Safety and efficacy of REP 2139 and pegylated interferon alfa-2a for treatment-naive patients with chronic hepatitis B virus and hepatitis D virus co-infection (REP 301 and REP 301-LTF): a non-randomised, open-label, phase 2 trial

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Summary

Background REP 2139 clears circulating hepatitis B virus (HBV) surface antigen (HBsAg), enhancing the restoration of functional control of HBV infection by immunotherapy. We assessed the safety and efficacy of REP 2139 and pegylated interferon alfa-2a in patients with chronic HBV and hepatitis D virus (HDV) co-infection.

Methods In this open-label, non-randomised, phase 2 trial, patients aged 18–55 years, who were treatment naive, hepatitis B e antigen [HBeAg] negative, anti-hepatitis D antigen [HDAg] positive, and HDV RNA positive, with serum HBsAg concentrations of more than 1000 IU/mL, and a history of HDV infection for 6 months or more before treatment, were recruited at Toma Ciorbă Hospital of Infectious Diseases in Chișinău, Moldova. Patients were excluded if they had HDV superinfection, liver infections other than HBV and HDV, or liver cirrhosis. Patients received 500 mg intravenous REP 2139 once per week for 15 weeks, followed by combined therapy with 250 mg intravenous REP 2139 and 180 µg subcutaneous pegylated interferon alfa-2a once per week for 15 weeks, then monotherapy with 180 µg pegylated interferon alfa-2a once per week for 33 weeks. The primary endpoints assessed at the end of treatment were the safety and tolerability of the treatment regimen, analysed in the intention-to-treat population. Secondary outcomes included the proportion of patients with serum HBsAg less than 50 IU/mL, the proportion of patients with suppressed HBV DNA, and the proportion of patients who maintained these responses through follow-up. The REP 301 trial is registered with ClinicalTrials.gov, number NCT02233075. We also did an additional follow-up at 1 year after the end of treatment, as an interim analysis of the REP 301-LTF trial (planned duration 3 years), registered with ClinicalTrials.gov, number NCT02876419, which is ongoing but not recruiting patients.

Findings Between Sept 8, 2014, and Jan 27, 2015, we enrolled 12 patients into the REP 301 study. All 12 patients experienced at least one adverse event during treatment: two (17%) patients experienced anaemia, eight (67%) neutropenia, and ten (83%) thrombocytopenia. Five (42%) patients had raised alanine aminotransferase levels, four (33%) had raised aspartate aminotransferase levels, and two (17%) had increased bilirubin concentrations. Four (33%) patients had a serious adverse event, and 12 (100%) patients had treatment-emergent lab abnormalities. Six patients had HBsAg levels less than 50 IU/mL by the end of treatment (all <0·05 IU/mL); five maintained this level of suppression at the end of 1 year follow-up. Six patients had hepatitis B surface antibody (anti-HBs) titres above 10 mIU/mL at the end of treatment (five had maximum anti-HBs concentrations of 7681–86 532 mIU/mL during treatment), which were maintained at the end of 1 year follow-up in these five patients. Elevated alanine and aspartate aminotransferase concentrations and profound elevations of anti-HBs titres were restricted to patients who had HBsAg levels of less than <1 IU/mL before the introduction of pegylated interferon alfa-2a. Nine patients had suppressed HBV DNA (<10 IU/mL]) at the end of treatment, which was maintained by seven patients and newly established in an eighth patient at the end of 1 year follow-up. 11 patients became HDV RNA negative during treatment, with nine remaining HDV RNA negative at the end of treatment; seven of these patients remained HDV RNA negative by the end of 1 year follow-up. By the end of 1 year follow-up, normalisation of serum aminotransferases occurred in nine of 12 patients.

Interpretation Combined REP 2139 and pegylated interferon alfa-2a therapy is safe, well tolerated, and establishes functional control of HBV and HDV co-infection and normalisation of serum aminotransferases in a high proportion of patients 1 year after therapy. This combination therapy approach might provide a new treatment option for patients with HBV and HDV co-infection.

Funding Replicor.

Lancet Gastroenterol Hepatol 2017; 2: 877–89

Published Online September 27, 2017 http://dx.doi.org/10.1016/S2468-1253(17)30288-1

This online publication has been corrected. The corrected version first appeared at thelancet.com/gastrohep on Dec 8, 2017

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Introduction

Chronic hepatitis D virus (HDV) infection affects between 15 and 20 million patients worldwide[1,2] and is highly prevalent in eastern Europe, the Middle East, sub-Saharan Africa, South America, and parts of Asia,[3] with increasing prevalence in developed parts of the

Research in context

Evidence before this study

Chronic hepatitis B virus (HBV) and hepatitis D virus (HDV) co-infection affects a considerable proportion of patients with HBV infection and results in rapid progression of liver disease. Treatment of HBV and HDV co-infection with pegylated interferon alfa-2a has been shown to control HDV infection in a small subset of patients, but rarely achieves control of HBV infection (ie, loss of hepatitis B surface antigen [HBsAg]). We searched PubMed from inception to May 1, 2017, using the search terms "interferon", "hepatitis delta", "hepatitis D", and "HDV" for studies that included patients with HBV and HDV co-infection who were exposed to at least 48 weeks of treatment with pegylated interferon alfa-2a. Studies without HBsAg serum concentration and HDV RNA data were excluded.

Added value of this study

Nucleic acid polymers inhibit the release of HBV subviral particles, which share the same secretory mechanisms as HDV virions. We tested the nucleic acid polymer REP 2139 as monotherapy with transition to combined therapy with pegylated interferon alfa-2a and then pegylated interferon alfa-2a monotherapy in patients with chronic HBV and HDV co-infection and showed potent antiviral effects against HBV (ie, achieving HBsAg loss), which persisted for 1 year after treatment in five of 12 patients, and against HDV (ie, achieving HDV RNA negativity), which persisted for 1 year after treatment in seven of 12 patients. To the best of our knowledge, the high rates of HBsAg loss and HDV RNA negativity achieved during therapy and after treatment in these studies are unique antiviral effects, which have not been previously observed with other therapeutic interventions in white patients with HBV and HDV co-infection to date.

Implications of all the available evidence

REP 2139 has antiviral effects against both HBV and HDV, which when used in combination with pegylated interferon alfa-2a, result in the establishment of functional control of both infections that persists in the absence of therapy. These effects suggest a previously uncharacterised, highly potent direct antiviral activity of nucleic acid polymers against HDV. These proof-of-concept clinical data suggest better outcomes might be achieved with the use of longer-term REP 2139-based combination regimens, which are currently being tested in clinical studies of HBV mono-infection.

world.[4,5] Co-infection with hepatitis B virus (HBV) and HDV is more aggressive than HBV mono-infection, with up to 80% of untreated patients developing cirrhosis within 10 years after co-infection.[6]

Elimination of the HBV surface antigen (HBsAg) is a crucial feature of a successful therapeutic outcome: HBsAg is abundant in HBV and HDV co-infection and has immunoinhibitory activities[7] that block immune function and inhibit immunotherapies used to treat HBV and HDV infection.[8,9] Treatment with pegylated interferon alfa-2a alone or with HBV reverse transcriptase inhibitors clears HDV RNA in up to 50% of patients during therapy with only minor effects on HBsAg, and thus recurrence of HDV infection is common.[10–13] The hepatitis D antigen (HDAg) prenylation inhibitor lonafarnib and the HBV and HDV entry inhibitor myrcludex B also decrease circulating HDV RNA concentrations, but have no clinically significant effect on HBsAg.[14–19]

REP 2139 is a nucleic acid polymer that clears circulating HBsAg by blocking the release of subviral particles.[8,20–24] In-vitro and animal studies have shown that the selective inhibition of subviral particle release by nucleic acid polymers does not increase intracellular HBsAg[24] but is associated with the clearance of surface antigen and viruses from the blood and reductions in total viral DNA and covalently closed circular (ccc) DNA in the liver during therapy.[25] Inhibition of viral replication in the liver is accompanied by clearance of surface and core antigens in the liver, which persists after nucleic acid polymers are withdrawn.[22,26] REP 2139 also clears serum HBsAg in human patients[8,21] and allows the establishment of functional control of HBV infection (ie, control of serum HBsAg and HBV DNA) that persists after removal of therapy.[8]

HDV virions are secreted by the same mechanism as HBV subviral particles,[27] suggesting that REP 2139 might also inhibit HDV virion secretion and have a therapeutic effect on HDV infection. In the REP 301 study, we assessed the safety and efficacy of combined therapy with REP 2139 and pegylated interferon alfa-2a in patients with chronic HBV and HDV co-infection. We present the on-treatment responses and 24 month follow-up from the completed REP 301 study and 1 year interim follow-up results from the ongoing REP 301-LTF study, which extended patient follow-up to 1 year.

