

Research Article

Open Access

CrossMark

# Occult hepatitis C infection: viruses with infectious potential in Huh7.5 and MDBK cell lines suggest HCV/OCI transmission

## Abstract

**Background:** Occult hepatitis C infection (OCI) is characterized by the detection of HCV-RNA in patient's peripheral blood mononuclear cells (PBMCs) without detection in serum. We aim to evaluate the capacity of viruses presented in OCI patients to infect and replicate in Huh7.5 (human hepatoma-derived Huh7 cell line) and in naïve Mardin-Darby Bovine Kidney (MDBK) cell lines questioning HCV/OCI transmission.

**Methods:** Huh7.5 cells were infected with serum and PBMCs samples of 4 OCI patients. Native MDBK cells were infected with the same serum samples. Huh7.5 and naïve MDBK cells were also infected and reinfected, with an *inoculum* of naïve MDBK cell cultures previously infected with the same 4 serum samples, respectively. All infected/reinfected Huh7.5 and MDBK cell cultures were screened for HCV/OCI-RNA detection by droplet digital-PCR (ddPCR). MDBK cell cultures infected with the serum samples were evaluated for HCV/OCI specific proteins by proteomic liquid chromatography-mass spectrometry (LC-MS) analysis. Huh7.5/infected and MDBK/reinfected cell cultures with the *inoculum* serum samples were also evaluated for the E2, NS3 and NS4a HCV/OCI specific regions by immunogold electron microscopy (IEM) analysis.

**Results:** HCV/OCI-RNA was detected in Huh7.5 cell cultures infected with serum and PBMCs samples of the OCI patients 4 and 3, respectively, and in Huh7.5/infected and MDBK/reinfected cell cultures with the *inoculum* serum samples of the 4 OCI patients by ddPCR. HCV specific proteins peptides were detected in MDBK cell cultures (first infection) infected with 1 serum sample by LC-MS. HCV specific immunostaining was shown in Huh7.5 cell cultures infected with PBMCs samples of the OCI patients' 3- NS3 and 4-NS4a, and in MDBK cell cultures reinfected with *inoculum* serum sample of the OCI patient 4-E2 and -NS4a by IEM analysis.

**Conclusions:** Infectious potential of HCV/OCI viruses was demonstrated in Huh7.5 cell cultures infected with serum and PBMCs samples of the 4 OCI patients. Infectious potential of HCV/OCI viruses was also demonstrated in MDBK cell cultures infected with the serum samples, as well as, in Huh7.5/infected and MDBK/reinfected cell cultures with the *inoculum* serum samples. Overall results suggest HCV/OCI transmission, and could contribute for a better understanding of the HCV/OCI life cycle, infection and transmission.

**Keywords:** HCV, OCI, Huh7.5 cell line, MDBK cell line, HCV/OCI transmission, Droplet digital PCR, LC-MS, IEM

## Introduction

Occult hepatitis C infection (OCI) is characterized by the detection of hepatitis C virus (HCV)-RNA in liver biopsy patient's samples and in peripheral blood mononuclear cells (PBMCs) without detection in serum samples.<sup>1-3</sup> It is a common condition worldwide, all HCV genotypes can be involved in this form of infection,<sup>4</sup> and no HCV vaccine is currently available.<sup>5</sup> OCI has been described in patients with chronic liver disease, coinfections, comorbidities and in population with risk factors, such as, in patients infected with hepatitis B virus or human immunodeficiency virus, with tattoos, drugs users, and apparently in healthy population.<sup>6-11</sup> The primary goal of HCV therapy is to cure the infection, i.e. to achieve a sustained virological response (SVR) defined as undetectable HCV-RNA in serum after treatment completion, and the use of direct-acting antiviral agents (DAAs) markedly improved the SVR rates.<sup>12-14</sup> Although, the detection of OCI in patients who achieved a SVR with DAAs was previously described,<sup>15-17</sup> and we have recently presented OCI detection in people with or without DAAs therapy.<sup>18</sup>

HCV present tropism to replicate in the liver-hepatocytes, and also have capacity to replicate in PBMCs and lymphoid cells.<sup>19,20</sup> While, in

2003 Kato et al cloned a HCV-2a consensus genome with the capacity to produce infectious HCV in human hepatoma-derived Huh7 cell line.<sup>21</sup> Furthermore, Huh7.5 (human hepatoma-derived Huh7 cell line) cell line has also been widely used for the propagation of HCV in vitro, and both cell cultures demonstrated to be suitable experimental substitutes for primary hepatocytes.<sup>22-25</sup> Moreover, the replication of HCV homologs in non-hepatoma cells, such as in mosquito, African green monkey, Vero cells, and Mardin-Darby Bovine Kidney (MDBK) cell lines have also been previously described.<sup>26-28</sup> The aim of this study was to evaluate the capacity of viruses detected in OCI patients to infect and replicate in Huh7.5 and in naïve MDBK cell lines by droplet-digital PCR (ddPCR), proteomic liquid chromatography-mass spectrometry (LC-MS) and immunogold electron microscopy (IEM) analysis questioning HCV/OCI transmission.

