV-IA remained alive during the study period (and beyond 6 months after vector administration; data not shown), all mice that received AAV-IFN died within 60 days (Fig. 1B). In these animals, a dramatic and significant decline in erythrocytes, leukocytes and platelets was observed while the red blood cell and platelet counts remained unchanged in AAV-IA treated mice (Fig. 1C). Neutrophil and monocyte counts decreased significantly in mice treated with AAV-IFN but not in those which received AAV-IA while lymphocytes showed a decline in mice treated with either vector (Supplementary Fig. 1).

The antiviral activity of AAV-IA is comparable to that of AAV-IFN

We then analysed if the antiviral activity of AAV-IA was similar to that of AAV-IFN. To this aim, mice were injected intravenously with 5 × 10^11 vg/mouse of AAV-IA, AAV-IFN or the control vector AAV-Luc, and 1 month later we measured the hepatic expression of IFN-stimulated genes. We found that both, AAV-IFN and AAV-IA but not AAV-Luc, stimulated the expression of IFN-stimulated genes at similar levels (Fig. 2A). Next, we tested the antiviral effect of AAV-IA or AAV-IFN by injecting mice with a lethal dose of EMCV. We found that animals treated with either of the two IFN-vectors were equally protected against EMCV infection provoked one month after the vector administration (Fig. 2B). These data show that AAV-IFN and AAV-IA exhibit comparable antiviral activity but hematological toxicity is much attenuated with the latter vector. Also it should be noted that no histological changes were observed in the liver of animals 6 months after AAV-IA administration (Supplementary Fig. 2).

Antiviral effects and hematological toxicity of AAV-IA and AAV-IFN in HBVTg mice

For evaluating the therapeutic effects of AAV-IA in chronic HBV infection, we tested this vector in HBVTg mice, an animal model that exhibits levels of viremia comparable to those observed in patients with CHB (7). HBVTg mice received a single dose of 1.5 × 10^11 vg of AAV-IA, AAV-IFN or AAV-Luc control vector via the tail vein. Blood samples were collected 7 days after the AAV injection, and animals were sacrificed thereafter.

Immunoreactive IFNα was not detected in the serum of control mice (AAV-Luc) while detectable amounts of this cytokine were present in the blood of mice that received AAV-IFN or AAV-IA (Supplementary Fig. 3). IFNα serum levels were higher with AAV-IA than with AAV-IFNα and both groups demonstrated a marked and significant decline in serum viral loads and in serum HBsAg in comparison to control animals (Fig. 3A). The reduction in circulating HBV viral particles was associated with a decline in hepatic HBV DNA abundance and disappearance of HBV DNA replicative forms in the liver (Supplementary Fig. 4) accompanied by a marked reduction of HBV core antigen mRNA levels in the liver (Fig. 3A). Similarly to our observations in normal mice, leukocyte, red cell and platelet counts dropped significantly in HBVTg mice that received AAV-IFN being the descent of leukocytes and platelets significantly less pronounced in AAV-IA treated mice (Fig. 3B).
that was euthanized because of the development of hepatocellular carcinoma, a frequent finding in woodchucks with chronic WHV infection. Notably, in AAV-wIA-treated animals, we observed no remarkable changes in red blood cell counts and only a moderate reduction of leukocytes and platelets in some woodchucks followed by posterior recovery in some of them. In all groups treated with AAV-wIA or AAV-wIFN, ALT levels did not show significant changes with respect to basal levels (data not shown).

