Research Article
Viral Hepatitis

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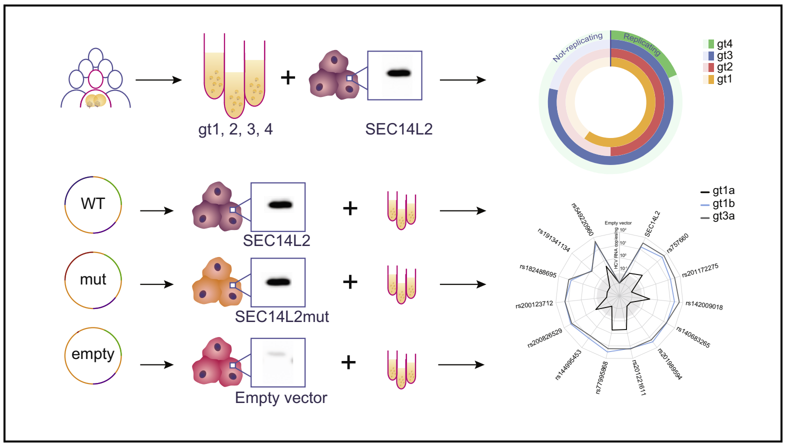
SEC14L2, a lipid-binding protein, regulates HCV replication in culture with inter- and intra-genotype variations

Graphical abstract



Highlights

- SEC14L2 was shown to allow natural HCV isolates to replicate *in vitro.*
- Natural isolates from genotypes 1,2,3 and 4 replicate in cells overexpressing SEC14L2.
- Replication does not occur in 100% of the cases and varies with genotype.
- SNP rs191341134 causes lower levels of SEC14L2 in the cytosol and lower viral replication.
- SNP rs757660 recapitulates the wild-type protein phenotype *in vitro* and *in vivo*.

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Lay summary

Until the year 2015, consistent replication of patient-derived isolates of hepatitis C virus (HCV) in an *in vitro* model remained a limitation in HCV research. In 2015 a group of authors identified a protein named SEC14L2 that enabled the replication of HCV isolates in cell culture. We performed a large screen encompassing 73 isolates of 4 different HCV genotypes. Additionally, we replaced the natural SEC14L2 with 13 different mutants to test if the protein variation significantly altered its HCV replication enhancing functions. We showed that different genotypes of HCV react differently to the presence of this protein and the variants of the protein mimic the behavior of the wild-type.

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SEC14L2, a lipid-binding protein, regulates HCV replication in culture with inter- and intra-genotype variations

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Background & Aims: The lipid-binding protein, SEC14L2, is crucial for the efficient viral replication of clinical hepatitis C virus (HCV) isolates in cell culture. Given the role of SEC14L2 in HCV replication, we aimed to study a large number of HCV positive sera carrying genotypes 1–4, to identify viral factors associated with efficient replication in culture. Additionally, we investigated whether 13 single nucleotide polymorphisms (SNPs) of SEC14L2 have an impact on RNA replication of naturally occurring HCV isolates.

Methods: We generated Huh-7.5 cell lines overexpressing SEC14L2 or 13 coding SNPs and tested 73 different HCV positive sera for *in vitro* replication. Furthermore, we genotyped a cohort of 262 patients with chronic HCV for the common SNP (rs757660) and investigated its effect on the clinical phenotype.

Results: HCV isolates from genotype 1, 2, 3 and 4 replicate in Huh-7.5 cells overexpressing SEC14L2. Interestingly, only subgenomic replicons from genotypes 1 and 3 showed enhanced replication whereas genotypes 2 and 4 remained unaffected. Furthermore, replication was independent of viral load. Importantly, all tested SNPs supported HCV RNA replication *in vitro*, while 1 SNP was associated with decreased SEC1L2 expression and viral RNA. All SNPs exhibited comparable cellular cholesterol and vitamin E abundance in naïve Huh-7.5 cells.

Conclusions: This large screen of natural HCV isolates of 4 genotypes underscores the relevance of SEC14L2 as an *in vitro* HCV host factor. Additionally, SEC14L2 variants appear to recapitulate the wild-type enhancement of HCV replication. Variant rs191341134 showed a decreased effect due to lowered stability, whereas variant rs757660, a high prevalence mutant, showed a similar phenotype to the wild-type.

Lay summary: Until the year 2015, consistent replication of patient-derived isolates of hepatitis C virus (HCV) in an *in vitro* model remained a limitation in HCV research. In 2015 a group of authors identified a protein named SEC14L2 that enabled the replication of HCV isolates in cell culture. We performed a large screen encompassing 73 isolates of 4 different HCV genotypes. Additionally, we replaced the natural SEC14L2 with 13 different mutants to test if the protein variation significantly altered its HCV replication enhancing functions. We showed that different genotypes of HCV react differently to the presence of this protein and the variants of the protein mimic the behavior of the wild-type.

Introduction

Hepatitis C virus (HCV) is a positive stranded RNA *Hepacivirus* in the *Flaviviridae* family. Today, HCV remains an important element in the aetiology of chronic liver disease and according to the World Health Organization there are estimated to be 71 million chronically infected patients worldwide.[1] Since the discovery of the virus in 1989, the development of tools such as a subgenomic replicon system, cell culture adapted variants and highly permissive hepatoma cell lines have enabled researchers to study different aspects of the HCV life cycle. This paved the way for the generation of effective antiviral therapies against the virus.[2,3] Nevertheless, replication of non-cell culture adapted variants remained a difficult achievement and later facets of the viral life cycle are yet to be fully described. This status quo was challenged in 2015 when pan-genotype (GT) replication of patient-derived isolates was shown by Saeed *et al.* They were able to show that the overexpression of the lipid-binding protein SEC14L2 in a Huh-7.5 hepatoma cell line allowed efficient replication of non-cell culture adapted HCV isolates, through a vitamin E mediated mechanism of lipid peroxidation resistance.[4] Curiously vitamin E supplementation was

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Keywords: Hepatitis C virus; SEC14L2; Host factors; Polymorphisms; Viral and cellular determinants of HCV replication.

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not sufficient to allow replicon colony formation in the absence of SEC14L2 which would suggest an alternative mechanism may be at play. The context of lipid peroxidation as a regulatory mechanism of HCV had already been suggested by Yamane *et al.* Yamane and co-authors showed that sphingosine kinase-2 partly regulated lipid peroxidation and the data implied a significant role of this regulation upon HCV replication. The EC50 of tested HCV antivirals was shown to increase in the presence of vitamin E or the sphingosine kinase inhibitor.[5] Further data on the subject of non-cell culture adapted virus replication were made available in 2016 when a mechanism for viral dependence on host phosphatidylinositol 4-kinase IIIα (PI4KA) was reported to be a determinant factor in hepatoma cell lines. Harak *et al.* were able to show that adaptive mutations with loss of function in NS5A-NS5B were necessary for efficient replication in hepatoma cell lines. The difference in the expression of PI4KA, and consequently, the abundance of PiP4, were pointed out as being detrimental for viral replication as they were present in excess when comparing model hepatoma cell lines with primary human hepatocytes.[6] Together these new data provided an important understanding of the role of host factors and lipid peroxidation in the life cycle of HCV, chief among them, SEC14L2.

SEC14L2 is a human homologue of a lipid-binding protein first described in mice in 1977 as supernatant protein factor.[7] This protein is closely related to yeast sec14p proteins and it is characterized by a CRAL-TRIO domain. It has been reported to function as a putative transporter of lipophilic molecules between cellular compartments and to be intimately connected to the availability of vitamin E.[8,9] It is characterized by high expression levels in the brain, prostate and the liver.[10] Several authors have attributed a role in vitamin E distribution in the cytosol and in the abundance of vitamin E in serum to SEC14L2/hTAP.[11–13]

A common coding single nucleotide polymorphism (SNP) in SEC14L2 (rs757660) has been associated with alpha-tocopherol levels in the serum and the risk of prostate cancer.[13] but the impact of this SEC14L2 variant on HCV has not been addressed so far. At least 13 validated coding non-synonymous SNPs with a minor allele frequency (MAF) of at least 0.0002, each resulting in the exchange of a single amino acid in the SEC14L2 protein have been deposited in the National Center for Biotechnology Information dbSNP database (www.ncbi.nlm.nih.gov/snp) (Table 1). If these SNPs have a clinical phenotype is presently unknown.

Given the central role of SEC14L2 in HCV replication we aimed to study a larger number of sera from HCV RNA positive individuals infected with HCV GT 1–4 in order to identify factors that are associated with efficient replication in cell culture. In addition, we investigated if genetic variants of SEC14L2 may have an impact on RNA replication of natural occurring HCV isolates. As previously stated, there are unanswered questions regarding HCV replication in the presence of SEC14L2, chief among them, the variation of efficiency in inter- and intragenotypic HCV replication and the inability of vitamin E to independently allow for replicon colony formation.[4] For this purpose, we generated Huh-7.5 cell lines overexpressing wild-type isoform 1 SEC14L2 or 13 coding SNPs selected by MAF. Furthermore, we genotyped a cohort of 262 patients with chronic HCV for a common SNP (rs757660) and investigated its effect on the clinical phenotype. Overall, our data indicate that SEC14L2 elicited HCV replication varies within HCV GT and that all tested SEC14L2 SNPs support HCV RNA replication *in vitro*, while 1 SNP was associated with lower SEC1L2 expression and decreased viral titers.