## Patients and methods

### Patients

OCI patients that were referred to the Coimbra Hospital and University Center (CHUC) were identified in a previous study we have performed.<sup>18</sup> Here, we randomly select 4 OCI patients from the

previously identified, patient 1, 2, 3 and 4, and performed the cell lines investigation attending to the HCV/OCI transmission possibility. Two HCV positive patients (patient 5 and 6) were also included in the study.

## Samples

Blood samples from 4 OCI patients were collected in dry and in lithium heparin tubes, and serum was recovered from the dry tubes as previously described.<sup>29</sup> PBMCs were isolated using lymphoprep<sup>TM</sup> (Alere Technologies AS, Norway) following the manufacturer's instructions, and recovered PBMCs were resuspended in 200 µL of water molecular grade (G-Biosciences, USA) and also in 200 µL of RPMI 1640 culture medium (Thermo Fisher Scientific, USA) supplemented with 50% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 20% dimethyl sulfoxide (Thermo Fisher Scientific, USA). All samples were stored at -80°C. Then, Huh7.5 cell line was infected with serum and PBMCs resuspended in water molecular grade samples. Naïve MDBK cell line was infected with serum samples, and Huh7.5 and naïve MDBK were also infected and reinfected with an *in vitro* serum samples, respectively. All infected/reinfected Huh7.5 and MDBK cell cultures were screened for HCV/OCI-RNA detection by droplet digital-PCR (ddPCR). MDBK cell cultures infected with the serum samples (first infection) were evaluated for HCV/OCI specific proteins by proteomic liquid chromatography-mass spectrometry (LC-MS) analysis. Huh7.5/infected and MDBK/reinfected cell cultures with the *inoculum* serum samples were also evaluated for the E2, NS3 and NS4a HCV/OCI specific regions by immunogold electron microscopy (IEM) analysis. Blood samples from the 2 HCV positive patients were also collected and evaluated as for the 4 OCI patients.

## Cell lines

Huh 7.5 cell line was kindly provided by Professor Charles Rice (Apath, L.L.C and Rockefeller University, USA) and maintained in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, USA) supplemented with 10% FBS (Thermo Fisher Scientific, USA), 0.1% penicillin (100 U/mL)–streptomycin (100 µg/mL) (GRISP, Porto, Portugal), 200 mM L-glutamine (Thermo Fisher Scientific, USA) and 1% of non-essential amino acids (Thermo Fisher Scientific, USA) at 37°C and 5% CO<sub>2</sub>, similarly as previously described.<sup>25,30</sup> MDBK cell line was purchased at the European Collection of Authenticated Cell Cultures and were maintained as previously described.<sup>28,31</sup>

## Viral infection in Huh7.5 and in MDBK cell lines

Huh7.5 cells were infected with serum and PBMCs resuspended in water molecular grade samples (4 OCI patients and 2 HCV positive patients) to evaluate the infectivity and efficient production of HCV/OCI particles following previously described methods for naïve MDBK cells infection.<sup>28,31</sup> Naïve MDBK cell line was infected with serum samples (4 OCI patients and 2 HCV positive patients), and Huh7.5 and naïve MDBK cells were also infected and reinfected with an *inoculum* serum samples (4 OCI patients and 2 HCV positive patients), respectively, for the same evaluation similarly as previously described.<sup>28,31</sup> Briefly, 2 mL of each serum and PBMCs resuspended in water molecular grade samples filtered through a 0.2 µm membrane were adsorbed onto Huh7.5 cells in T25 flasks and incubated at 37°C and 5% CO<sub>2</sub> for 3 h. Monolayers were washed twice with 1X PBS, the culture medium was replaced, and flasks were maintained at 37°C and 5% CO<sub>2</sub> for 7 days (S-Huh7.5-II-P0 and PBMCs-Huh7.5-II-P0 passages, 7 days' post infection). After, 1 (P1) and 2 (P2) subcultures, respectively, were performed and maintained at the same conditions and for the same time, S-Huh7.5-II-P1, PBMCs-Huh7.5-II-P1 and PBMCs-Huh7.5-II-P2, respectively. Supernatants and cells recovered

separately from each S-Huh7.5-II-P1 and PBMCs-Huh7.5-II-P2 flask/sample were then evaluated for HCV/OCI-RNA detection by ddPCR, and the cell cultures from each PBMCs-Huh7.5-II-P2 flask/sample were also evaluated for HCV/OCI specific immunostaining by IEM. Uninoculated Huh7.5 cells (negative control) were maintained and also evaluated by ddPCR and IEM.