Methods

Study design

We did a proof-of-concept, open-label, non-randomised, phase 2 study to assess the safety and efficacy of 15 weeks of monotherapy with 500 mg intravenous REP 2139 followed by 15 weeks of combined therapy with 250 mg intravenous REP 2139 and 180 µg subcutaneous pegylated interferon alfa-2a, then 33 weeks of monotherapy with 180 µg subcutaneous pegylated interferon alfa-2a. The study was based at the Toma Ciorbă Hospital of Infectious Diseases in Chișinău, Moldova. Eastern Europe was chosen as the site to conduct this trial since the prevalence of HBV and HDV co-infection is high in this region. Enrolment of treatment-naive patients was facilitated by the absence of subsidisation for antiviral therapy. The REP 301 study protocol can be found online. The REP 301 and

For the **REP 301 study protocol** see www.replicor.com/wp-content/uploads/2017/09/REP301protocol.pdf

REP 301-LTF protocols comply with current Good Clinical Practice guidelines, the Declaration of Helsinki, and regulations in Moldova, and the study was approved by the National Ethics Committee of Moldova and the Health Ministry of Moldova.

Participants

Eligible patients (aged between 18 and 55 years) were treatment-naive, hepatitis B e antigen (HBeAg) negative, anti-HDAg positive, HDV RNA positive, and had serum HBsAg concentrations of more than 1000 IU/mL and a body-mass index of more than 16 kg/m². All eligible patients had HDV infection for 6 months or more before treatment, with no HDV superinfection at the start of treatment. Patients were excluded if they had clear evidence of cirrhosis or any other liver disease, any liver infections other than HBV and HDV, alanine aminotransferase concentrations higher than ten times the upper limit of the normal range (10–50 U/L), or altered liver synthetic function (bilirubin concentration higher than the upper limit of the normal range [0–17·1 µmol/L] and albumin concentrations less than the lower limit of normal [35–52 g/L]). Diagnosis of cirrhosis was based on evaluation of hepatic and haematological parameters, including abdominal ultrasound according to accepted practice at the trial site. Complete enrolment criteria are provided in the REP 301 study protocol. All patients provided written informed consent at enrolment.

Procedures

REP 2139 is a phosphorothioate oligoribonucleotide[8,20] with the sequence (2′OMeA, 2′OMe-5-MeC)$_{20}$.[20] The REP 2139 drug product was provided in 3 mL polystyrene syringes containing 2 mL of 25 mg/mL REP 2139 calcium chelate complex in normal saline.[8] REP 2139 was diluted in a 250 mL bag of normal saline and infused for 1–2 h. Pegylated interferon alfa-2a (Pegasys; F Hoffmann La-Roche AG, Basel, Switzerland) was administered according to prescribing information. REP 2139 infusion was done before the administration of pegylated interferon alfa-2a.

Patients received 500 mg REP 2139 monotherapy once per week for 15 weeks via 2 h intravenous infusion, followed by 15 weeks of combined treatment with 250 mg REP 2139 once per week via 1 h intravenous infusion and 180 µg subcutaneous pegylated interferon alfa-2a once per week, then 33 weeks of 180 µg subcutaneous pegylated interferon alfa-2a monotherapy once per week. REP 2139 monotherapy allowed the confirmation of serum HBsAg clearance, which has been described previously in the REP 102 protocol.[8] All patients were followed up for 24 weeks after cessation of protocol treatment.

Protocol deviations approved jointly by the sponsor (MB and AV) and the attending physicians (VP, VC, LC, and PJ) enabled continued dosing in patients with an antiviral response and grade 4 alanine aminotransferase or aspartate aminotransferase elevations that were otherwise asymptomatic and not accompanied by changes in liver function including elevated prothrombin time, elevated international normalised ratio, or elevated bilirubin, or reduced albumin at any time during these aminotransferase elevations.

We also present the results of the first assessment in the REP 301-LTF protocol (follow-up 1 year after withdrawal of therapy in the REP 301 trial). Because of the enhanced mineral elimination that is common following the administration of phosphorothioate oligonucleotides (including nucleic acid polymers), mineral supplementation with calcium, magnesium, zinc, and vitamin D3 was provided throughout treatment and follow-up to maintain optimum mineral concentrations. Patients who completed treatment in the REP 301 trial were enrolled in the REP 301-LTF follow-up study to be monitored for 3 years.

We assessed the safety and tolerability of the treatment regimen via weekly physical evaluation, and periodic electrocardiogram, ophthalmic and liver ultrasound evaluations, which were accompanied by weekly or biweekly complete biochemical and haematological assays. Hepatic stiffness was measured during treatment and follow-up using Fibroscan (Echosens, Paris, France). In accordance with Good Clinical Practice guidelines, safety data and findings were subject to independent audit and verification. Safety data cited but not presented in the article are available in the appendix.

See Online for appendix

We did virological assessments every 2 or 4 weeks at the Institute for Virology, University of Duisburg-Essen (Essen, Germany). The assessments measured serum HBsAg and hepatitis B surface antibody (anti-HBs; Abbott Architect quantitative assay; Abbott, Abbott Park, IL, USA), hepatitis B e antibody (anti-HBe; Abbott Architect qualitative assay), HBV DNA (Abbott Realtime HBV viral load assay), anti-HDAg (ETI-AB-DELTAK-2; DiaSorin, Saluggia, Italy), and HDV RNA (Robogene 1.0; AJ Roboscreen, Leipzig, Germany). We validated HDV RNA response with Robogene-extracted RNA using a non-commercial validated assay (Institute of Virology, Technical University of Munich, Munich, Germany) and in frozen serum samples using an in-house validated assay (National Genetics Institute, Los Angeles, CA, USA), the Eurobioplex HDV kit[28] (Eurobio, Paris, France), and an ultrasensitive in-house validated nested RT-PCR assay (Centre national de référence des hépatites B, C et Delta, Hôpitaux universitaires de Paris-Seine-Saint-Denis, Paris, France). HBV RNA was assessed by DDL Diagnostics (Rijswijk, Netherlands) with an in-house validated quantitative RT-PCR assay, and hepatitis B core-related antigen (HBcrAg) was assessed via a quantitative chemiluminescent enzyme immunoassay (Lumipulse; Fujirebio, Ghent, Belgium) in frozen serum samples by DDL Diagnostic.