Materials and methods

Patient samples

The study was approved by the Ethic committee of the University Hospital Frankfurt and Hannover Medical School required the written approval of the recipients. Informed consent was obtained from all participants. The study was conducted in accordance with the ethical guidelines of the 1975 Helsinki Declaration. Samples were stored at −80 °C (BM, GM and GD) and −20 °C (GE) and −20 °C (DNA) before use.

Genetic constructs

The SEC14L2 isoform 1 open reading frame was cloned into the lentiviral construct pWPI-GUN and pWPI-BLR using restriction endonuclease recognition sites at 5′ BamHI (NEB, Germany) and 3′ SpeI (NEB, Germany) as described previously.[14] SNP variants were generated through standard PCR based mutagenesis and the inserts were sequenced (Eurofins, Germany) using primers for the flanking regions of the SEC14L2 ORF. Plasmids encoding the subgenomic firefly

Table 1. SEC14L2 non-synonymous polymorphisms.

Ref SNP nomenclature	MAF (1,000 genomes)	Amino acid exchange	mRNA nucleotide exchange	exon:
rs757660	0.37	R11K	208	1
rs201172275	0.0002	R20Q	235	1
rs142009018	0.0024	D69N	381	2
rs140683265	0.0002	I72V	390	2
rs201989594	0.0006	F113L	515	5
rs201221611	0.0002	E129G	562	5
rs77995868	0.0134	H137P	586	5
rs144995453	0.0002	R188C	738	6
rs200826529	0.0002	P248S	918	8
rs200123712	0.0002	Y268H	978	9
rs182488695	0.0002	R286H	1,033	10
rs191341134	0.0002	G359D	1,252	11
rs549220960	0.0010	F19Y	232	1

SNP identifiers for the 13 selected polymorphisms of SEC14L2 as found in NCBI's SNP database. MAF, amino acid exchange, exchange location in the primary structure of the protein, mRNA location of the non-synonymous mutation and the exon where the mutation manifests are shown. Amino acid changes are represented using standard single letter IUPAC code. MAF, minor allele frequency; SNP, single nucleotide polymorphism.

luciferase reporter replicons of GT1a (H77), GT1b (ConI), GT2a (JFH-1), GT3a (S52), GT4a (ED43), or GT5a (SA1) have been described previously.[15] Plasmids encoding for S52, ED43 and SA1 additionally carry a selectable neomycin phosphotransferase gene (Feo replicons). A replication deficient variant harboring a deletion in NS5B (ΔGDD) was used as negative control for replication of subgenomic replicons. A full-length replication deficient variant of Jc1 (harboring a deletion in NS5B (ΔGDD) was used as negative control for replication in tandem with serum RNA replication.

Compounds

Sofosbuvir (S2794) was obtained from Selleckchem (Houston, USA) and Daclatasvir (D101500) was obtained from Toronto Research Chemicals (Toronto, Canada).

Cell lines and cell culture

Cell lines were maintained in Dulbecco's modified eagle medium (DMEM, Thermo Fisher, Germany) supplemented with 10% fetal bovine serum (Sigma, Germany) penicillin/streptomycin (100 U/ml) (Biochrom, Germany) L-glutamine (2 mM) and non-essential amino acids (Thermo Fisher, Germany). Transduced Huh-7.5-pWPI-GUN-SEC14L2 cell lines were maintained in DMEM further supplemented with 800 ug/ml G418 (Thermo Fisher, Germany). Transduced Huh-7.5-pWPI-BLR-SEC14L2 cell lines were maintained in DMEM further supplemented with 10 ug/ml Blasticidin (Thermo Fisher, Germany). Huh-7.5 cell line lineage authenticity was confirmed by Eurofins with the sample number CL180117_019.

Transduction of target cell lines with pseudotyped lentiviral particles/generation of SEC14L2 expressing cell lines

Pseudotyped particles for the transduction of target cell lines with the desired transgene were produced as previously described.[4] Briefly, HEK-293T were co-transfected with 3 plasmids, a provirus encoding the transgene (pWPI-GUN-SEC14), a plasmid encoding the HIV gag pol proteins and a plasmid encoding the G glycoprotein from the vesicular stomatitis virus (VSV-G), in the presence of polyethylenimine. Supernatants were collected after 48 h and 72 h. Target cells were transduced by incubation for 6 h with supernatant supplemented with 4 µg/ml polybrene (Sigma, Germany). After the incubation period the supernatant was replaced with DMEM. Cells were maintained for 48 h and the medium was then supplemented with selection antibiotic.

In vitro transcription of HCV RNA, electroporation of cells and luciferase activity assay

In vitro transcription, electroporation and luciferase assays were performed as described previously.[16] In brief, 5–10 µg of *in vitro*-transcribed viral RNA was electroporated into 1×10^7 Huh7-Lunet derived cells harboring SEC14L2, its variants or only the selection marker. The cells were seeded into 6-well plates and harvested at the indicated time points, and luciferase activity was determined in duplicates using a tube luminometer (Lumat LB9507; Berthold Technologies). Luciferase activity at 4 h after transfection was used for normalization to account for varying transfection efficiency.

Western blot

Transduced cell lines were lysed, SEC14L2 expression was then detected using a standard immunoblot protocol. Briefly, cells were lysed using 1 ml of Cell Lytic M (Sigma Aldrich, Germany) and the protein content was measured using a Pierce Comassie Blue assay (Thermo Fisher, Germany), 25 µg of protein were then loaded onto an 11% (w/v) polyacrilamide gel and separated through electrophoresis at 100 V for 1 h 30 m (Biorad Laboratories, USA). The protein was then blotted into a PVDF membrane for 40 min at 100 V using a Mini Trans-Blot® (Biorad, Germany). The proteins were detected using monoclonal mouse and monoclonal rabbit anti-human antibodies (ab119050 and ab137013 respectively, abcam, UK) and anti-mouse and anti-rabbit Horseradish Peroxidase conjugated secondary antibodies (A4416 and A6154 respectively, Sigma Aldrich, Germany) using ECL chemistry (GE Healthcare, Germany). Images were acquired using a LAS4000R system (Fujifilm, Germany).

Flow cytometry

Cells were trypsinized, resuspended in DMEM and refrigerated on ice for the duration of the experiments. GFP expression was measured using a BD FACS Canto II (Beckman Coulter, Germany) and mean fluorescence intensity was calculated using the geometric mean of the fluorescence in the FITC channel of 10–30,000 recorded events using the software FlowJo (FlowJo LLC, USA).

Infection of Huh-7.5-hSEC14 with patient sera

Infection of Huh-7.5-hSEC14L2 cell lines was performed as described previously.[17] Briefly, cells were seeded in a 24-well plate at a density of 2×10^4 the day before infection and incubated overnight. The infection was performed by inoculating the cells with 500 µl of DMEM (Thermo Fisher, Germany) supplemented with 10% FBS (Sigma Aldrich, Germany), penicillin/streptomycin (Biochrom GmbH, Germany), L-glutamine (Thermo Fisher, Germany), non-essential amino acids (Thermo Fisher, Germany), G418 800 µg/ml (Thermo Fisher, Germany), 50 nM EGF (Thermo Fisher, Germany), 250 nM SOF (Selleckchem, USA), combined with 50 nM Daclatasvir (Toronto Research Chemicals, Canada) or DMSO (SERVA GmbH, Germany) and 25 µl patient serum. Following inoculation cells were incubated overnight. The infection medium of DMEM supplemented with G418 was replaced 24 h post infection. At 5 days post infection the medium was refreshed and 7 days post infection the cells were lysed.

Extraction and quantification of HCV RNA

Ribonucleic acids were extracted from cell lysates using Qiagen RNAeasy (Qiagen, Netherlands) according to the manufacturer's instructions and quantified using Nanodrop™ 2000 spectrophotometer (Thermo Fisher, Germany). An adapted Xtail RT-qPCR was performed on all samples to quantify HCV copy number.[18] Briefly, reactions of 20 µl containing 3 µl of sample RNA and 17 µl of Master Hydrolysis Probes (Roche, Switzerland), 200 nM of primer XTF5, 300 nM of primer HCMgR2 and 100 nM of an HCV custom probe HCVMGB2 were run for 45 cycles on a Light Cycler 480 thermocycler (Roche, Switzerland). Copy number was obtained by interpolation of the reaction Ct with a standard curve obtained in run by quantification of standard copy number 10-fold dilutions with *in vitro*-transcribed HCV Con1 RNA (pFKi 389 Neo EI Core 3‘ JFH1wt). Cycle 3 was used for baseline determination and cycle 40 was elected as cut out cycle for evaluation. Biological replicates were analyzed in dual technical replicates. Copy number was normalized to the RNA quantity in each sample.