In naïve MDBK cells a first inoculation (II) of serum samples was performed. Two mL of each serum sample filtered through a 0.2 µm membrane were adsorbed onto naïve MDBK cells in T25 flasks and incubated at 37°C and 5% CO<sub>2</sub> for 3 h. Monolayers were washed twice with 1X PBS, the culture medium was replaced, and flasks were maintained at 37°C and 5% CO<sub>2</sub> for 7 days (S-MDBK-II-P0, 7 days' post infection). After, 1 subculture (P1) of these cell cultures was performed and maintained at the same conditions and for the same time, S-MDBK-II-P1 passage. Supernatants from each S-MDBK-II-P1 cell cultures flask/sample, and from uninoculated MDBK cells (negative controls) flasks were recovered and evaluated for HCV/OCI specific proteins by LC-MS analysis, and remaining cell cultures were stored at -80°C for 8 days. After, cell cultures from S-MDBK-II-P1 were evaluated for HCV/OCI-RNA detection by ddPCR and used later as *inoculum* to reinfect naïve MDBK and infect Huh7.5 cell lines. For the reinfection in naïve MDBK cells and the infection in Huh7.5 cells with the serum *inoculum* samples, 2 mL of the *inoculum* from each cell culture sample were also filtered through a 0.2 µm membrane, adsorbed onto naïve MDBK and Huh7.5 cells in T25 flasks and incubated at 37°C and 5% CO<sub>2</sub> for 3 h. Monolayers were washed twice with 1X PBS, the culture medium was replaced and flasks were maintained at 37°C and 5% CO<sub>2</sub> for 7 days, S-MDBK-RI-P0 and S-Huh7.5-I-P0, respectively. Then 2 and 1 subcultures for the MDBK-RI-P0 and S-Huh7.5-I-P0, were performed, S-MDBK-RI-P1, S-MDBK-RI-P2 and S-Huh7.5-I-P1, respectively, and supernatants and cells recovered separately from each S-MDBK-RI-P0, S-MDBK-RI-P1, S-MDBK-RI-P2, S-Huh7.5-I-P0 and S-Huh7.5-I-P1 flask/sample were screened for HCV/OCI-RNA detection by ddPCR. Cell cultures from each S-MDBK-RI-P2 and S-Huh7.5-I-P1 flask/sample were also evaluated for HCV/OCI specific immunostaining by IEM. Uninoculated MDBK and Huh7.5 cells (negative controls) were maintained and also evaluated by ddPCR and IEM.

A flowchart of the study design for the Huh7.5 and naïve MDBK cells infection/reinfection assays is presented in Figure 1.

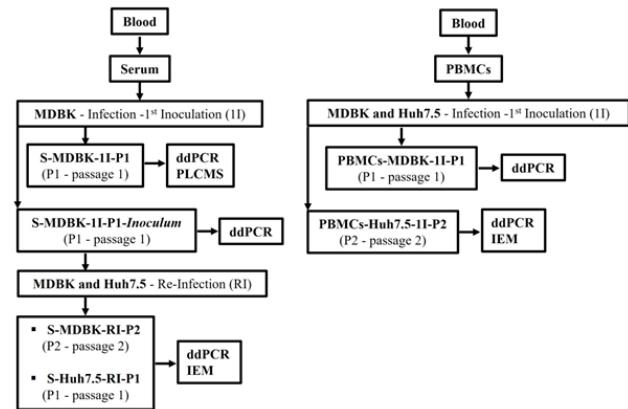


Figure 1 Flowchart of the study design for the MDBK and Huh7.5 cells infection and reinfection assays.

## RNA extraction, cDNA synthesis and ddPCR

Total RNA from cell cultures was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The cDNA synthesis was performed using random

hexamers included in the Xpert cDNA synthesis kit (GRISP Porto, Portugal) following the manufacturer's instructions. DdPCR was performed as previously described<sup>8,32</sup> with some modifications. Briefly, HCV/OCI-RNA was detected for the HCV core region using only the sense 5'-GCGTTAGTAYGAGTGTYG-3' and antisense 5'-CRATTCCGGTGTACTCAC-3' primers, that were able to amplify HCV genotypes 1, 2, 3 and 4.<sup>30</sup> The reaction mix contained 10 µL of ddPCR™ Supermix for Probes (Bio-Rad, USA), 900 nM of each primer, 250 nM of FAM-labeled HCV probe (5'-FAM-CCGCAGACCACTATGGCTC-BHQ1-3') and 2 µL of template in a final volume of 22 µL. Amplicons are of 89 bp. The reactions mix were placed on a Bio-Rad QX200 Droplet Digital PCR System for droplets generation following the manufacturer's instructions, and then HCV/OCI-RNA amplification was performed on a Bio-Rad Thermal Cycler C1000 (Bio-Rad, USA) under the cycling conditions of 95°C for 10 min, 45 cycles at 94°C for 30 s and 55°C for 1 min, and 98°C for 10 min, with a hold step at 4°C. After, the plate was read on a Bio-Rad QX200™ Droplet Reader for droplets analysis, and data was analysed using the Bio-Rad QuantaSoft™ Analysis Pro Software v. 1.0.596, following manufacturer's instructions. The fluorescence amplitude threshold was automatically adjusted in individual wells and HCV/OCI-RNA concentration was achieved in copies/µL attending to the number of observed accepted droplets (10.000 or greater). For each tested sample, two runs were performed and in duplicate to adjust ddPCR sensitivity. The plasmid pGNN-non-replicative (p7-nsGluc2A) kindly provided by Professor Charles Rice (Apath, L.L.C and Rockefeller University, USA) was used as positive control in ddPCR reactions.