	Age (years)	Sex	ALT (U/L)	Median hepatic stiffness* (kPa)	HBeAg status	Anti-HBe status	HBsAg (IU/mL)	HBV DNA (IU/mL)	HBV RNA (log copies per mL)	HBcrAg (log U/mL)	HDV RNA (IU/mL)	HDV genotype†	Duration of HDV infection before treatment
Patient 001-01	33	Female	188	8·4	Negative	Positive	13 988	<10	TND	<LLoD	$3{\cdot}94\times10^{5}$	1	1 year, 5 months
Patient 001-02	29	Female	98	7·7	Negative	Positive	27 264	<10	TND	<LLoD	$4{\cdot}71\times10^{7}$	1	3 years, 6 months
Patient 001-03	40	Male	53	14·8	Negative	Positive	28 261	<10	TND	<LLoD	$6{\cdot}97\times10^{5}$	1	18 years
Patient 001-06	37	Male	95	6·8	Negative	Positive	17 511	726	TND	4·1	$5{\cdot}49\times10^{6}$	1	12 years
Patient 001-09	22	Male	85	12·0	Negative	Positive	16 426	104	<LLoQ	4·4	$2{\cdot}11\times10^{5}$	1	4 years, 7 months
Patient 001-11	35	Male	200	9·6	Negative	Positive	12 382	<10	TND	3·2	$1{\cdot}21\times10^{7}$	1	9 years
Patient 001-14	32	Male	143	11·6	Negative	Positive	20 869	<10	TND	<LLoD	$2{\cdot}30\times10^{7}$	1	6 years, 1 month
Patient 001-17	34	Male	62	9·5	Negative	Positive	8314	350	TND	<LLoD	$1{\cdot}69\times10^{6}$	1	10 months
Patient 001-20	44	Female	29	8·8	Negative	Positive	13 430	<10	TND	4·5	$2{\cdot}74\times10^{4}$	1	12 years
Patient 001-22	36	Male	101	11·9	Negative	Positive	7836	16	<LLoQ	5·0	$1{\cdot}09\times10^{6}$	1	1 year, 6 months
Patient 001-24	39	Male	160	7·8	Negative	Positive	20 473	<10	TND	2·8	$1{\cdot}89\times10^{6}$	1	4 years, 10 months
Patient 001-26	39	Male	85	30·7	Negative	Positive	5854	256	TND	4·5	$3{\cdot}76\times10^{6}$	1	9 years

ALT=alanine aminotransferase. HBeAg=hepatitis B e antigen. Anti-HBe=hepatitis B e antibody. HBsAg=hepatitis B surface antigen. HBV=hepatitis B virus. HBcrAg=hepatitis B core-related antigen. HDV=hepatitis D virus. TND=target not detected. LLoD=lower limit of detection (log 2 U/mL). LLoQ=lower limit of quantification (log 2·49 copies per mL). *Measured with Fibroscan (Echosens, Paris, France). †HDV genotype analysis is shown in the appendix.

Table 1: **Baseline characteristics**

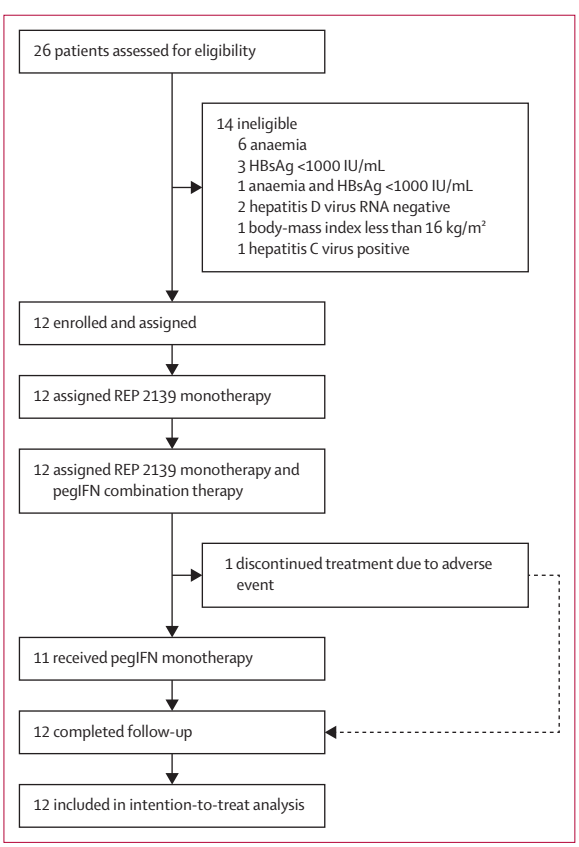

Figure 1: **Patient selection**
HBsAg=hepatitis B surface antigen. pegIFN=pegylated interferon alfa-2a.

HDV genotyping was based on the whole open reading frame of large-HDAg. We extracted viral RNA from the serum samples of patients, and complementary DNA was prepared by reverse transcription using HDV specific primers followed by a two-step, nested PCR. PCR products (approximately 750 base pairs) were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and sequenced by conventional sequencing. We constructed a phylogenetic tree using the Tamura-Nei substitution model with MEGA software version 6.0. The reliability of tree construction was verified by the use of 1000 bootstrap replicates. Genotypes were established by comparison of the sequences obtained with the HDAg sequences retrieved from GenBank, which includes all known HDV genotypes.[29,30]

To investigate assay interference, REP 2139 was added to pre-characterised serum samples collected from human patients with HBV and HDV co-infection who had provided consent. REP 2139 was pre-diluted in normal saline to produce a 5% dilution in serum for all concentrations. Clinical REP 2139 doses were approximately 3·5 mg/kg (250 mg) and 7 mg/kg (500 mg), therefore we tested 0·1, 1·0, 10·0, and 150·0 µg/mL REP 2139, which exceeded the C_{max} of 9 mg/kg REP 2139 after 2 h intravenous infusion.[25] Spiked serum samples for each REP 2139 concentration were tested in triplicate for changes in test output versus normal saline on the following test platforms: Abbott Architect HBsAg quantitative and qualitative assays, Abbott Architect quantitative anti-HBs assay, Abbott Architect qualitative HBeAg and anti-HBe assay, Abbott Architect qualitative anti-HBc total and IgM assay, Abbott Realtime HBV DNA assay, Robogene HDV RNA assay (version 1.0; AJ Roboscreen), the in-house HDV RNA assay (Technical University of Munich), the in-house HDV RNA assay (National Genetics Institute), and the Eurobioplex HDV RNA assay (Eurobio).

Outcomes

The primary outcome was whether REP 2139 and pegylated interferon alfa-2a could be combined safely in a well tolerated manner in patients with chronic HBV and HDV co-infection. Measures of this primary outcome were the incidence of treatment-emergent cytopenic abnormalities, liver dysfunction, renal impairment, adverse events, serious adverse events, and treatment-emergent laboratory abnormalities. Secondary outcomes were the proportion of patients with serum HBsAg less than 50 IU/mL, the proportion of patients with anti-HBs titres above 10 mIU/mL, the proportion of patients who suppress HBV DNA, the proportion of patients who suppress HDV RNA, and the proportion of patients who maintained HBsAg suppression, HBV DNA, and HDV RNA suppression through follow-up.

Statistical analysis

No formal sample size calculation was done; because of the very strong antiviral response of nucleic acid polymers in previous trials,[8] 26 patients were screened and enrolment was limited to 12 patients in anticipation of similar effects in the current patient population. We analysed all patients in accordance with the intention-to-treat analysis. Statistical analysis was done in Microsoft Excel version 365. REP 301 and REP 301-LTF are registered with ClinicalTrials.gov, numbers NCT02233075 and NCT02876419, respectively.

Role of the funding source

MB and AV (as employees of Replicor) designed the studies and were involved in the analysis and interpretation of the data, in the writing of this report, and in the decision to submit the paper for publication. All authors had access to all of the raw data and certify its accuracy. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between Sept 8, 2014, and Jan 27, 2015, we screened 26 patients with HBV and HDV co-infection for eligibility, of whom 12 were enrolled into the REP 301 study. 63 weeks of treatment and 1 year of follow-up was complete for all patients in May, 2017. Baseline characteristics of enrolled patients are shown in table 1 and the trial profile is shown in figure 1. Pre-treatment platelet counts ranged between 96–236 × 10^9 per mL, white blood cell counts ranged between 4·19–9·11 × 10^9 per mL, and serum albumin ranged between 39·6–47·4 U/mL. All 12 patients received REP 2139 monotherapy for 15 weeks followed by REP 2139 and pegylated interferon alfa-2a combination therapy for 15 weeks. 11 patients received subsequent pegylated interferon alfa-2a monotherapy for 33 weeks; one patient discontinued treatment because of adverse events. All 12 patients completed follow-up and were assessed for the primary outcome. In patients with elevated median hepatic stiffness, the absence of cirrhosis was confirmed by abdominal ultrasound. Protocol deviations occurred in patients 001-01 and 001-02 and had a positive effect on efficacy endpoints.