Statistical analysis

No statistical methods were used to predetermine sample size. Except where mentioned otherwise all experimental data are the result of at least 2 biological replicates of the same conditions. The mean of technical replicates was taken where available. Statistical analysis was performed using Graph Pad Prism 7.02 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) and results are represented as an average of the biological replicates with error bars representing SD. Statistical significance analysis was performed using standard ANOVA with a Bonferroni post-test for multiple comparison correction where applicable. For epidemiological data, medians 25% and 75% percentiles are presented. Statistical comparison was performed using a 2-tailed Mann-Whitney *U* test. Correlation was inferred using Spearman r.

Genotyping of SEC14L2 variant rs757660

Genotyping of DNA samples from the INDIV cohort was performed using custom LightSNP probes (TIB, Germany) and FastStart DNA Master HybProbe (Roche, Germany) master mix according to the manufacturer's instructions. Briefly, a reaction containing the sample DNA, custom LightSNP probes and the master mix was run on a Light Cycler 480 thermocycler (Roche, Germany). These reactions produce a signature melting curve after amplification by PCR that can be used to identify nucleotide differences according to the dissociation temperature of the probe.

Analysis of HCV non-structural protein variants

Partial NS3 and NS5A sequences were retrieved by Sanger sequencing. A detailed protocol is available upon request. These are mapped to the N terminal regions of NS3 and NS5A and map to relevant resistance determinant regions of the virus. Using multiple sequence alignment software Ugene,[19] we compared the amino acid sequences of the replicating and non-replicating viruses and elected residues based on comparative analysis between replicating and/or non-replicating and the consensus sequence from the alignment with database sequences from the European HCV database (euHCVdb).[20] The selected residue frequency was then calculated. Phylogenetic analysis was conducted with MEGA7[4] using maximum likelihood method with the Poisson amino acid substitution model. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model. A discrete gamma distribution was used to model evolutionary rate differences among sites (6 categories and the rate variation model allowed for some sites to be evolutionarily invariable). Robustness of the trees was tested via bootstrap method with 1,000 replicates. The analysis involved 17 NS3 and 21 NS5A amino acid sequences. All positions containing gaps and missing data were eliminated, leaving a total of 169 positions in the final datasets.

Quantification of intracellular vitamin E content

Vitamin E quantification was performed using a reverse ELISA protocol according to the manufacturer's instructions (Lifespan, USA). Briefly, cells were trypsinized, washed 3 times with PBS and resuspended in 500 µl cold PBS. Lysis was achieved by 3 cycles of freezing at −20 °C and thawing at room temperature followed by dissociation with a pestle. The cells were then centrifuged at 4 °C 1,500 g for 10 min and the supernatant was collected and stored at −80 °C. Pellets were stored at −80 °C for protein quantification. The supernatants were thawed at room temperature and the assay was performed. Incubation times were reduced from the manufacturer's recommendation down to 8 min. Protein content in the pellets was measured using a Pierce Comassie Blue assay (Thermo Fisher, Germany). Vitamin E concentration was normalized to pellet protein content. Standard curves were constructed using the supplied standard by interpolating unknowns on a polynomial standard curve on GraphPad Prism.

Quantification of intracellular cholesterol content

Intracellular cholesterol quantification was performed using Sigma (Cholesterol Assay Kit Sigma Aldrich, Germany) according to the manufacturer's instructions modified by replacing IGEPAL with Tritonx100. Briefly, Lipids were extracted from cell pellets. Lipid extracts were then used as samples for the colorimetric assay based on a cholesterol oxidase and esterase reaction for 30 min at 37 °C. Following the reaction in the presence of the reagent, absorbance was quantified at 560 nm wavelength. Standard curves were constructed using the supplied standard by interpolating unknowns on a polynomial standard curve on GraphPad Prism 5.0. Protein content for normalization was measured using a Pierce Comassie Blue assay to quantify aliquoted lysates of the cell pellets (Thermo Fisher, Germany).

Quantification of ROS

Quantification of reactive oxygen species was performed using a commercial malondialdehyde assay according to the manufacturer's instructions (Abcam, UK). Briefly, 1×10^6 cells were washed in cold PBS and lysed in 600 µl buffer. Cells were then centrifuged for 13,000 g for 10 min and the fluid part of the supernatant was collected and frozen at −80 °C. A total of 200 µl of supernatant was supplemented with 600 µl malondialdehyde-thiobarbituric acid and incubated at 95 °C for 60 min. Samples were then cooled down on ice for 10 min and measured in 3 technical replicates on a 96-well plate reader at 532 nm. A standard curve was constructed using the supplied standard by interpolating unknowns on a polynomial standard curve in GraphPad Prism.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

Sufficient HCV RNA replication of isolates from HCV positive patients in Huh-7.5 cells expressing SEC14L2 depends on HCV GT

Saeed *et al.* showed replication of 6 high-titer HCV sera from HCV GT1a, GT1b and GT3a in SEC14L2 expressing cells.[16,21,22] However, data encompassing a broader range of viral loads and HCV GTs would complement this interesting experiment. Therefore, we first investigated replication of 73 patient-derived HCV isolates from GT1 to 4 in Huh-7.5 cells expressing SEC14L2. Firstly, a lentiviral expression system was adapted to generate novel cell lines stably expressing SEC14L2. Afterwards, Huh-7.5 harboring an empty vector as well as the novel cell line expressing SEC14L2 were infected with different patient sera. Overall, 45% of the tested isolates replicated in Huh-7.5_SEC14L2 cells (Fig. 1A). Cells infected with GT1 GT2 and

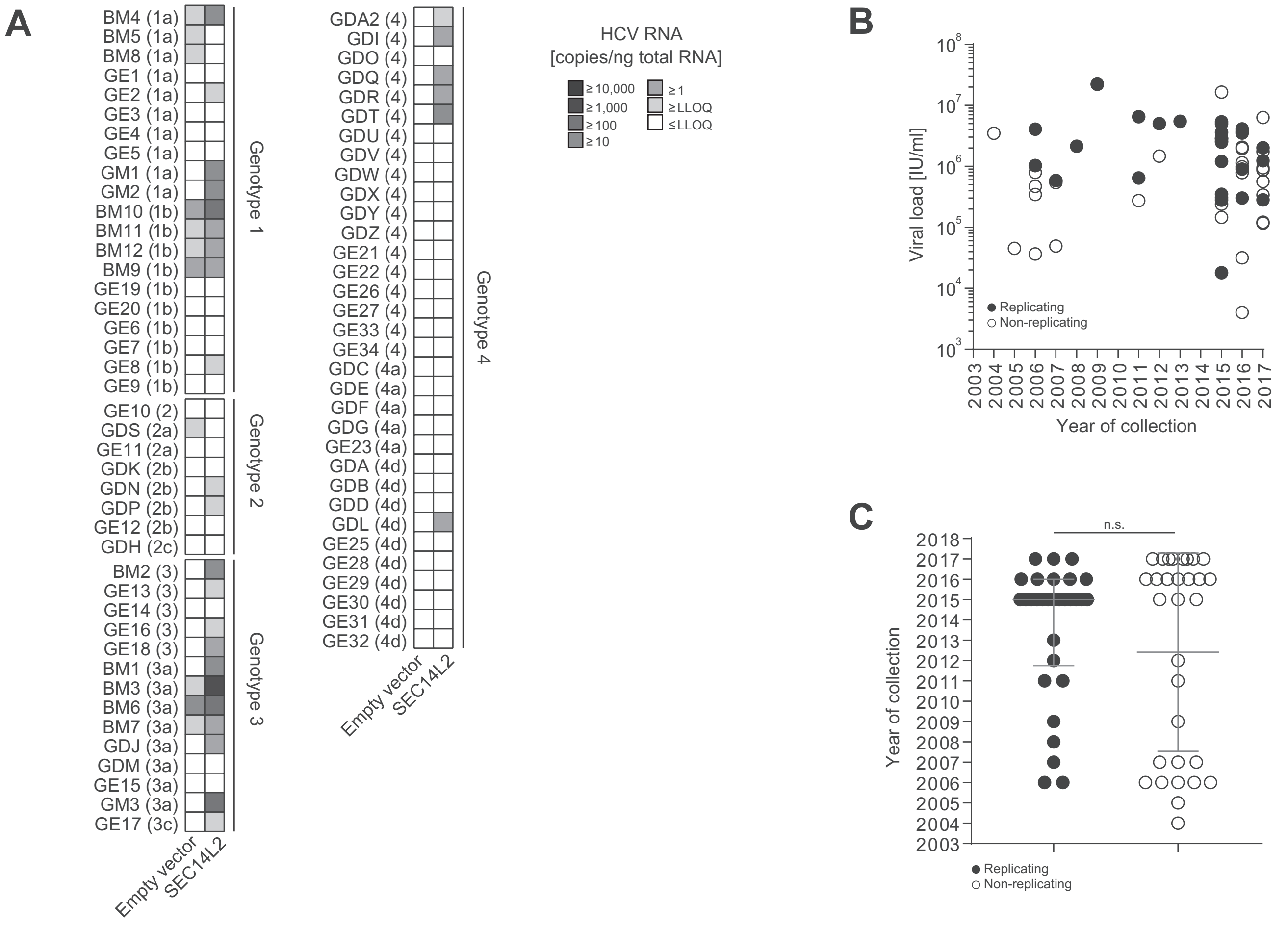


Fig. 1. RNA replication of HCV in cells harboring SEC14L2 wild-type. (A) Huh-7.5 cells harboring SEC14L2 wild-type were infected with serum from HCV infected patients in the presence of DMSO or DAA and lysed 7 days post infection. HCV RNA was quantified through RT-qPCR, results are expressed as mean RNA copy number per ng total RNA after subtraction of DAA control values to the corresponding DMSO samples to account for potential input RNA molecules present in the final lysates (2 biological replicates GE, GM, GD and 3 biological replicates BM). (B) No correlation of HCV viral load and the year of collection. Only samples where both collection year and viral load were considered. (C) Comparison of year of sample collection between replicating and non-replicating sera. Shown are the medians and the 95% confidence intervals. Statistical significance of the differences in medians was tested using Mann-Whitney *U* test (n.s. $p > 0.05$). Only samples where both collection year and viral load were considered. DAA, direct-acting antivirals; HCV, hepatitis C virus; RT-qPCR, quantitative reverse transcription PCR.