### LC-MS analysis

Supernatants from S-MDBK-1I-P1 cell cultures/flasks and from un-inoculated MDBK cells (negative controls) flasks were recovered and evaluated for HCV/OCI specific proteins by LC-MS analysis as previously described<sup>33</sup> with some modifications. Briefly, 300 µL of each cell culture supernatant were recovered to 1.5 mL low protein binding microcentrifuge tubes (Thermo Fisher Scientific, USA) and were placed in a water bath at 95°C for 10 min. Protein extractions from each cell culture supernatant were performed and recovered proteins were digested with trypsin. The resulting peptides were then analysed by LC-MS. In data analysis the HCV/OCI protein peptides identification was performed with the data available in the UniProt protein sequence database for the Homo sapiens Reviewed Proteome 2021\_03 with 20,371 entries, hepatitis C virus Proteome 2021\_04 with 220,291 entries, Bos taurus Reviewed Proteome 2021\_03 with 6,014 entries and a common contaminant database from MaxQuant (version 1.6.2.6, Max Planck Institute of Biochemistry, Munich, Germany). The mass spectrometry proteomics data have been deposited to the Proteome Xchange Consortium via the PRIDE (<http://www.ebi.ac.uk/pride/archive/>) partner repository with dataset identifier PXD033680 and 10.6019/PXD033680. This procedure was performed at the i3S Proteomics Scientific Platform.

### IEM analysis

Huh7.5 cell cultures infected with PBMCs samples, passage PBMCs-Huh7.5-1I-P2, were evaluated for HCV/OCI specific immunostaining for the E2, NS3 and NS4a HCV specific regions by IEM analysis as previously described<sup>28,31</sup> with some modifications. Moreover, Huh7.5 and MDBK cell cultures that were infected and re-infected with the *inoculum* serum samples, S-Huh7.5-I-P1 and S-MDBK-RI-P2, respectively, were also evaluated. Briefly, cell culture suspensions were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, USA) and 2% paraformaldehyde (Merck, Germany) in tris buffered saline. Cell cultures were evaluated using

primary mouse monoclonal antibodies anti-E2, anti-NS3 and anti-NS4a HCV proteins at dilutions 1:10 and/or 1:25. Huh7.5 and MDBK uninoculated cells (negative controls) were evaluated using anti-E2 mouse monoclonal antibodies at dilution 1:10.

## Results

### DdPCR Huh7.5 and MDBK cell cultures

Huh7.5 cells were infected with serum and PBMCs re-suspended in water molecular grade samples of the 4 OCI patients to evaluate the infectivity and efficient production of HCV/OCI. Huh7.5 cells were also infected with samples of the 2 HCV positive patients and evaluated. From these cell cultures, supernatants and cells recovered separately from each S-Huh7.5-1I-P1 and PBMCs-Huh7.5-1I-P2 flask/samples were evaluated for HCV/OCI-RNA detection by ddPCR. Considering supernatants plus cells ddPCR results, positive results were detected in S-Huh7.5-1I-P1 and in PBMCs-Huh7.5-1I-P2 infected with the 4 OCI patients' serum and PBMCs samples, respectively, with the exception for PBMCs-Huh7.5-1I-P2-OCI patient 4 (Table 1). Positive results were also detected in S-Huh7.5-1I-P1 and in PBMCs-Huh7.5-1I-P2 infected with the 2 HCV positive patients' serum and PBMCs samples, with the exception for S-Huh7.5-1I-P1-HCV positive patient 6 (Table 1). In S-Huh7.5-1I-P1 infected with the 4 OCI patients' serum samples, HCV/OCI-RNA was detected in a range of 0.14 to 1.89 copies/µL with 17963 and 18088 number of accepted droplets, respectively, and for the S-Huh7.5-1I-P1 infected with the sample of the HCV positive patient was of 0.14 copies/µL with 17123 number of accepted droplets (Table 1). In PBMCs-Huh7.5-1I-P2 infected with the 4 OCI patients' PBMCs samples HCV/OCI-RNA was detected in a range of 0.21 to 0.56 copies/µL with 16953 and 16919 number of accepted droplets, respectively, and for PBMCs-Huh7.5-1I-P2 infected with the samples of the HCV positive patients was of 0.32 to 1.01 copies/µL with 15156 and 15998 number of accepted droplets, respectively, (Table 1). Negative results were obtained for the uninoculated Huh7.5 cells (negative control).

The S-MDBK-1I-P1 cells cultures (SN + cells) that were used as the *inoculum* serum to reinfect new naïve MDBK and to infect Huh7.5 cells were evaluated for HCV/OCI-RNA detection by ddPCR. HCV/OCI-RNA was detected only in the cell cultures infected with the serum of the OCI patient 3, and negative results were shown in cell cultures infected with the serum samples of the HCV positive patients (Table 2). In S-MDBK-1I-P1 cells cultures infected with the serum of the OCI patient 1 HCV/OCI-RNA was detected with 0.98 copies/µL and 15594 number of accepted droplets (Table 2). Negative results were obtained for the uninoculated MDBK cells (negative control).

Moreover, S-MDBK-RI-P0, S-MDBK-RI-P1 and S-MDBK-RI-P2 reinfected cell cultures (SN and cells recovered separately) were analysed for HCV/OCI-RNA detection by ddPCR. Considering supernatants plus cells and the 3 evaluated passages (S-MDBK-RI-P0, -P1 and -P2) ddPCR results, positive results were detected in all tested reinfected MDBK cell cultures that were infected with the MDBK *inoculum* serum samples-4 OCI patients (Table 2). Negative or not determined results were shown in the tested MDBK cell cultures reinfected with the MDBK *inoculum* serum samples-2 HCV positive patients (Table 2). HCV/OCI-RNA (S-MDBK-RI-P0, -P1 and -P2) was detected in a range of 0.15 to 3.14 copies/µL with 15771 and 15887 number of accepted droplets, respectively (Table 2). Negative results were obtained for the uninoculated MDBK cells (negative control).