All woodchucks in this study exhibited very high viremia, with levels between \(10^{11}\) and \(10^{13}\) vg/ml. In agreement with reports indicating that an elevated viral load is associated with poor response to IFN therapy in the woodchuck model [14], we observed little changes in circulating WHV DNA in all treatment groups (Fig. 5). Only the woodchuck that was euthanized...
Fig. 4. IFNα serum levels and hematological parameters in woodchucks with chronic WHV infection treated with AAV-wIFN (left) or AAV-wIA (right). (A) IFNα serum levels in woodchucks treated with a single dose of $1.25 \times 10^{11}$ (open symbols) or $1.25 \times 10^{12}$ vg (closed symbols) of AAV-wIFN or AAV-wIA by direct intrahepatic injection. (B) Number of leukocytes (WBC), (C) erythrocytes (RBC) and (D) platelets in treated woodchucks. †, dead woodchucks.
from pretreatment), accompanied by a moderate reduction in viral load of greater than 2 logs from pretreatment values. Notably, these antiviral effects were achieved with serum IFNα levels that were below the detection threshold of our assay (data not shown). Consistent with these observations, no significant changes in blood cell counts (leukocytes, erythrocytes, and platelets) nor in other biochemical alterations including renal and liver function parameters were found in treated or control animals (data not shown). Thus, combination treatment with ETV and AAV-wIA was safe and achieved significant antiviral effects in a very difficult-to-treat model of chronic hepadnavirus infection.

**Discussion**

The introduction of effective NAs possessing a high barrier to viral resistance such as ETV or tenofovir has undoubtedly represented a formidable progress in the fight against HBV infection. However, treatment of CHB is still challenging since these drugs do not affect transcription of viral proteins from covalently-closed circular DNA molecule of HBV [15], nor exert direct immunostimulatory effects to facilitate immune clearance of HBV-infected hepatocytes (15). Persistent antigenemia despite NAs therapy favours the maintenance of immune tolerance to the virus and, as a result, rebound of HBV replication is frequently observed following the cessation of therapy [3]. Thus, treatment needs to be maintained for an indefinite period of time to ensure sustained control of HBV infection. PegIFNα is an effective drug for treatment of chronic HBV infection as it inhibits HBV replication as well as HBV transcription. In addition, IFNα possesses immunostimulatory properties that facilitate the activation of anti-HBV T cell immune responses. However, the use of IFNα is limited by severe toxicity in a majority of patients [16]. Thus, in the present work, we have tested a protein that is formed by the fusion of IFNα to apoA-I (termed IA) as a potential therapy for CHB. Previously, we have shown that IA displays immunostimulatory activities without inducing hematological toxicity [4]. Because of the favourable toxicology profile of IA, we have now investigated if this protein could be administered to the liver by gene transfer. In this study we used a serotype 8 AAV vector. This is a hepatotropic long-term expression vector that has been used in the clinic for the treatment of hemophilia B with remarkable success and excellent tolerance [17]. We found that the AAV vector encoding IA (AAV-IA) was able to protect normal mice against a lethal challenge of EMCV as efficiently as an AAV vector encoding IFNα only (AAV-IFN). AAV-IA was also comparable to AAV-IFN in the control of HBV transcription and replication in HBV Tg mice. Notably, AAV-IFN induced severe leukopenia and thrombocytopenia and moderate anemia in treated animals while AAV-IA therapy was associated with minor changes in the platelet count, slight anemia and moderate leukopenia. Because of these encouraging results, AAV-IA was compared to AAV-IFN in woodchucks with chronic WHV infection using the appropriate woodchuck apoA-I and IFNα gene sequences. In the first study, the vectors (AAV-wIA and AAV-wIFN) were administered at two different doses by intrahepatic injection (a route which ensures high transduction efficiency) [18]. We could again confirm the hematological toxicity risk associated with the intrahepatic expression of IFNα at high levels that caused death of all animals. In contrast, AAV-wIA did not produce relevant hematological changes, and all animals survived suggesting that this transgene may be suitable for
clinical application. However, neither AAV-wIFN nor AAV-wIA promoted any significant decline in viremia in the treated woodchucks. This was not surprising as these animals exhibited extremely high viral loads and it has been shown that the level of viremia is the main factor that negatively predicts the antiviral response to IFN [14]. As viremia is in the range of $10^{11}$ and $10^{13}$ vg/ml in woodchucks with chronic WHV infection while viral load in patients with CHB rarely exceeds $10^7$ vg/ml [6], we designed a new protocol combining AAV-IA (or the control vector AAV-Apo) with 4 weeks of treatment with ETV. In this protocol, we selected the intravenous route for vector administration as this would be more acceptable for clinical use. Using this route, the transduction efficiency was lower compared to previous protocols, and in fact serum IFNα levels were below the detectable threshold. In this study, we did not observe any toxicity related to vector administration but we found a significant enhancement and prolongation of the antiviral effect of ETV in such a way that the decrease of viremia persisted during several weeks after ETV interruption in animals that received AAV-IA while viremia relapsed rapidly to basal values in animals treated with the control vector. It seems possible that by increasing liver transduction with AAV-IA (using a higher vector dose or a different administration route) or by prolonging ETV treatment, we may control chronic WHV infection in a more efficient and sustained manner.