GT3 isolates showed SEC14L2 enhancement of replication, importantly, some isolates exhibited replication in the absence of SEC14L2 (Fig. 1A). 79% of all tested HCV GT3 isolates, 50% of the tested GT2 isolates and 60% of tested GT1 isolates were able to replicate in Huh-7.5 SEC14L2. In contrast, only 19% of GT4 samples replicated in culture.

Overall these data highlight that HCV GT3, GT2 and GT isolates replicate in hepatoma cells expressing human SEC14L2. GT4 isolates exhibited limited ability to replicate in our model.

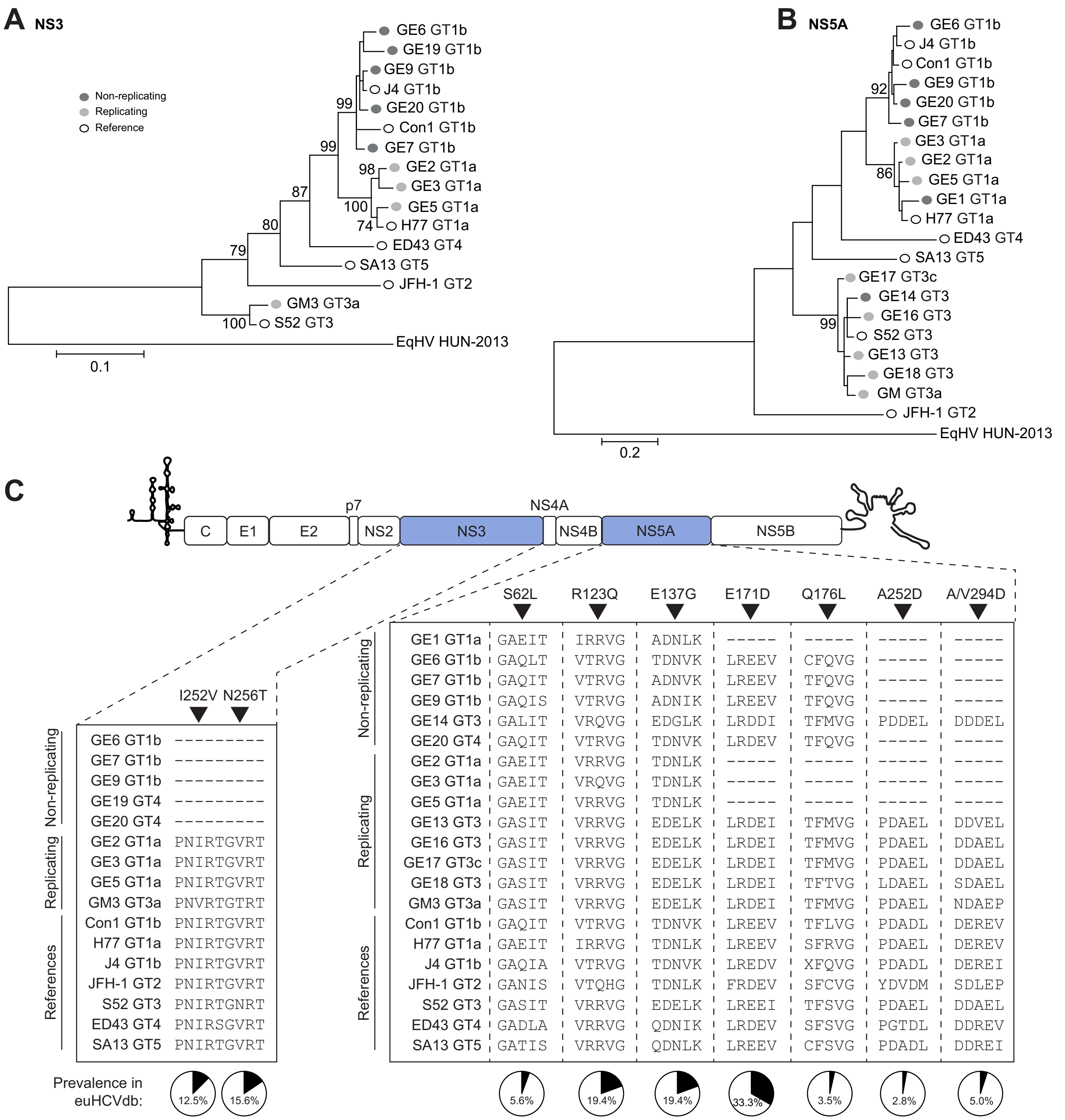
Viral replication in Huh-7.5 SEC14L2 is independent of viral load

We next aimed to understand why some natural occurring isolates can replicate in cell culture and others do not. To gather data to approach this question we compared the viral load (Fig. 1B) and storage time (Fig. 1C) of the 75 sera and matched it with the ability to replicate in Huh-7.5 SEC14L2 cells. We observed that even isolates with older collection dates (GDH to GDT) were able to replicate in our model. Crucially, we extracted RNA from high-titer serum samples GDS and GDT, which exhibited detectable replication in the empty vector and SEC14L2 cell lines respectively, and electroporated Huh-7.5 cells harboring SEC14L2 with these RNAs. We did not observe enhancement of replication when compared to the replication deficient Jc1ΔGDD (Fig. S1).

Taken together these data suggest that viral load is not a reliable predictor of the replicative phenotype of isolates used to infect cells harboring SEC14L2. Interestingly, storage conditions post collection may negatively influence the stability of the viral particle, especially in the case of GT4 isolates from the GDE collection, where samples collected in 2007 and before, when coupled with storage at −20 °C, failed to replicate in the presence of SEC14L2.

Comparative analysis of partial NS3 and NS5A sequences from replicating *vs.* non-replicating isolates

We compiled partial NS3 and NS5A sequence data obtained from resistance clinical testing and recorded amino acid differences. Firstly, we compared replicating samples *vs.* non-replicating samples and used phylogeny to establish relationships between the coding sequences. The phylogenetic tree exhibited GT determined clustering both for NS3 (Fig. 2A) and



		I252V N256T
Non-replicating	GE6 GT1b	----------
	GE7 GT1b	----------
	GE9 GT1b	----------
	GE19 GT4	----------
	GE20 GT4	----------
Replicating	GE2 GT1a	PNIRTGVRT
	GE3 GT1a	PNIRTGVRT
	GE5 GT1a	PNIRTGVRT
	GM3 GT3a	PNVRTGTRT
References	Con1 GT1b	PNIRTGVRT
	H77 GT1a	PNIRTGVRT
	J4 GT1b	PNIRTGVRT
	JFH-1 GT2	PNIRTGVRT
	S52 GT3	PNIRTGNRT
	ED43 GT4	PNIRSGVRT
	SA13 GT5	PNIRTGVRT
Prevalence in euHCVdb:		12.5% 15.6%