Furthermore, S-Huh7.5-I-P0 and S-Huh7.5-I-P1 infected cell cultures (SN and cells recovered separately) were analysed for HCV/OCI-RNA detection by ddPCR. Considering supernatants plus cells

and the 2 evaluated passages (S-Huh7.5-I-P0 and -P1), positive results were detected in all tested Huh7.5 infected with the MDBK *inoculum* serum samples of the 4 OCI and the 2 HCV positive patients (Table 2). HCV/OCI-RNA (S-Huh7.5-I-P0 and -P1, -4 OCI patients) was detected in a range of 0.13 to 3.44 copies/µL with 18402 and 14044 number of accepted droplets, respectively (Table 2). HCV/OCI-RNA (S-Huh7.5-I-P0 and -P1, -HCV positive patients) was detected in a range of 0.79 to 1.77 copies/µL with 16394 and 15998 number of accepted droplets, respectively (Table 2). Negative results were obtained for the uninoculated Huh7.5 cells (negative control).

Positive result was obtained for the tested plasmid pGNN-non-replicative-p7-nsGluc2A (ddPCR positive control) by ddPCR, with 22.4 copies/µL and 17423 number of accepted droplets.

**Table 1** HCV/OCI-RNA detected in Huh7.5 cell line infected with serum and PBMCs samples of the 4 OCI and 2 HCV positive patients by ddPCR

Cell cultures	Patients	OCI Patients			HCV positive patients	
		1	2	3	4	5
SN-S-Huh7.5-II-PI	HCV/OCI-RNA (Copies/µL)	0	0.46	1.89	0.67	0.14
	Accepted droplets	14383	17963	18088	15847	17123
Cells-S-Huh7.5-II-PI	HCV/OCI-RNA (Copies/µL)	0.62	1	1.08	0.9	0.14
	Accepted droplets	17104	11717	16288	16991	17123
SN + Cells - S-Huh7.5-II-PI		+	+	+	+	-
SN-PBMCs-Huh7.5-II-P2	HCV/OCI-RNA (Copies/µL)	0.5	0.56	0.35	0	0.32
	Accepted droplets	16380	16919	13638	15325	15156
Cells-PBMCs-Huh7.5-II-P2	HCV/OCI-RNA (Copies/µL)	0	0.21	0.39	0	0
	Accepted droplets	16712	16953	17780	12273	16394
SN + Cells - PBMCs-Huh7.5-II-P2		+	+	+	-	+

SN, supernatant; S, serum; P, passage; PBMCs, peripheral blood mononuclear cell; HCV, hepatitis C virus; OCI, occult hepatitis C infection; (+) positive result; (-) negative result.

**Table 2** HCV/OCI-RNA detected in infected/reinfected MDBK and infected Huh7.5 cell cultures with the serum samples of the 4 OCI and 2 HCV positive patients by ddPCR

Cell cultures	Patients	OCI Patients			HCV positive patients	
		1	2	3	4	5
Inoculum serum-S-MDBK-II-PI SN + Cells	HCV/OCI-RNA (Copies/µL)	0	0	0.98	0	0
	Accepted droplets	12518	14868	15594	15537	15568
SN-S-MDBK-RI-P0	HCV/OCI-RNA (Copies/µL)	0	0.15	0	0	N.D
	Accepted droplets	14075	15771	15057	16797	N.D
Cells-S-MDBK-RI-P0	HCV/OCI-RNA (Copies/µL)	0.28	0.9	0	0	N.D
	Accepted droplets	16950	14378	16632	14375	N.D
SN + Cells - S-MDBK-RI-P0		+	+	-	-	N.D
SN-S-MDBK-RI-P1	HCV/OCI-RNA (Copies/µL)	1.03	0	0	0	N.D
	Accepted droplets	12563	10925	15799	12896	N.D
Cells-S-MDBK-RI-P1	HCV/OCI-RNA (Copies/µL)	0.22	0	0.21	0	N.D
	Accepted droplets	16265	14440	11313	15537	N.D
SN + Cells - S-MDBK-RI-P1		+	-	+	-	N.D
SN-S-MDBK-RI-P2	HCV/OCI-RNA (Copies/µL)	3.41	0	0.49	2.11	N.D
	Accepted droplets	15887	14192	19027	17722	N.D
Cells-S-MDBK-RI-P2	HCV/OCI-RNA (Copies/µL)	2.71	0	0	0	N.D
	Accepted droplets	16525	16893	17542	16099	N.D
SN + Cells - S-MDBK-RI-P2		+	-	+	+	N.D
SN-S-Huh7.5-I-P0	HCV/OCI-RNA (Copies/µL)	0	3.44	3.18	0.47	0
	Accepted droplets	17006	14044	15978	10021	15156
Cells-S-Huh7.5-I-P0	HCV/OCI-RNA (Copies/µL)	0	1.07	0	0.18	0.79
	Accepted droplets	15214	16737	12137	18513	16394
SN + Cells - S-Huh7.5-I-P0		-	+	+	+	-
SN-S-Huh7.5-I-P1	HCV/OCI-RNA (Copies/µL)	0	0	0.13	0	0
	Accepted droplets	16406	18102	18402	15617	15156
Cells-S-Huh7.5-I-P1	Copies/µL	1.54	0	0	0.72	0.79
	Accepted droplets	16024	16431	17466	16268	16394
SN + Cells - S-Huh7.5-I-P1		+	-	+	+	+

SN, supernatant; S, serum; P, passage; PBMCs, peripheral blood mononuclear cell; HCV, hepatitis C virus; OCI, occult hepatitis C infection; (+) positive result; (-) negative result.