	REP 2139 monotherapy	Combination therapy	Pegylated interferon monotherapy	REP 2139 infusion	Total treatment
Patients with one or more adverse event	11 (92%)	12 (100%)	9 (75%)	7 (58%)	12 (100%)
Blood and lymphatic system disorders					
Thrombocytopenia	1 (8%)	8 (67%)	7 (58%)	0	10 (83%)
Neutropenia	0	8 (67%)	5 (42%)	0	8 (67%)
Anaemia	1 (8%)	1 (8%)	1 (8%)	0	2 (17%)
Eye disorders					
Conjunctival hyperaemia*	3 (25%)	0	0	3 (25%)	3 (25%)
Gastrointestinal disorders					
Upper abdominal pain	1 (8%)	1 (8%)	3 (25·0)	0	3 (25·0)
Nausea	1 (8%)	0	1 (8%)	1 (8%)	2 (17%)
Vomiting	1 (8%)	1 (8%)	0	1 (8%)	2 (17%)
General disorders and administration-site conditions					
Pyrexia*	11 (92%)	9 (75%)	0	2 (17%)	12 (100%)
Chills*	6 (50%)	7 (58%)	0	1 (8%)	9 (75%)
Asthenia*	2 (17%)	8 (67%)	0	0	8 (67%)
Clinical investigations					
Increased alanine aminotransferase concentrations	1 (8%)	5 (42%)	3 (25%)	0	5 (42%)
Increased aspartate aminotransferase concentrations	0	4 (33%)	3 (25%)	0	4 (33%)
Weight loss	0	0	4 (33%)	0	4 (33%)
Increased conjugated bilirubin concentrations	0	1 (8%)	1 (8%)	0	2 (17%)
Increased γ-glutamyltransferase concentrations	0	1 (8%)	0	0	1 (8%)
Increased hepatic enzyme concentrations	0	1 (8%)	0	0	1 (8%)
Musculoskeletal and connective tissue disorders					
Arthralgia	0	2 (17%)	1 (8%)	0	2 (17%)
Nervous system disorders					
Headache*	2 (17%)	0	0	1 (8%)	2 (17%)
Dizziness	1 (8%)	0	0	0	1 (8%)
Skin and subcutaneous tissue disorders					
Pruritus	6 (50%)	0	0	6 (50%)	6 (50%)
Puntis (generalised)	1 (8%)	0	0	1 (8%)	1 (8%)
Urticaria	1 (8%)	0	0	1 (8%)	1 (8%)
Vascular disorders					
Hyperaemia*	5 (42%)	2 (17%)	0	5 (42%)	5 (42%)
Hypotension*	1 (8%)	0	0	1 (8%)	1 (8%)

Data are n (%) of the 12 enrolled patients. Only system organ class symptoms defined by the Medical Dictionary for Regulatory Activities that occurred in at least two patients during treatment are included. *Symptoms presented after completion of REP 2139 infusion, but were considered to be infusion related.

Table 2: **Treatment-emergent adverse events**

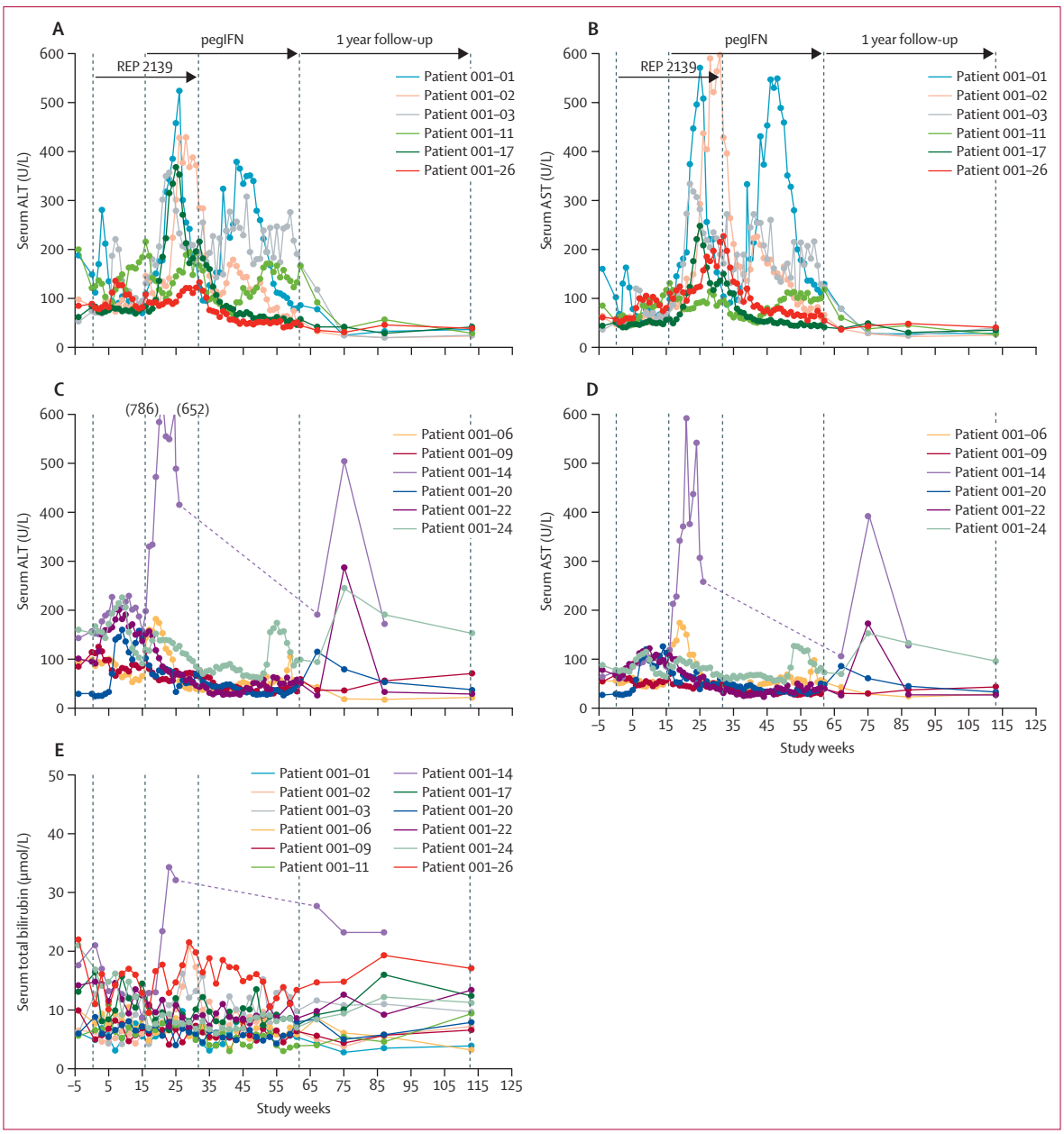

Figure 2: **Liver function of patients during treatment and follow-up**
Individual, colour-coded patient responses during treatment and at 1 year follow-up are shown for serum ALT (A) and AST (B) in patients with serum HBsAg concentrations of less than 1 IU/mL before treatment with pegIFN, serum ALT (C) and AST (D) in patients with serum HBsAg concentrations of more than 1 IU/mL before treatment with pegIFN, and serum total bilirubin (E) for all patients. Dotted black lines indicate the upper limit of normal or normal ranges. Dotted segment for patient 001-14 indicates early transition to follow-up. Study weeks equal to or less than 0 indicate the pre-treatment baseline. ALT=alanine aminotransferase. AST=aspartate aminotransferase. HBsAg=hepatitis B surface antigen. pegIFN=pegylated interferon alfa-2a.

Treatment-related adverse events are shown in table 2; briefly, all 12 patients experienced an adverse event during treatment. Two (17%) patients experienced anaemia, eight (67%) neutropenia, and ten (83%) thrombocytopenia during treatment. Five (42%) patients had raised alanine aminotransferase levels, four (33%) had raised aspartate aminotransferase levels, and two (17%) had increased bilirubin concentrations. 12 (100%) patients had treatment-emergent lab abnormalities.