		S62L	R123Q	E137G	E171D	Q176L	A252D	A/V294D
Non-replicating	GE1 GT1a	GAEIT	IRRVG	ADNLK	-----	-----	-----	-----
	GE6 GT1b	GAQLT	VTRVG	TDNVK	LREEV	CFQVG	-----	-----
	GE7 GT1b	GAQIT	VTRVG	ADNVK	LREEV	TFQVG	-----	-----
	GE9 GT1b	GAQIS	VTRVG	ADNIK	LREEV	TFQVG	-----	-----
	GE14 GT3	GALIT	VRQVG	EDGLK	LRDDI	TFMVG	PDDEL	DDDEL
	GE20 GT4	GAQIT	VTRVG	TDNVK	LRDEV	TFQVG	-----	-----
Replicating	GE2 GT1a	GAEIT	VRRVG	TDNLK	-----	-----	-----	-----
	GE3 GT1a	GAEIT	VRQVG	TDNLK	-----	-----	-----	-----
	GE5 GT1a	GAEIT	VRRVG	TDNLK	-----	-----	-----	-----
	GE13 GT3	GASIT	VRRVG	EDELK	LRDEI	TFMVG	PDAEL	DDVEL
	GE16 GT3	GASIT	VRRVG	EDELK	LRDEI	TFMVG	PDAEL	DDAEL
	GE17 GT3c	GASIT	VRRVG	EDELK	LRDEI	TFMVG	PDAEL	DDAEL
	GE18 GT3	GASIT	VRRVG	EDELK	LRDEI	TFTVG	LDAEL	SDAEL
	GM3 GT3a	GASIT	VRRVG	EDELK	LRDEI	TFMVG	PDAEL	NDAEP
References	Con1 GT1b	GAQIT	VTRVG	TDNVK	LREEV	TFLVG	PDADL	DEREV
	H77 GT1a	GAEIT	IRRVG	TDNLK	LREEV	SFRVG	PDAEL	DEREV
	J4 GT1b	GAQIA	VTRVG	TDNVK	LREDV	XFQVG	PDADL	DEREI
	JFH-1 GT2	GANIS	VTQHG	TDNLK	FRDEV	SFCVG	YDVDM	SDLEP
	S52 GT3	GASIT	VRRVG	EDELK	LREEI	TFSVG	PDAEL	DDAEL
	ED43 GT4	GADLA	VRRVG	QDNIK	LRDEV	SFSVG	PGTDL	DDREV
	SA13 GT5	GATIS	VRRVG	QDNLK	LREEV	CFSVG	PDADL	DDREI
		5.6%	19.4%	19.4%	33.3%	3.5%	2.8%	5.0%

Fig. 2. Phylogeny and multiple sequence alignment of NS3 and NS5A. (A) and (B) Using UGENE we translated and performed multiple sequence alignment of partial sequences for NS3 and NS5A. Phylogeny was determined via maximum likelihood method. Branch values represent the consensus after 1,000 bootstrap passes (only values above 70% shown). Reference sequences were obtained from deposited data at eHCVdb.org, the equine hepacivirus was used as outgroup to root the tree. (C) Location of relevant observed amino acid permutations after comparative analysis of primary protein structure of NS3 and NS5A in serum samples with sequences obtained from euHCVdb. Black arrows represent relevant changes. Amino acid changes are represented using standard single letter IUPAC code. Pie charts indicate the prevalence of the respective amino acid changes in the euHCVdb cohort. Dashes represent regions of the sequence for which no nucleotide information was available. Isolates where no sequences were available were omitted from the analysis. euHCVdb, European HCV database; GT, genotype.

NS5A (Fig. 2B). Following these steps, we determined the prevalence of selected residues by multiple sequence alignment with data collected from the euHCVdb and checked for correlations between the replication phenotype and prevalence of the mutations in the analyzed populations. Additionally, we compared a subset of adaptive mutations from the literature to the available regions.[4,5] The selected mutations from the literature were not present in the surveyed regions of the serum samples. Using comparative analysis, we analyzed the sequences and noted 7 residues in NS5A which were differentially distributed among replicating and non-replicating clusters which may be of relevance to the phenotype, substantiating the observed variation in Fig. 1A. Additionally, we identified 2 residues in the NS3 of the highly replicating GM3 isolate that were of low prevalence in the remaining available NS3 sequences. (Fig. 2C).

Ultimately, single point mutations in NS3 and/or NS5A that correlated with efficient *in vitro* replication were absent in our analysis, nevertheless, we identified several residues that warrant further study, *i.e.* S62L was absent from replicating samples and present in the non-replicating GT3 sample GE14 (Fig. 2C).

Generation of Huh-7.5 cell lines expressing SEC14L2 polymorphisms

The SNP database (dbSNP) contains 13 coding non-synonymous SNPs in the human *SEC14L2* gene that are each expected to result in the exchange of a single amino acid in the SEC14L2 protein (Table 1, Fig. 3A). Polymorphisms were selected using a biased approach based on protein localization and minor allele frequency, with preference given to higher reported MAF. Interestingly, rs757660 has a MAF of 0.37 of the screened population according to the 1000 genomes database (www.1000genomes.org; Table 1). Other coding variants had lower MAF with an incidence ranging from 0.0002 to 0.0134. To study the impact of these SEC14L2 polymorphisms on HCV replication we generated Huh-7.5 cell lines expressing SEC14L2 isoform 1 variants. To achieve persistent expression of the desired proteins we employed a lentiviral monocistronic and a lentiviral bicistronic expression system carrying the gene of interest (GOI) or the GOI and a GFP protein where the GFP expression was downstream of an IRES. Both vectors carried an antibiotic resistance gene. SEC14L2 variant expression levels were measured by western blot and flow cytometry (Fig. 3B). While all cell lines have been successfully transduced, we were only able to detect SEC14L2 SNP rs191341134 protein in non-denaturing conditions (Fig. S2).

Huh-7.5 SEC14L2 variant rs191341134 is associated with reduced viral replication *in vitro*

Each of the cell lines with different SEC14L2 variants were infected with high viral titer GT1a, GT1b or GT3a patient sera normalized to 3x10^6 IU/ml. Afterwards, viral RNA content was quantified using a qPCR approach. Specificity of replication was controlled through the use of biological replicates supplemented with direct-acting antivirals (DAAs, sofosbuvir and daclatasvir). All SNPs exhibited a marked enhancement in replication when compared to the levels found in the empty vector control (Fig. 3C) when infected with a GT1b or GT3a serum sample. However, SNPs rs201172275, rs140683265, rs201989594, rs144995453, rs200123712, rs182488695 and rs191341134 did not yield detectable viral replication when infected with our GT1a serum sample. Interestingly, in GT1b and GT3a infected cells, replication levels were equivalent in 12 of the 13 polymorphism harboring cell lines and the wild-type. In the case of rs191341134, replication was 1 log$_{10}$ inferior when compared to the wild-type SEC14L2 cell lines.

Overall these data indicate that all SEC14L2 SNPs can support HCV RNA replication of HCV GT1b and GT3a. Importantly, rs191341134 was associated with reduced viral replication.