**Table 3** HCV/OCI protein peptides identified in the supernatants of MDBK cell cultures infected with serum samples of the 4 OCI and 2 HCV positive patients by LC-MS analysis

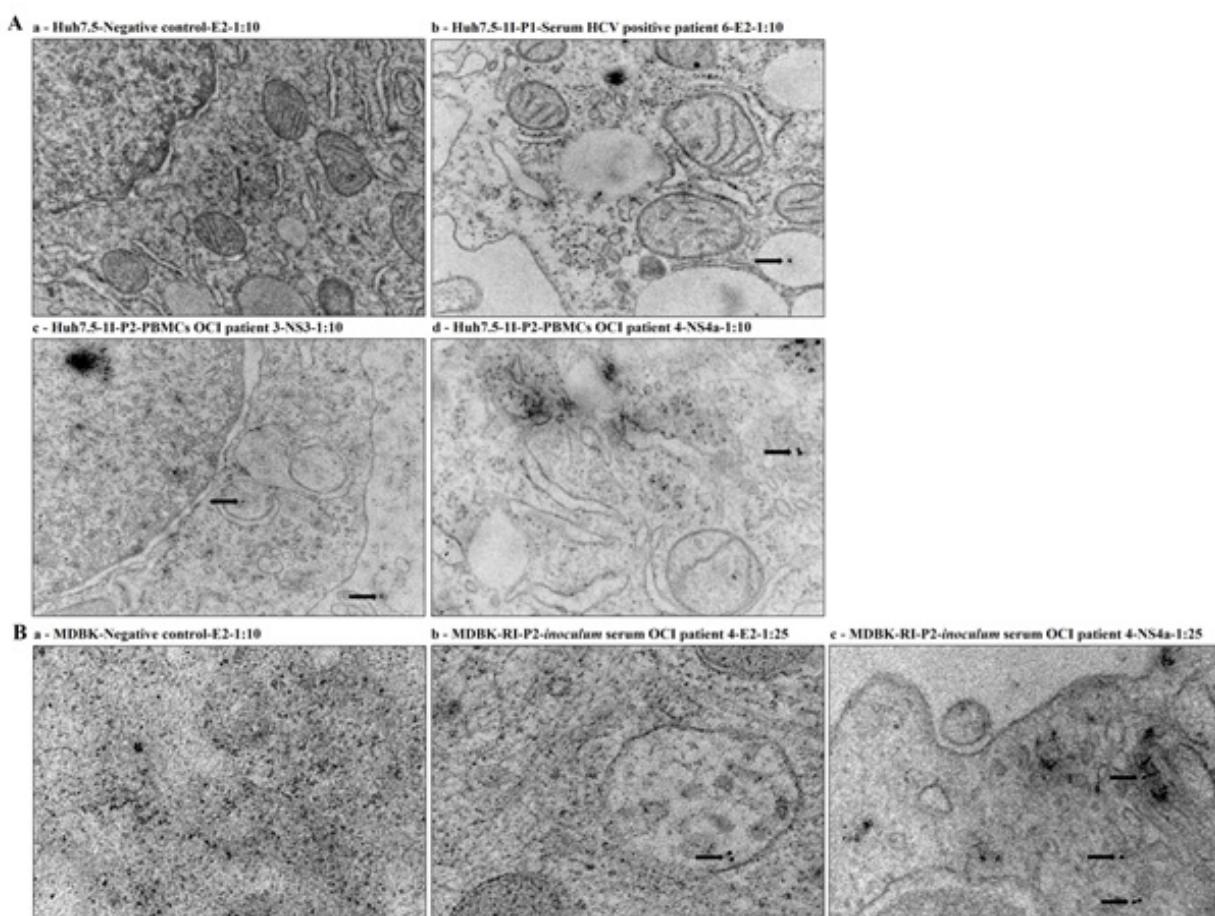
Patients	UniProt accession no. (Genotype)	HCV Protein	Best peptide sequence	Genome position (aa)	Exp. Q-value
3	A0A650C4D1 (N/A)	Protein F	ADPGRSLSGMLGLFMAMR	60-76	0
6	K0A6T5 (1b)	Core	FAQGWGPITYAEPPK	465-479	0.005
6	A0A1B1UY2 (N/A)	Core	DAIILLTCAVYPELIFDITK	871-890	0.005
6	X2BYG4 (N/A)	Core	LGNEILLGPADTEDTAGWR	1006-1024	0.006
6	A0A139ZG90 (N/A)	Core	LPTAAMGAAYGFQYSRKQR	2608-2626	0.005
6	B1NE60 (N/A)	E1	VAIIMVMFSGVDATHTVGSSTAR	53-76	0.006
6	A0A1I9RGK9 (N/A)	E1	THIDMVVMSASLCSALY	260-276	0.005
6	I1UZB4 (1)	RdRp	LQDCTMLVCGDDLVVICESAGVHEDAASLR	90-119	0.006
6	A0A2H4YRE0 (N/A)	RdRp	LPGAVMGAAYGFQYSRKER	182-200	0.005
6	B1PBT9 (N/A)	RdRp	LQVLDDHYQDVFK	532-544	0.005

HCV, hepatitis C virus; aa, amino acids; N/A, not available.