Four (33%) of 12 patients had serious adverse events. Serious adverse events attributed to pegylated interferon alfa-2a included elevated alanine and aspartate aminotransferase concentrations in patients 001-01 and 001-02 (figure 2A, figure 2B), which resolved without dose reduction, elevated alanine aminotransferase

concentration in patient 001-14 (figure 2C), and thrombocytopenia in patient 001-17 (figure 3), which resolved without dose reduction. In patient 001-14, elevated alanine aminotransferase and bilirubin concentrations occurred with the introduction of pegylated interferon alfa-2a (figure 2C, figure 2E), and thus the patient required early discontinuation of treatment, after which liver function improved. No serious adverse events were attributed to treatment with REP 2139. Serious adverse events unrelated to therapy were tendon injury (n=1), acute pyelonephritis (n=1), and food poisoning (n=1).

The most frequent adverse events observed during REP 2139 monotherapy were pyrexia and chills, and less frequently conjunctival hyperaemia, headache, and asthenia near the end of the infusion (table 2), which resolved after infusion in the absence of supportive therapy and were infrequent after the first 6–8 weeks of dosing. Reductions in platelet and white blood cell counts occurred in seven of 12 patients near the end of REP 2139 monotherapy (figure 3), and became significant in those patients with low platelet and white blood cell counts at baseline. These reductions were stable with pegylated interferon alfa-2a or were effectively managed with pegylated interferon alfa-2a dose reduction in seven of 12 patients (appendix) and eltrombopag administration in two of 12 patients (001-22 and 001-26). Patient 001-22 was given 25 mg eltrombopag daily for 2 weeks (weeks 23–25) and patient 001-26 was administered eltrombopag intermittently after the first dose reduction of pegylated interferon alfa-2a, receiving 25 mg eltrombopag daily between weeks 17 and 18, 22 and 24, and 29 and 32, and 50 mg eltrombopag during the last 5 days of treatment. These haematological alterations either rebounded or fully recovered during follow-up (figure 3B, figure 3D).

The introduction of pegylated interferon alfa-2a induced transient elevations in alanine and aspartate aminotransferase, which were substantially more pronounced in HBsAg rapid responder patients with HBsAg concentrations less than 1 IU/mL (figure 2A, figure 4A). These strong aminotransferase elevations were otherwise asymptomatic, self-resolving, and not accompanied by other changes in liver function (figure 2E, appendix) or the appearance of jaundice, and were not linked to the extent of patient exposure to pegylated interferon alfa-2a (appendix). Of the seven patients who maintained control of HDV RNA at 1 year follow-up, aminotransferase concentrations normalised in six patients and median hepatic stiffness was improved in five patients (table 3). No alterations in renal function, serum calcium, or lipid metabolism were observed during treatment or follow-up (appendix). All treatment emergent adverse events are listed in the appendix.

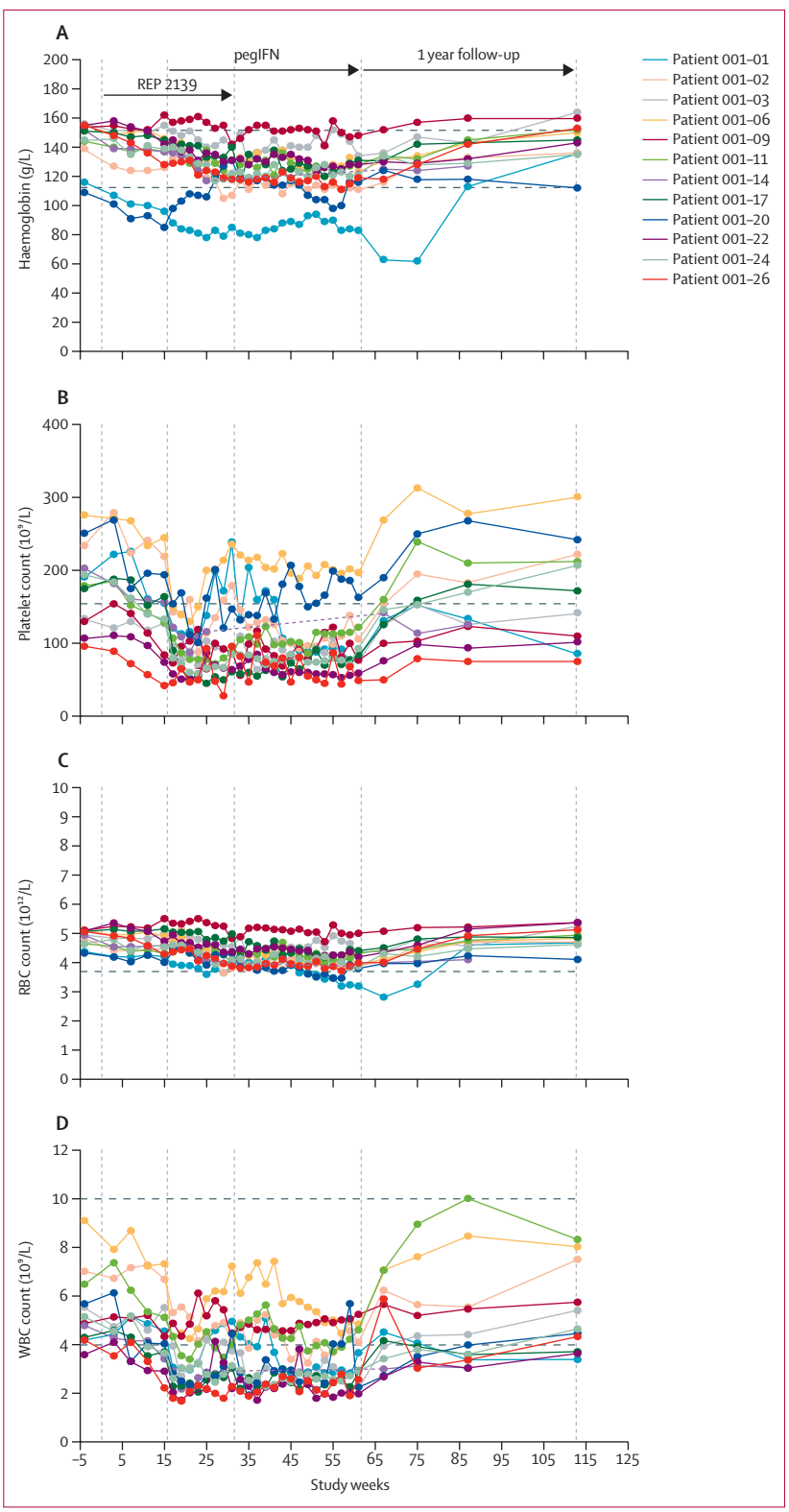

Figure 3: **Haematological changes during treatment and follow-up**
Individual, colour-coded patient responses during treatment and 1 year follow-up are shown for haemoglobin (A), platelet count (B), RBC count (C), and WBC count (D). Dotted black lines indicate lower limit of normal or normal ranges. Dotted segment for patient 001-14 indicates early transition to follow-up. Study weeks equal to or less than 0 indicate the pre-treatment baseline. pegIFN=pegylated interferon alfa-2a. RBC=red blood cell. WBC=white blood cell.