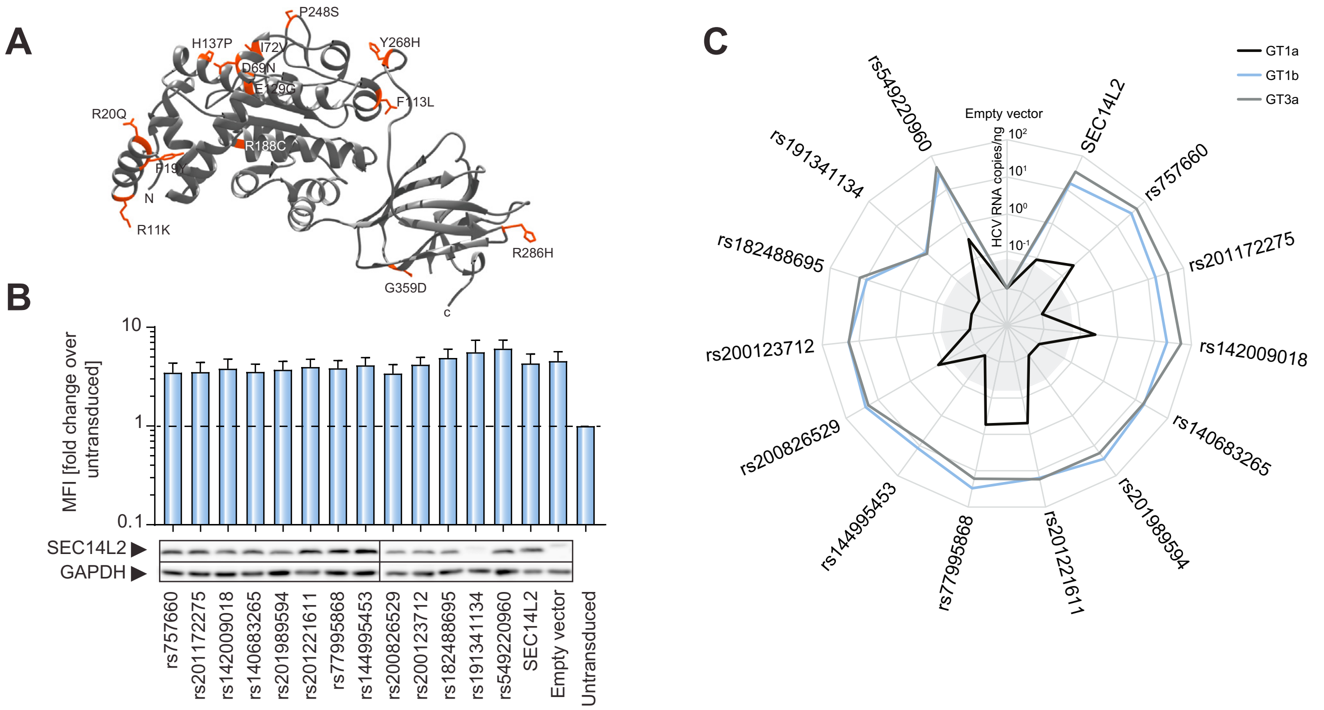


Fig. 3. Infection of Huh-7.5 cell lines harboring SEC14L2 variants. (A) The SEC14L2 protein database submission lo6u was used as template and using the PyMol software selenomethionines were manually removed. Mutated amino acids were substituted in the primary structure and manually highlighted and annotated. The 3D representation was rendered using the free version of POV-Ray. (B) Huh-7.5 cell lines were transduced with lentiviral particles carrying a payload coding for SEC14L2 variants, GFP and an antibiotic selection marker. SEC14L2 presence was verified via immunoblot with an anti-SEC14L2 monoclonal antibody. Anti-GAPDH antibodies were used as housekeeping genes to control for protein input. Flow cytometric control was conducted by quantification of the mean fluorescence intensity in the green channel of a control population, results are expressed as mean + SD MFI of 3 biological replicates. (C) Huh-7.5 cells stably expressing SEC14L2 polymorphisms were infected with 3 high-titer patient isolates in the presence of DMSO or DAA and lysed 7 days post infection. HCV RNA was quantified via RT-qPCR, results are shown as a spider web plot of the mean of 6 biological replicates (GT1a) and 4 biological replicates (GT1b and GT3a). DAA, direct-acting antiviral; GT, genotype; HCV, hepatitis C virus; MFI, mean fluorescence intensity; RT-qPCR, quantitative reverse transcription PCR.

Additionally, several SNPs of SEC14L2 exhibited altered replication of a unique GT1a isolate.

A common SNP rs757660 can promote viral replication *in vitro*

SNP rs757660 exhibits a very high prevalence in the human population. Comparatively the remaining selected 12 SNPs have a much lower allele frequency (Table 1). Taking this observation into consideration we investigated the effect of this SNP on HCV replication of GTs 1 to 5 by using a subgenomic replicon model. Replication was measured at 24 h, 48 h and 72 h postexposure and normalized to 4 h post electroporation. As shown in Fig. 4A, an enhancing effect of SEC14L2 wild-type or SNP rs757660 on viral replication was seen for GT1a, GT1b and GT3a isolates, while there was no significant difference in viral replication of GT2a, GT4a and GT5a. Overall, the enhancing effect of rs757660 was comparable with SEC14L2 wild-type.

To further investigate if viral replication of natural occurring viruses is affected by this SNP, we infected Huh-7.5 cell lines expressing wild-type SEC14L2 and variant rs757660 with a panel of 35 different sera derived from HCV infected patients (GT 1–4). Specificity of replication was controlled using biological replicates supplemented with a DAA cocktail containing sofosbuvir and daclatasvir. In line with Fig. 1A and B, viral replication was observed in 31% of the tested sera without any significant differences between SEC14L2 wild-type and the SNP (Fig. 4B). For GT2, we again observed no detectable replication, although the small number of samples containing this GT must be considered when interpreting these data. Interestingly, 2 HCV isolates (GT 3a and 4a) showed viral replication in Huh-7.5 cells expressing the SNP in contrast to SEC14L2 wild-type.

Overall these data indicate that rs757660 exhibits a phenotype comparable to that of wild-type SEC14L2.

SNPs in SEC14L2 exhibit equivalent vitamin E, ROS and cholesterol phenotypes

Next, we wanted to further investigate if these SNPs in SEC14L2 may alter its function as a vitamin E binding protein and may change the amount of intracellular vitamin E concentrations. We measured the content of vitamin E of all cell lines expressing either wild-type SEC14L2 or its variants using a competitive ELISA. We did not observe any statistically significant vitamin E content in the analyzed samples (Fig. 5A). In addition, we also quantified the presence of reactive oxygen species in rs757660 to investigate a relationship between the variation of lipid peroxidation, previously reported to be a significant factor inhibiting viral replication in Huh-7.5 cells,[4,5] and the presence of this SNP. Importantly, we did not observe a statistically significant variation in the cytosolic presence of reactive oxygen species in rs757660 (Fig. 5B). Additionally, we also performed total cholesterol quantification. Overall, we did not observe significant differences in total cholesterol content between wild-type SEC14, polymorphism harboring cell lines and empty vector control (Fig. 5C).

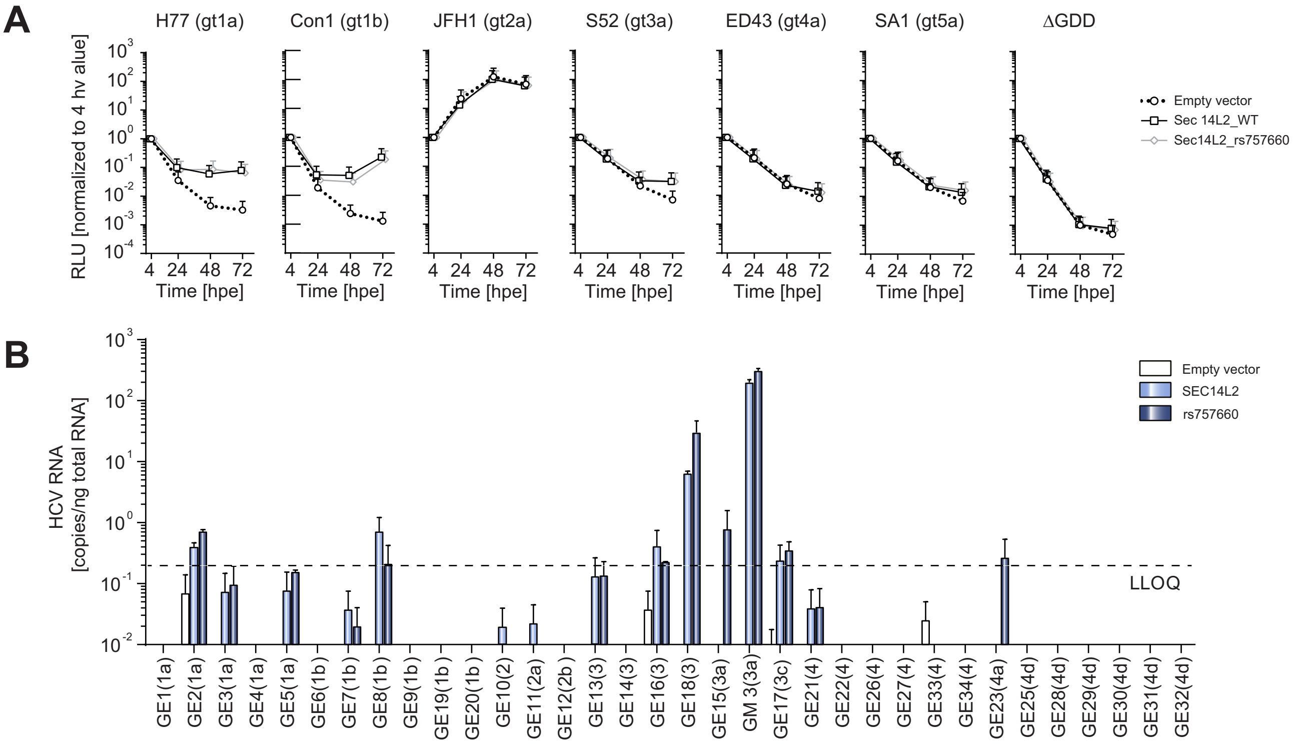


Fig. 4. rs757660 allows viral replication *in vitro*. (A) Huh-7 Lunet cells harboring SEC14L2wild-type or rs757660 were transfected with polycistronic wild-type HCV subgenomes, harboring a luciferase reporter gene in the first cistron. (GT1a H77, GT1b ConI WT, GT2a JFh-1, GT3a S52, GT4a ED43, GT5a SA1). Cells were lysed at 4 h, 24 h, 48 h and 72 h postexposure and luciferase activity was quantified. Results are presented as mean values normalized to 4 h postexposure from 2 technical replicates of 2 biological replicates. (B) Huh-7.5 cells stably expressing SEC14L2 wild-type or rs757660 were infected with patient isolates in the presence of DMSO or DAA and lyzed 7 days post infection. HCV RNA was quantified via RT-qPCR, results are shown as mean + SD of 2 biological replicates. GT, genotype; HCV, hepatitis C virus; RT-qPCR, quantitative reverse transcription PCR; WT, wild-type.