### IEM in Huh7.5 and MDBK cell cultures

Cell cultures suspensions S-Huh7.5-1I-P1, PBMCs-Huh7.5-1I-P2 and S-MDBK-RI-P2 that were infected or reinfected with the 4 OCI and 2 HCV positive patients' samples were also evaluated by IEM using mouse monoclonal antibodies anti-HCV E2, anti-HCV NS3 and anti-HCV NS4a. HCV/OCI specific immunostaining was shown in PBMCs-Huh7.5-1I-P2 cell cultures infected with the PBMCs samples

of the OCI patients 3 (NS3) and 4 (NS4a), and serum samples of the HCV positive patient 6 (E2) (Figure 2A). In S-MDBK-RI-P2 cell cultures HCV/OCI specific immunostaining was shown in cell cultures infected with serum sample of the OCI patient 4 (E2 and NS4a) (Figure 2B). Uninoculated Huh7.5 and MDBK cells (negative controls) were also evaluated with the anti-HCV E2 monoclonal antibodies and no HCV/OCI specific immunostaining was shown (Figure 2A and 2B).



**Figure 2** HCV specific immunostaining for the HCV E2, NS3 and NS4a specific regions by IEM. (A) IEM detection in Huh7.5 cell cultures infected with serum and PBMCs samples of the 4 OCI and 2 HCV positive patients. (a) Huh7.5-Negative control-E2-1:10, (b) Huh7.5-1I-P1-Serum HCV positive patient 6-E2-1:10, (c) Huh7.5-1I-P2- PBMCs OCI patient 3-NS3-1:10 and (d) Huh7.5-1I-P2-PBMCs OCI patient 4-NS4a-1:10. The immunogold particles were 10 nm in diameter. Scale bars: (a and c 0,5 μm); (b 0,2 μm); (d 200 nm). (B) IEM detection in MDBK cell cultures reinfected with *inoculum* serum samples. (a) MDBK-Negative control-E2-1:10, (b) MDBK-RI-P2-*inoculum* serum OCI patient 4-E2-1:25, (c) MDBK-RI-P2-*inoculum* serum OCI patient 4-NS4a-1:25. The immunogold particles were 10 nm in diameter. Scale bars: (a) 200nm; (b and C) 100 nm.

## Discussion

OCI is normally characterized by the detection of HCV in PBMCs patient samples without detection in serum samples,<sup>1-3</sup> that could be negative due to the presence of low viral load not easily detected by the current methods and/or technologies. Furthermore, data where the possibility to detect HCV in serum samples after ultracentrifugation preventing low HCV viral load detection in OCI patients was also previously described.<sup>34,35</sup> In this line, we have performed a previous study in OCI where we have characterized OCI patients with HCV/OCI negative and positive results in serum and PBMCs samples, respectively, by ddPCR.<sup>18</sup> Here, we have randomly selected 4 OCI patients from our previous study and performed the work. Huh7.5 and naïve MDBK cell lines were infected with the serum of the 4 OCI identified patients in order to evaluate if serum samples could have HCV/OCI viruses that were not detected by ddPCR in our previous study. We also infect Huh7.5 cells with PBMCs samples of the 4 OCI patients that tested positive for HCV/OCI by ddPCR in our previous study. We aimed to investigate the possibility of the viruses presented in both samples to be infectious in both cell lines and understand if they could also have the capacity of HCV/OCI transmission. When Huh7.5 cell line was infected with the serum and PBMCs samples of the 4 OCI patients' positive results were shown in all infected cell cultures by ddPCR. These results demonstrated that HCV/OCI viruses are presented in serum samples previously determined as HCV/OCI negative using the same methods and ddPCR reaction.<sup>18</sup> Moreover, these results demonstrated also the infectious potential of the presented viruses in the serum and PBMCs samples in Huh7.5 cell line. This achievement in infected Huh7.5 cells was not previously described, while the HCV capacity to infect and replicate in Huh7.5 cell line was previously described.<sup>21-25</sup>