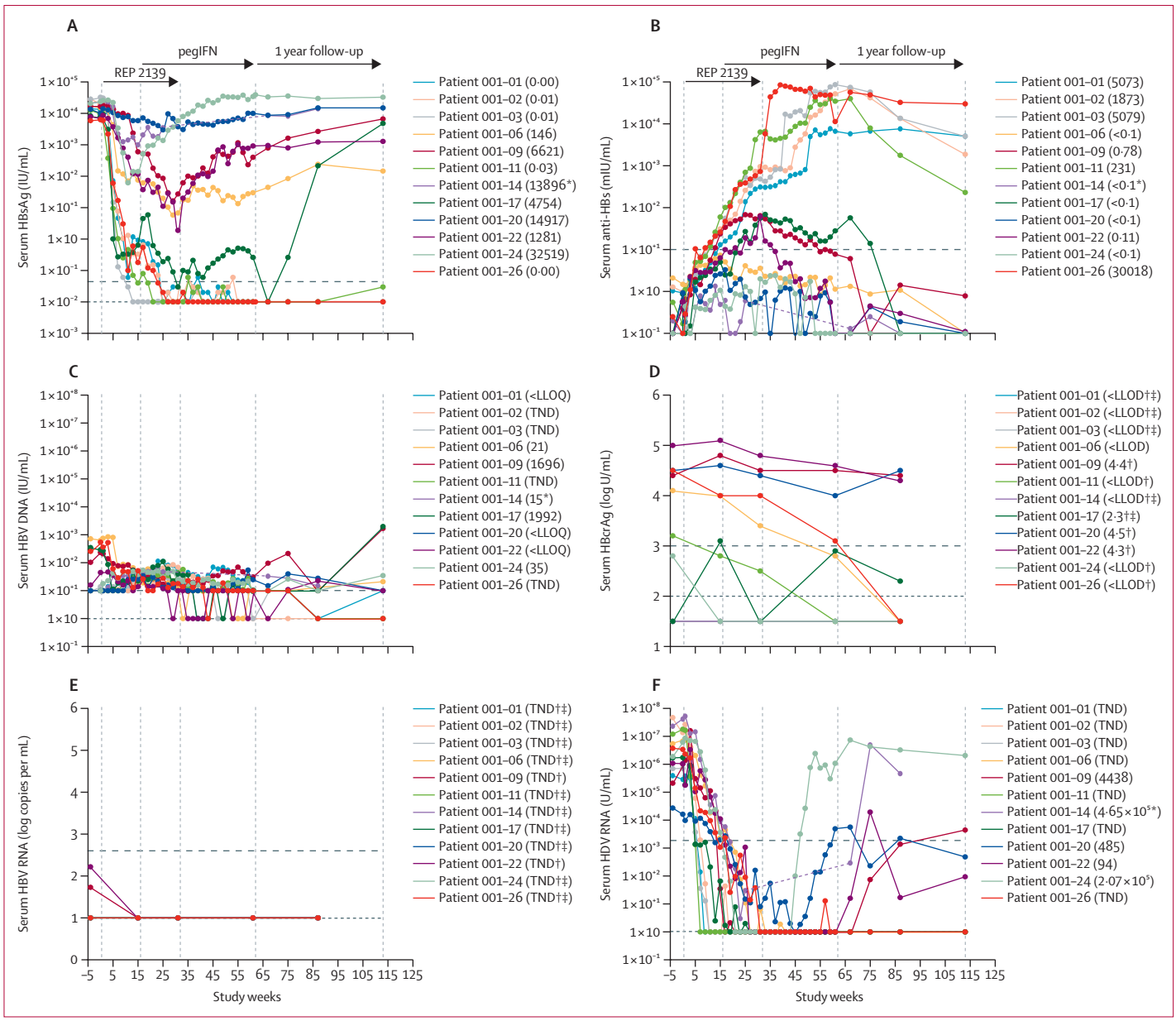

Figure 4: **Changes in HBV and HDV serum viraemia during treatment and follow-up**

Individual, colour-coded patient responses during treatment and follow-up are shown for HBsAg (A), anti-HBs (B), HBV DNA (C), HBcrAg (D), HBV RNA (E), and HDV RNA (F). Numbers in parentheses are quantitative values for each patient at 1 year follow-up. Dashed black lines indicate the LLoQ and dotted black lines indicate TND for HBsAg, HBV RNA, HBV DNA, and HDV RNA, and the LLoD for HBcrAg. Double dashed line in (B) indicates the establishment of protective immunity (10 mIU/mL). Dotted segment for patient 001-14 indicates early transition to follow-up. Study weeks equal to or less than 0 indicate the pre-treatment baseline. LLoQ was 0·05 IU/mL (A), 10 IU/mL (C), $\log_{10}$ 3 U/mL (D), $\log_{10}$ 2·49 copies per mL (E), and 1800 U/mL (F). LLoD was $\log_{10}$ 2 U/mL. HBcrAg=hepatitis B core-related antigen. HBsAg=hepatitis B surface antigen. HBs=hepatitis B surface antibody. HBV=hepatitis B virus. HDV=hepatitis D virus. LLoD=lower limit of detection. LLoQ=lower limit of quantification. pegIFN=pegylated interferon alfa-2a. TND=target not detected. *Value taken from 24 week follow-up result (001-14 was not enrolled in the REP 301-LTF study). †Value taken from 24 week follow-up result. ‡These patients had values below the LLoQ (HBV RNA) or LLoD (HBcrAg) at baseline. At –4 weeks, overlapping values were present in five patients (D) and ten patients (E). Quantitative baseline values in A–F are available in the appendix.

Studies to exclude potential REP 2139 interference in virological assays used in the REP 301 study showed no clinically relevant assay interference with REP 2139, with the exception of one HDV RNA validation assay (appendix). However, because sampling was done 1 week after REP 2139 administration, when plasma levels are below the threshold for interference,[25] the validity of the data from this assay was not affected.

Six patients had HBsAg levels less than 50 IU/mL by the end of combined treatment; five patients maintained this level of suppression at the end of 1 year follow-up. By the end of REP 2139 monotherapy, mean HBsAg reduction was $\log_{10}$ 3·31 (1·99; table 4). HBsAg reduction of more than $\log_{10}$ 1 from baseline occurred in 11 of 12 patients and more than $\log_{10}$ 2 from baseline in eight of 12 patients (figure 4A, appendix). In six of these

	EOT status		Functional control at 1 year follow-up			Serum ALT:AST ratio at follow-up liver assessment (U/L)				Median hepatic stiffness at follow-up liver assessment (kPa)*			
	Serum HBsAg reduction (log)†	HDV RNA reduction (log)†	Serum HBsAg reduction (log IU/mL)†	HBV‡	HDV§	Baseline	EOT	24 week follow-up	1 year follow-up	Baseline	EOT	24 week follow-up	1 year follow-up
Patient 001-01	6·15	5·60¶	6·15¶	Yes	Yes	188:160	80:11	33:29	37:29	8·4	17·1	12·0	10·9
Patient 001-02	6·44	7·67¶	6·44¶	Yes	Yes	98:64	53:61	21:23	24:26	7·7	9·9	7·3	6·1
Patient 001-03	6·45¶	5·84¶	6·45¶	Yes	Yes	53:36	191:129	20:24	25:40	14·8	17·1	14·6	12·0
Patient 001-06	2·77	6·74¶	1·86	No	Yes	95:54	53:57	17:24	21:30	6·8	7·1	8·1	6·3
Patient 001-09	1·61	5·32¶	0·79	No	No	85:55	34:29	56:38	71:44	12·0	12·0	10·2	19·8
Patient 001-11	6·09	7·08¶	5·62‖	Yes	Yes	200:85	133:100	57:46	29:27	9·6	10·3	6·9	6·6
Patient 001-14	0·78	5·88**	0·18	No	No	143:64	415:258**	172:128	NE	11·6	27·0	44·3	NE
Patient 001-17	4·50	6·23 ¶	0·24	No	Yes	62:44	46:45	29:30	42:35	9·5	9·8	7·8	8·4
Patient 001-20	0·13	0·81††	–0·05	No	No	29:27	47:50	53:45	37:33	8·8	10·2	15·0	9·0
Patient 001-22	0·93	6·04¶	0·79	No	No	101:78	58:42	33:28	29:27	11·9	11·8	8·9	11·8
Patient 001-24	–0·22	0·24††	0·26	No	No	160:88	97:82	191:133	153:96	7·8	8·2	10·4	10·8
Patient 001-26	5·77¶	6·58¶	5·77¶	Yes	Yes	85:61	51:65	46:48	39:40	30·7	27·0	34·3	33·3

ALT and AST normal range, 10–50 IU/mL. EOT=end of treatment. ALT=alanine aminotransferase. AST=aspartate aminotransferase. HBsAg=hepatitis B surface antigen. HDV=hepatitis D virus. HBV=hepatitis B virus. NE=not enrolled in REP 301-LTF study. *Measured with Fibroscan (Echosens, Paris, France). †Log reduction versus baseline value. ‡Serum HBsAg less than the lower limit of quantification and HBV DNA less than lower limit of quantification at 1 year follow-up. §Serum HDV RNA not detected by Robogene (AJ Roboscreen, Leipzig, Germany) at 1 year follow-up. ¶Target not detected (0 IU/mL for serum HBsAg). ‖Less than lower limit of quantification (<0·05 IU/mL). **Last reading taken before patient terminated treatment early and entered follow-up. ††HDV RNA in these patients became undetectable during REP 2139 exposure but rebounded during pegylated interferon alfa-2a monotherapy.