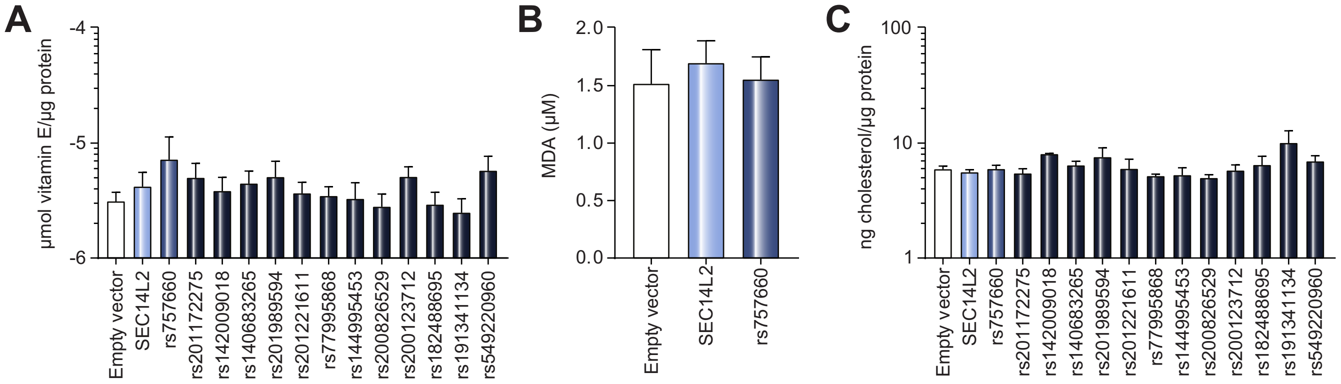


Fig. 5. Characterization of Huh-7.5 cell lines harboring *SEC14L2* SNPs. Huh-7.5 cells stably expressing SEC14L2 variants were lysed and vitamin E contents were quantified through a reverse ELISA commercial kit, results are expressed as mean + SD of 3 biological replicates (A). Huh-7.5 cells stably expressing SEC14L2 wild-type or rs757660 were lysed and MDA contents were quantified using a commercial kit, results are expressed as mean + SD of 5 biological replicates (panel B). Huh-7.5 cells stably expressing SEC14L2 variants were lysed and cholesterol contents were quantified through a fluorescence commercial assay. Results are expressed as mean + SD of 3 biological replicates. MDA, malondialdehyde; SNP, single nucleotide polymorphism.

Effects of SNP rs757660 on clinical parameters

As SEC14L2 has been shown to be an important host factor for HCV RNA replication, we questioned if SNPs in the protein may alter the clinical course of HCV infection. To answer this, we genotyped a subset of 262 samples of the INDIV2 cohort of patients infected with HCV GT1 for the most frequent coding SNP in SEC14L2, rs757660, which exhibits a prevalence of 37% according to 1000genomes. We correlated all available clinical data to the SEC14L2 genotype of the analyzed patients to uncover any relationship between SEC14L2 and high-density lipoprotein, low-density lipoprotein, sustained virologic response, end of treatment response, body mass index, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, hemoglobin, white blood cells and HCV load. In line with our *in vitro* data we did not observe an association between the SEC14L2 rs757660 polymorphism and the selected markers (Table 2).

Discussion

This study offers a perspective on the correlation between variation of SEC14L2 and pan-GT viral replication *in vitro*. We aimed to extend the available information on the effect of SEC14L2 on the viral life cycle using patient and laboratory derived strains. Following the work of Saeed *et al*, SEC14L2 emerged as a promising target for research for its role in enabling pan-GT replication of HCV in cell culture. These data reaffirmed that HCV was susceptible to lipid peroxidation and the virus would replicate more efficiently in the presence of vitamin E, a powerful lipophilic antioxidant. In the same study, the influence of SEC14L2 in the cholesterol synthesis was ruled out as a justification for the observed replicative phenotype as well as the influence of the AKT/PI3K pathway in Huh-7.5 cells.[23–25]

At a first stage we aimed to reproduce the pan-GT replication while employing a larger number of patient samples of 4 distinct GTs (Table 3). Interestingly, replication was highly efficient

Table 2. Clinical parameters of genotyped INDIV2 cohort.

Variable	Wild-type, median (25th–75th percentile)	Heterozygous, median (25th–75th percentile)	Homozygous rs757660, median (25th–75th percentile)
Cholesterol, mg/dl (n = 248)	178 (159–214)	182 (155–201)	189 (155–251)
HDL, mg/dl (n = 249)	41.00 (34.00–50.00)	39.00 (31.25–48.75)	39.50 (31.75–54.50)
LDL, mg/dl (n = 249)	117.0 (96.0–141.0)	116.5 (96.0–143.0)	138.0 (99.0–182.8)
Triglycerides, mg/dl (n = 247)	86.00 (66.00–119.50)	88.00 (67.00–125.00)	81.50 (56.25–111.00)
SVR *vs.* no SVR (n = 262)	54.05%	53.66%	60.71%
ETR *vs.* no ETR (n = 262)	71.17%	73.17%	78.57%
BMI, w/h^2 (n = 262)	24.40 (22.10–26.80)	25.60 (23.00–28.10)	24.55 (22.20–26.00)
Age, yr (n = 262)	45.00 (36.00–51.00)	43.00 (35.00–50.00)	41.00 (32.00–45.75)
ALT, U/L (n = 260)	67.00 (45.75–95.00)	66.00 (43.75–105.30)	54.00 (42.50–87.25)
AST, U/L (n = 259)	47.50 (38.00–65.00)	45.00 (33.75–64.00)	39.00 (36.00–50.00)
GGT, U/L (n = 260)	47.00 (23.75–88.50)	42.50 (23.00–82.00)	45.00 (18.25–88.50)
Hb, g/dl (n = 261)	14.90 (14.00–15.90)	14.70 (13.90–15.80)	15.00 (14.03–15.70)
WBC, Kcells (n = 261)	6.30 (5.40–8.01)	6.45 (5.27–8.20)	6.70 (4.77–8.20)
HCV viral load, $\log_{10}$ (n = 258)	5.77 (5.23–6.09)	5.73 (5.22–6.10)	5.67 (5.00–6.10)

Collection of clinical data for 262 samples of the INDIV2 cohort of patients infected with HCV genotype 1. Columns Indicate rs757660 genotype: Allele (G) wild-type, heterozygous and allele (A) mutation. Statistical significance of the differences in medians was tested using Mann-Whitney *U* test (*n.s.* $p > 0.05$). AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; ETR, end of treatment response; GGT, gamma-glutamyltransferase; Hb, hemoglobin; HCV, hepatitis C virus; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SVR, sustained virologic response; WBC, white blood cell count.

Table 3. Collection of data corresponding to all the serum samples used in the study, with viral loads at collection date, collection date and genotype information.

Sample name	Genotype	Year of collection	Viral load (IU/ml)
BM1(3a)	3a	2015	3,600,000
BM2(3)	3	2015	5,000,000
BM3(3a)	3a	2015	2,900,000
BM4(1a)	1a	2015	310,000
BM5(1a)	1a	2015	2,800,000
BM6(3a)	3a	2015	5,400,000
BM7(3a)	3a	2015	350,000
BM8(1a)	1a	2015	1,200,000
BM9(1b)	1b	2015	18,000
BM10(1b)	1b	2015	2,600,000
BM11(1b)	1b	2015	2,500,000
BM12(1b)	1b	2015	280,000
GM3(3a)	3a	2015	2,900,000
GE1(1a)	1a	2017	873,000
GE2(1a)	1a	2016	3,736,000
GE3(1a)	1a	2016	787,700
GE4(1a)	1a	2011	275,700
GE5(1a)	1a	2016	3,535,000
GE6(1b)	1b	2015	16,500,000
GE7(1b)	1b	2016	987,500
GE8(1b)	1b	2017	1,240,000
GE9(1b)	1b	2015	241,500
GE10(2)	2	2017	n.d.
GE11(2a)	2a	2016	n.d.
GE12(2b)	2b	2016	n.d.
GE13(3)	3	2017	282,300
GE14(3)	3	2017	338,600
GE15(3a)	3a	2016	2,061,000
GE16(3)	3	2016	304,000
GE17(3c)	3c	2016	4,136,000
GE18(3)	3	2017	2,032,000
GE19(1b)	1b	2012	1,479,000
GE20(1b)	1b	2015	146,400
GE21(4)	4	2017	947,400
GE22(4)	4	2016	4,029
GE23(4a)	4a	2016	31,840
GE25(4d)	4d	2003	n.d.
GE26(4)	4	2004	3,493,000
GE27(4)	4	2009	22,300,000
GE28(4d)	4d	2005	45,420
GE29(4d)	4d	2006	345,300
GE30(4d)	4d	2006	36,790
GE31(4d)	4d	2006	1,035,000
GE32(4d)	4d	2006	469,200
GE33(4)	4	2007	543,800
GE34(4)	4	2007	588,800
GDA (4d)	4d	2017	6,328,676
GDB (4d)	4d	2016	1,958,584
GDC (4a)	4a	2017	121,557
GDD (4d)	4d	2017	566,812
GDE (4a)	4a	2016	1,150,338
GDF (4a)	4a	2017	1,745,306
GDG (4a)	4a	2017	116,642
GDH (2c)	2c	2007	49,240
GDI (4d)	4d	2007	588,800
GDJ (3a)	3a	2011	647,700
GDK (2b)	2b	2006	795,400
GDL (4d)	4d	2006	1,035,000
GDM (3a)	3a	2012	1,745,306
GDN (2b)	2b	2008	2,153,000
GDP (2b)	2b	2006	4,088,000
GDQ (4d)	4d	2012	5,049,000
GDR (4)	4	2013	5,480,000
GDS (2a)	2a	2011	6,535,000
GDT (4d)	4d	2009	22,300,000
GDU (4)	4	2018	n.d.
GDV (4)	4	2017	n.d.
GDW (4)	4	2017	n.d.
GDX (4)	4	2017	n.d.
GDY (4)	4	2017	n.d.
GDZ (4)	4	2017	n.d.
GDA2 (4)	4	2017	n.d.
GM1 (1a)	1a	2016	very high
GM2 (1a)	1a	2016	900,000

n.d., no data.