To conclude, our results show in two different animal models that a non-toxic IFNα-based therapy can be administered in combination with NAs for more stringent control of hepadnavirus infection. In our study, we have used a gene therapy approach to ensure sustained intrahepatic expression of IA, but it seems possible that similar results could be obtained by combining ETV with repeated periodic injections of recombinant IA which is easier to use in clinical practice. However, the gene therapy option should not be ruled out for difficult-to-treat cases considering the excellent tolerance demonstrated by AAV vectors in different clinical trials [19,20], the possibility of employing inducible promoters and the fact that liver-directed gene therapy would allow attaining high intrahepatic IA concentration without unduly increasing systemic levels.

Financial support

This work was funded in part under IDIQ contract HHSN272201000111L, task order HHSN272000002 (D06) for the Department of Microbiology & Immunology, Georgetown University Medical Center from the Division of Microbiology and Infectious Diseases (DMID) of the National Institute of Allergy and Infectious Diseases (NIAID). This work was also supported by grants UTE project CIMA, CIBERehd Instituto de Salud Carlos III, SAF SAF2009-08524 and SAF2012-39578 (to GGA) from the Spanish Department of Science, Fondo de Investigación Sanitaria PI10/00264 and PI13/00207 (financed by the FEDER program of the European Union) and Fundación Mutua Madrileña (to PB). P.B. was supported by a Miguel Servet contract from Spanish Fondo de Investigación Sanitaria. L.S. was in receipt of FPI grant.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.
Research Article

Authors’ contributions

Study concept and design: PB, SM, JP, and GGA; acquisition of data: PB, MDS, KK, LMT, IO, LS, JF, FA, NA, JY, BVK, RDT, SM, MV; analysis and interpretation of data: PB, SM, JP, and GGA; drafting of the manuscript: PB, SM, JP, and GGA; statistical analysis: PB; obtained funding: PB, SM, JP, and GGA; study supervision: PB, SM, JP, and GGA.

Acknowledgements

The authors thank Diana Berard and Dr. Roldolo for continued discussion and support. The authors thank Dr. Francis V. Chisari for kindly providing us with the HBV transgenic mouse. The authors thank Elena Ciordia, Alberto Espinal, and CIFA staff for animal care and vivarium management. This work was funded in part under IDIQ contract HHSN27220100011L, task order HHSN27200002 (D06) for the Department of Microbiology & Immunology, Georgetown University Medical Center from the Division of Microbiology and Infectious Diseases (DMID) of the National Institute of Allergy and Infectious Diseases (NIAID). This work was also supported by grants UTE project CIMA, CIBERehd Instituto de Salud Carlos III, SAF SAF2009-08524 and SAF2012-39578 (to G. Gonzalez-Aseguinolaza) from the Spanish Department of Science, Fondo de Investigación Sanitaria (FIS PI10/00264) and PI13/00207 (financed by the FEDER program of the European Union) and Fundación Mutua Madrileña. P.B. was supported by a Miguel Servet contract from Spanish Fondo de Investigación Sanitaria. L.S. was in receipt of FPI grant.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2015.02.048.

References