Table 3: **Virological response during treatment and at 1 year follow-up**

eight patients, HBsAg reduction was rapid and profound (reductions of $\log_{10}$ 4·11 to $\log_{10}$ 6·45 from baseline; figure 4A) and decreased to less than 1 IU/mL before the introduction of pegylated interferon alfa-2a. These patients were deemed to be rapid HBsAg responders. During the transition to combination therapy with pegylated interferon alfa-2a, HBsAg rebounded in one patient (001-24), which was attributed to REP 2139 dose reduction, but continued to decline in the other nine HBsAg responsive patients, with a total of five patients achieving HBsAg loss (≤0·01 IU/mL). HBsAg loss was maintained in these five patients until the end of therapy and during 1 year of treatment-free follow-up (figure 4A, table 3, appendix). Slow rebound in HBsAg was observed in all other patients during pegylated interferon alfa-2a monotherapy and into the follow-up. Additional summaries of HBsAg responses are shown in table 4 and the appendix.

Six (50%) patients had anti-HBs titres above 10 mIU/mL at the end of treatment. With the introduction of pegylated interferon alfa-2a, rapid and profound increases in anti-HBs concentrations were only observed in five of the six HBsAg rapid responders with HBsAg concentrations of less than 1 IU/mL and were absent in all other patients with HBsAg concentrations higher than 1 IU/mL (figure 4B, appendix). Maximum anti-HBs concentrations of 7681–86 532 mIU/mL were observed during treatment in these five patients. These high anti-HBs titres were preserved during the 1 year of treatment-free follow-up but with slow, continuous declines. All patients were HBeAg negative and anti-HBe positive at baseline and throughout treatment and follow-up (data not shown). Additional summaries of anti-HBs responses are shown in table 4 and the appendix.

	REP 2139 monotherapy	End of combination therapy	End of treatment	24 week follow-up	1 year follow-up
HBsAg reduction (log from baseline)	3·31 (1·99)	4·15 (2·24)	3·45 (2·70)	2·99 (2·88)	3·06 (2·96)
HBsAg negative*	2 (17%)	4 (33%)	5 (42%)	5 (42%)	5 (42%)
Anti-HBs positive†	5 (42%)	6 (50%)	6 (50%)	5 (42%)	5 (42%)
HDV RNA reduction (log from baseline)	4·21 (1·99)	5·68 (1·14)	5·34 (2·34)	4·87 (2·55)	4·51 (3·47)
HDV RNA negative‡	4 (33%)	10 (83%)	9 (75%)	7 (58%)	7 (58%)

Data are mean (SD) or n (%). One patient (001-14) entered follow-up before completing treatment with REP 2139. HBsAg=hepatitis B surface antigen. HBs=hepatitis B surface antibody. HDV=hepatitis D virus. *HBsAg concentration less than 0·05 IU/mL. †Anti-HBs concentration 10 mIU/mL or higher. ‡Target not detected by Robogene assay (AJ Roboscreen, Leipzig, Germany).

Table 4: **Summary of virological responses during treatment, at 24 week follow-up, and 1 year follow-up**

Serum HBV DNA was very low or less than the lower limit of quantification in all 12 patients at baseline (figure 4C, table 1). Small increases in HBV DNA (reaching levels of 34–114 IU/mL) were observed during REP 2139 monotherapy, but decreased in all patients after the introduction of pegIFN. Nine patients had suppressed HBV DNA (<10 IU/mL) at the end of treatment. At 1 year follow-up, eight patients had no detectable HBV DNA. Two patients (001-09 and 001-17) had a minor HBV DNA rebound during follow-up (figure 4C).

HBcrAg and HBV RNA were not detected in five (42%) of 12 patients at baseline (figure 4D, figure 4E, table 1).

Serum HBV RNA became undetectable in all 12 patients by the end of REP 2139 monotherapy and remained undetectable throughout treatment and at 24 weeks of follow-up (figure 4C). HBcrAg did not significantly decline until pegylated interferon alfa-2a was introduced and was not correlated with HBsAg response (figure 4A, figure 4D). HBcrAg decline continued after therapy in two patients (001-06 and 001-26) and was absent in eight of 12 patients at 24 weeks of follow-up. HBcrAg and HBV RNA responses are summarised in the appendix.

A reduction in serum HDV RNA during REP 2139 monotherapy was observed in all patients (mean $\log_{10}$ 4·21 [1·99]; table 4), becoming undetectable in four patients during REP 2139 monotherapy and a total of nine patients by the end of REP 2139 and pegylated interferon alfa-2a combination therapy (figure 4F). Shortly after transition to pegylated interferon alfa-2a monotherapy, HDV RNA became undetectable in two additional patients (11 [92%] of 12 patients became HDV RNA negative overall). HDV RNA rebound was observed in two patients (001-20 and 001-24) near the end of pegylated interferon alfa-2a therapy and in two patients (001-09 and 001-22) during follow-up, but remained stably undetectable at 1 year follow-up in the remaining seven patients (figure 4F). Of these seven patients, five had no detectable evidence of HBV replication and abundant anti-HBs in the serum (figure 4B, table 3). Following early entry into follow-up, HDV RNA rebound also occurred in patient 001-014. HDV RNA responses observed on the Robogene platform were replicated by three other independent quantitative assays, with the same onset of HDV RNA reduction and negativity at 24 weeks of follow-up (appendix). An ultrasensitive nested RT-PCR analysis of HDV RNA-negative samples throughout treatment using the Eurobioplex platform (appendix) confirmed the absence of HDV RNA in these samples in all but three patients. In patient 001-03, trace amounts of HDV RNA were detectable at the end of therapy and at 24 weeks of follow-up, but were not detectable in any quantitative assay during these timepoints or at 1 year of follow-up (figure 4F, appendix). In patients 001-02 and 001-26, trace amounts of HDV RNA present at the end of therapy were not detectable at 24 weeks of follow-up. Thus, six (86%) of seven patients with undetectable HDV RNA at 24 weeks of follow-up by quantitative assay were also HDV RNA negative by ultrasensitive nested PCR. During REP 2139 exposure, HDV RNA declines occurred more quickly in rapid HBsAg responder patients than patients with a slower HBsAg response (appendix). HDV RNA responses are summarised in table 4 and the appendix.

Discussion

Combination therapy with REP 2139 and pegylated interferon alfa-2a was safe, with tolerability similar to monotherapy with pegylated interferon alfa-2a, and achieved stable control of HDV RNA in seven of 12 patients 1 year after the termination of treatment, accompanied by HBsAg loss, high anti-HBs titres, and suppressed HBV DNA in five of 12 patients, normalisation of serum aminotransferases in nine of 12 patients, and reduction of hepatic stiffness in five of 12 patients. Despite the absence of a control group, the high rate of HBsAg loss and HDV RNA negativity that was achieved during therapy and stably maintained after treatment in these studies represent antiviral effects, which to our knowledge, have not been previously observed with other therapeutic interventions in HBV and HDV co-infection to date.