in isolates from GT1, GT2 and GT3 (with the highest efficiency in GT3) whereas the replicating samples of GT4 were lower in number. These results were supported by the observations using the subgenomic replicon system. Furthermore, these data are in line with Saeed *et al.* who also found the largest number of infected cells for GT3a, followed by GT1b isolates.[4]

Data on the correlation between serum collection, storage date, viral load and the observed level of replication did not yield a clear link between these variables, suggesting that they are not the causative factors of the observed replicative phenotype. Nonetheless, none of the samples collected before 2007 and stored at –20 °C exhibited measurable replication. This implies that storage duration and conditions may be relevant to the success of patient-derived isolate replication in the cell culture model. Together these data point towards additional determinants, other than lipid peroxidation, that fully explain the discrepancies in the percentage of replicating isolates per GT. One can rule out at a first step, potential HCV core interactions as the patient-derived results are supported in the subgenomic replicon model. In this model, HCV core interaction with the host lipid machinery should be absent, which precludes endogenous peroxidation from enhanced lipid metabolism driven by core interaction.[6] This observation strongly supports the idea that any further viral requirement for replication, including naturally occurring adaptive mutations, should be located downstream of the structural proteins.

An interesting addition to this discussion was provided with the publication by Harak *et al.* where a mechanism for the modulation of HCV replication in the hepatoma cell culture system by the expression levels of PI4KA was described.[26–28] This study extended the outlook on HCV replication efficiency *in vitro* and showed that, for this application, replication is not an exclusive function of lipid peroxidation resistance but an interplay of multiple host pathways. This is in accordance with previous work that showed NS5A interactions with the phosphoinositide signaling pathways were necessary to establish efficient viral replication, specifically, the pan-GT requirement of PI4Kα expression and the GT1 specific requirement for PI4KIIIβ.[16,21,22] To further enrich this discussion, more experiments would have to be performed to research whether the enhancement achieved by tuning PI4KA expression levels would add or coalesce to the effects of vitamin E and SEC14L2 mediated lipid peroxidation resistance.

After successfully establishing the model for replication in the wild-type SEC14L2 cells, we aimed to extend the observations to 13 SEC14L2 SNPs that have been described in humans. Using high viral load, GT1a, GT1b and GT3a patient isolates we tested several SEC14L2 SNPs. Interestingly, rs191341134 elicited lower replication when compared to the wild-type SEC14L2. Our western blot data (Figs. 3B and S2) suggest that this SNP might be less stable than other SEC14L2 variants which result in lower levels of the protein, which in turn correlates

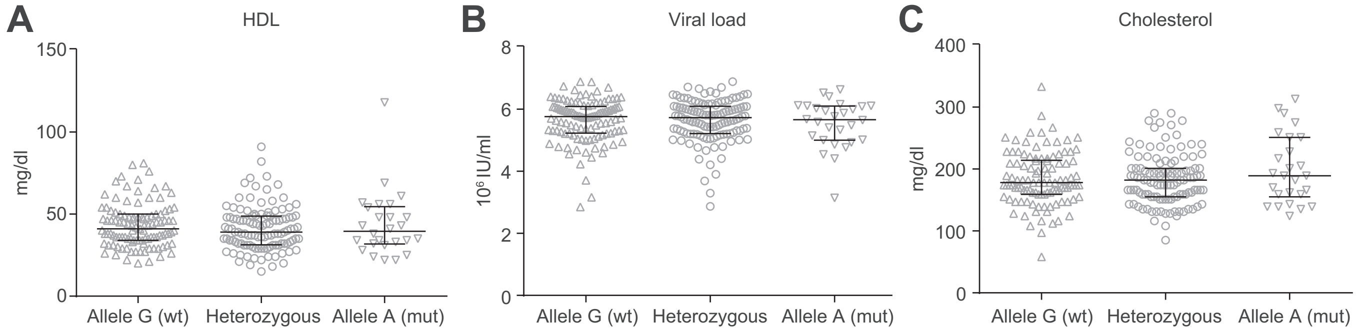


Fig. 6. Selected clinical parameters of the cohort apportioned due to *SEC14L2* SNP rs757660. No statistically significant differences regarding (A) HDL, (B) viral load or (C) cholesterol were observed between the individuals either homozygous for wild-type or rs757660, or heterozygous. Shown are the medians and the 25% and 75% percentiles. Statistical significance of the differences in medians was tested using Mann-Whitney *U* test (*n.s. p* >0.05). HDL, high-density lipoprotein; SNP, single nucleotide polymorphism.

with a lowered replication enhancing effect. Prompted by the observation that only a fraction of clinical isolates were able to replicate in the presence of SEC14L2, we compiled available viral sequences of NS3 and NS5A to perform genetic analysis of the employed viral isolates to attempt to understand this observation. Expectedly, proteins clustered according to GT in the phylogenetic analysis which indicates that there are no major changes in the surveyed regions of the proteins that would justify the observed phenotype. Additionally, adaptive mutations[13,29] from relevant laboratory strains were absent from the observed regions in the replicating samples, which suggests that this is not the explanation for the observed replicative phenotypes. Importantly, complete sequences were not available, so we cannot completely exclude the role of mutations in downstream regions of NS3, NS5A or the remaining non-structural proteins. After MSA comparing the distribution of the elected residues in the NS5A protein of GT3a from this study against the database sequences, we noted residues that occurred in replicating samples were consistently present in all replicating samples of the same GT and exhibited a relatively high prevalence in the database samples, whereas the residues identified in the non-replicating sample exhibited low overall prevalence in the database samples (Fig. 2), this distribution is consistent with the observed high number of GT3a isolates that exhibited enhanced replication in the presence of SEC14L2, nonetheless, further sequence data would be necessary to formulate a conclusion. In the case of NS5A from GT1 isolates, the elected residue although present in the replicating sample exhibited a low prevalence in the database samples, this is in accordance with the lower number of replicating samples observed from GT1 comparatively to GT3. Next generation sequencing as a follow-up would be an interesting tool to address amino acid sequence distribution. We could analyze sequences pre and post infection to further our understanding of the evolutionary dynamics of viral isolates in the presence of SEC14L2.

Given previous reports on the predictive role of vitamin E in prostate cancer patients and tocopherol concentration in the blood,[6] we decided to investigate the role of a high prevalence polymorphism of SEC14L2 in a clinical setting, as this protein enhances vitamin E mediated antioxidant activity during HCV infection and may exhibit clinically relevant phenotypes. Our cohort data showed that, consistent with the *in vitro* observations, SNP rs757660 did not significantly alter the course of HCV infection in the patients analyzed (Fig. 6). HDL, LDL and triglyceride levels exhibited non-significant differences, which is in accordance with the *in vitro* observations regarding cellular cholesterol abundance. Furthermore, liver inflammation markers and hematology also showed non-significant differences which suggest an equivalent course of infection. Finally, viral load values exhibited consistent values in all GTs. These data show that for GT1 isolates, SEC14L2 rs757660 exhibits similar function to the wild-type protein where the virus is concerned. These results provide an interesting framework when tackling SEC14L2 as a host factor. On the one side the most prevalent SNP is undistinguishable from the wild-type protein for the viral function, on the other hand we report on a second polymorphism, rs191341134 that maintains the protein ability to enable viral replication *in vitro* yet results in a significant decrease in replication, which suggests it may be an interesting target for further research.

Taken together, we have shown in this study that homogenous expression of SEC14L2 in hepatoma cell lines is a reliable model for the study of HCV patient isolates, but some limitations must be taken into consideration where storage conditions and GT are concerned. Furthermore, we have identified an SNP that results in reduced protein expression and HCV replication *in vitro*. Finally, we have shown that it is unlikely that rs757660 alters the course of HCV infection *in vivo* by providing *in vitro* and *in vivo* data that demonstrates that this polymorphism is phenotypically equivalent to the wild-type where the virus is concerned.

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Conflict of interest

The authors do not have any commercial or other association that might pose a conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

RC: acquisition of data, analysis and interpretation of data, drafting of the manuscript. FB, DT, CS: acquisition of data, analysis and interpretation of data, critical reading of the manuscript. OEA: analysis and interpretation of data. SR, LT, DB, AW: acquisition of data. BH, MR: analysis and interpretation of data. CS, JT, VL: interpretation of data. MPM: material support and obtained funding. ES: interpretation of data, critical reading of the manuscript. TVH: analysis and interpretation of data, critical reading of the manuscript, obtained funding. SC: study concept and design, analysis and interpretation of data, drafting of the manuscript, obtained funding.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2018.11.012.

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