The HBsAg clearance with REP 2139 in the current study is similar to that observed with REP 2139 in HBV mono-infection,[8,21] suggesting that REP 2139 might be reliably used to clear serum HBsAg in all patients with HBV infection. The high anti-HBs titres identified after the introduction of pegylated interferon alfa-2a (figure 4B) also replicate similar effects observed in HBV mono-infection,[8,21] suggesting that HBsAg clearance might synergistically improve the activity of other immunotherapies such as cytokine-based therapies (ie, pegylated interferon λ), toll-like receptor agonists, or therapeutic vaccines in all patient populations. The large titres of circulating anti-HBs in the patients are likely to have neutralising activity that blocks the infection of hepatocytes; however, the proportion of anti-HBs with neutralising activity is unknown and requires further investigation.

Rapid and profound reductions in HDV RNA occurred even when HBsAg response was attenuated or absent. However, an assessment of HBsAg and HDV RNA responses (appendix) revealed that HDV RNA reductions were fastest in patients who had rapid reduction in serum HBsAg concentrations. These observations suggest that blocking the secretion of HBV subviral particles affects HDV virion release, but also suggests that REP 2139 directly targets the replication of HDV via unrelated antiviral mechanisms. The HDV RNA responses observed in all patients also suggest that the attenuated HBsAg responses seen in a small proportion of patients in this study and previous studies of nucleic acid polymers[8] are not a result of a defect of their entry into hepatocytes, but instead a defect in the intracellular transit of nucleic acid polymers into the cellular compartment where HBV subviral particle assembly and secretion occur.[31] However, the mechanisms underlying this process, which are likely to be host-related, remain unclear.

HBV RNA and HBcrAg are new serum markers that measure HBV replication independently of HBV DNA via the detection of virions that do not contain HBV DNA or HBV RNA.[32–36] These markers are correlated with intrahepatic cccDNA and might indirectly indicate the level of cccDNA present. Because of the minimal amounts of HBV DNA present in patients in the REP

301 study and the clearance of HBsAg by REP 2139, HBV RNA and HBcrAg were used to assess the levels of cccDNA during therapy and follow-up. The absence of circulating HBeAg, HBcrAg, HBV RNA, and HBV DNA observed in five of 12 patients at baseline when substantial HBsAg concentrations were identified (table 1), suggests that the bulk of circulating HBsAg in these patients is derived from integrated HBV DNA and not from cccDNA, which appears sufficient to generate infectious HDV virions in these patients. Thus, nucleic acid polymers also appear to block the release of HBsAg and HDV from hepatocytes with viral DNA integrated in the host genome. In the five patients who were HBsAg negative at 24 week follow-up, the absence of HBV DNA, HBcrAg, and HBV RNA suggests the establishment of a profound functional control of HBV infection in these patients.

The aminotransferase flares in this study correlated with the introduction of pegylated interferon alfa-2a and appear to be therapeutic in nature because they were not accompanied by changes in liver synthetic function or other signs of liver dysfunction (ie, jaundice or hyperbilirubinaemia), resolved during therapy, and correlated with the maintenance of functional control of HBV and HDV. The features of the aminotransferase flares in the liver observed in this study are consistent with those reported with other therapies for HBV,[37–42] which have been associated with HBsAg loss during treatment and the establishment of functional control of chronic HBV infection that persists after treatment. The marked increases in alanine aminotransferase and aspartate aminotransferase in this study and the profound increases in anti-HBs were restricted to patients with HBsAg concentrations of less than 1 IU/mL, suggesting that a low threshold HBsAg concentration is required to to enable immunotherapy to stimulate immune function. However, additional larger trials will be needed to examine this issue.

HBsAg has been implicated in the progression of hepatocellular carcinoma,[43] and the potential carcinogenic effects due to inhibition of HBsAg release by nucleic acid polymers has been a point of some debate. However, the inhibition of HBsAg release in vitro is not accompanied by increased intracellular HBsAg concentrations,[24] and inhibition of viral replication in the liver occurs in vivo during therapy with REP 2139.[25] Moreover, in animals that achieve functional control of infection off treatment, surface antigen is persistently cleared from the liver.[22,26] The antiviral responses to nucleic acid polymers in vitro and in vivo are consistent with the clearance of serum antigenaemia and viraemia and the persistence of functional control of HBV infection after withdrawal of nucleic acid polymers in patients with HBV mono-infection and HBV and HDV co-infection in this current study and previous[8] and ongoing studies.[21] Although an increased risk of hepatocellular carcinoma with nucleic acid polymer therapy cannot be ruled out until studies with larger cohorts and longer follow-up are completed, the data available currently rebut this possibility.

Increased hepatic stiffness at the end of therapy was observed in nine of 12 patients and correlated with the magnitude of liver flares. Although hepatic stiffness has been associated with the necroinflammatory status of the liver in patients with chronic HBV and HDV co-infection, the association between aminotransferase elevations and increases in median hepatic stiffness and their significance with regard to therapy with nucleic acid polymers requires further examination. However, in five of these eight patients, hepatic stiffness declined during 24 weeks and 1 year follow-up, and median hepatic stiffness was below pre-treatment levels in four patients.

The enhanced mineral elimination that accompanies therapy with phosphorothioate oligonucleotides (including REP 2139) is normally well tolerated by patients, since minerals are mobilised from bone stores. However, in a previous clinical study[8] done in an area of Dhaka, Bangladesh, with substantial and chronic exposure to heavy metals,[44,45] REP 2139 exposure was associated with the onset of dysphagia, dysgeusia, and hair loss, which are classic features of heavy metal intoxication. These adverse events were attributed to heavy metal intoxication secondary to the liberation of heavy metals from bone stores in these patients.[8] Heavy metal exposure at our trial site in Chișinău, Moldova, is more representative of heavy metal exposures expected worldwide, and dysphagia, dysgeusia, and hair loss were absent in this study and ongoing[21] studies of REP 2139 in HBV mono-infection. These observations indicate that these side-effects represent an isolated occurrence with REP 2139 therapy in Bangladesh and are not expected to occur in most patients.

Although the antiviral responses observed with REP 2139 against HBV and HDV infection in this study replicate those observed previously[8] against HBV mono-infection, our trials are small in size and larger trials are needed to better define the antiviral potential of therapy with nucleic acid polymers. The safety and efficacy of 48 weeks of treatment with REP 2139 and a closely related nucleic acid polymer derivative (REP 2165) combined with tenofovir and pegylated interferon alfa-2a are currently being assessed in chronic HBV mono-infection in the REP 401 study (ClinialTrials.gov, number NCT02565719).[21] The REP 401 trial is establishing the safety of 48 weeks of combined therapy with REP 2139, tenofovir, and pegylated interferon alfa-2a, allowing future trials in patients with HBV and HDV co-infection to use longer-term combination regimens with the potential to establish higher rates of functional control of HBV and HDV infection. Additional trials are also planned to verify the efficacy of subcutaneously administered REP 2139 therapy to enable a more convenient route of administration.

Contributors
AV and MB designed the study. VP, VC, LC, and PJ collected data for the study. AK supervised all virological assessments done at the Institute for Virology, University of Duisburg-Essen (Essen, Germany). JA, PS, FLG, EG, HK, and MR were responsible for HDV RNA validation. HK was responsible for HDV genotype identification. HM, JA, and FLG did the interference studies. AV was responsible for data analysis and writing the manuscript with assistance from all other authors.

Declaration of interests
MB and AV are employees of and shareholders in Replicor; have patents (US 7358068, 8008269, 8008270, 8067385, 8513211, 8716259, 9133458, 9492506, 9533003, 9603865, and 9616083) issued to Replicor; and have additional patent issuances in numerous jurisdictions worldwide. HK and MR are paid consultants for Replicor. AK's institute received compensation from Replicor for performing virological diagnostics. All other authors have no interests to declare.

Acknowledgments
This study was funded by Replicor. We thank Tanya Korogodina and Sergiu Merioara for their assistance in the conduct of these studies.

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