Furthermore, when naïve MDBK cell line was infected with the serum *inoculum* S-MDBK-1I-P1 cell cultures samples (first infection) of the 4 OCI patients, positive results were just detected in cell cultures infected with the serum *inoculum* sample of the OCI patient 3 by ddPCR. Independently of this only detected positive result, infectious potential of the presented HCV/OCI viruses were demonstrated in the infected MDBK cell line, and HCV-homologs with the capacity to infect and replicate in this cell line were previously described.<sup>28,31</sup> Moreover, when naïve MDBK and Huh7.5 cell lines were reinfected and infected with the 4 OCI patients serum *inoculum* S-MDBK-1I-P1 cell cultures samples, positive results were detected in all tested reinfected MDBK and infected Huh7.5 cell cultures, meaning that serum samples of the 4 OCI patients have viruses with the capacity to infect MDBK cells, and that the viruses presented in the 4 OCI patients serum *inoculum* S-MDBK-1I-P1 cell cultures have also the capacity to internalize and replicate in reinfected MDBK cell cultures and in infect Huh7.5 cell cultures. These results are accordantly with the detected positive results shown in the analysed Huh7.5 cell cultures infected with the serum samples of the 4 OCI patients. Overall results demonstrated the infectious potential of the HCV/OCI viruses presented in the serum and PBMCs samples in Huh7.5 and MDBK cell lines suggesting the possibility of HCV/OCI transmission. To the best of our knowledge, no work was published describing the possibility of HCV/OCI transmission using Huh7.5 and MDBK cells infected with serum and PBMCs samples of OCI patients. Otherwise, an HCV cell-cell transmission assay using Huh7.5.1 cells was previously described.<sup>36</sup> Moreover, nowadays the HCV life cycle remains partly understood due to the difficulty of culturing HCV *in vitro* or obtaining fully susceptible yet immunocompetent *in vivo* models, nonetheless, an HCV life cycle in cells, infection and host response, and analysis of molecular markers influencing the outcome of infection and response to therapy was described in 2016.<sup>37</sup> Taking

in consideration our previously reported data and obtained results here we suggest that our work could support in the understanding of the HCV/OCI life cycle, HCV/OCI infection and HCV/OCI transmission. LC-MS analysis were performed in supernatants recovered from the S-MDBK-1I-P1 cell cultures (first inoculation) that were infected with the serum samples of the 4 OCI patients and an HCV specific protein peptide was identified in cell cultures infected only with the serum of the OCI patient 3. This result is accordantly with the obtained by ddPCR, while more positive results were achieved using the methodology and the ddPCR reaction. Although, a proteomic analysis by LC-MS reporting the first large-scale proteome analysis of the highly permissive Huh7.5 cells containing a full-length HCV replicon was previously described,<sup>38</sup> as well as, HCV specific proteins peptides identified in MDBK cells inoculated with rabbit and hare samples by proteomic analysis.<sup>28,31</sup>

Furthermore, HCV specific immunostaining for the NS3 and NS4a proteins was demonstrated in Huh7.5 cell cultures infected with the PBMCs samples of the OCI patient 3 and 4. In MDBK cell cultures reinfected with the serum *inoculum* sample-OCI patient 4 was demonstrated for the E2 and NS4a proteins. No HCV/OCI specific immunostaining was observed for all tested infected/Huh7.5 and reinfected/MDBK cell cultures, although achieved results are accordantly with the encountered by ddPCR-OCI patients 3 and 4 and LC-MS-OCI patient 3 analysis and are accordantly also with previously described data.<sup>22-25,28,31</sup>

## Conclusion

In this study infectious potential of HCV/OCI viruses was demonstrated in Huh7.5 cell cultures infected with serum and PBMCs samples of the 4 OCI patients. Infectious potential of HCV/OCI viruses was also demonstrated in MDBK cell cultures infected with the serum samples of the 4 OCI patients', as well as, in Huh7.5 and in MDBK cell cultures infected and reinfected with the *inoculum* serum samples-4 OCI patients, respectively. Overall results suggest HCV/OCI transmission and suggest also that our work could support in the understanding of the HCV/OCI life cycle, HCV/OCI infection and HCV/OCI transmission.

## Ethics approval and consent to participate

Informed consent was obtained from all subjects involved in the study under the project approval by the CHUC ethical committee (registration number CHUC-122-18).

## Consent for publication

None.

## Availability of data

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (<http://www.ebi.ac.uk/pride/archive/>) partner repository with the dataset identifier PXD033680 and 10.6019/PXD033680.

## Funding

This research was funded by the Foundation for Science and Technology (FCT) under the project PTDC/SAU-SER/30788/2017, FEDER.

## Author's contributions

Eliane Silva and Armando Carvalho designed the study. Armando Carvalho, Adélia Simão, João Madaleno and Bernardo Canhão participated in the patient's recruitment, data collection, and data

interpretation and all together with Eliane Silva participated in the sample's recruitment. Eliane Silva performed the ddPCR and the *in vitro* cells works, and prepared samples for LC-MS and IEM analysis, with Eliane Silva IEM results responsibility. Hugo Osório performed the LC-MS analysis and analysed data with Eliane Silva. Eliane Silva wrote the paper with Sara Marques support. All authors critically reviewed the manuscript and approved the final version of the manuscript for publication.

## Acknowledgments

The authors acknowledge the Foundation for Science and Technology (FCT) under the project PTDC/SAU-SER/30788/2017, FEDER. The authors acknowledge the Cancer Biology & Epigenetics Group, Research Center of IPO Porto, and the Molecular Pathology and Immunogenetic Laboratories, ICBAS-UP, for their availability to carry out the work. The authors also acknowledge the project NORTE-01-0246-FEDER- 000063, supported by Norte Portugal Regional Operational Programme (NORTE2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF) that co-funded the work.

## Competing interest

The authors declare that they have no competing interest.

## References

1. Castillo I, Pardo M, Bartolome J, et al. Occult hepatitis C virus infection in patients in whom the etiology of persistently abnormal results of liver-function tests is unknown. *J Infect Dis.* 2004;189(1):7–14.
2. Carreno V. Occult hepatitis C virus infection: a new form of hepatitis C. *World J Gastroenterol.* 2006;12(43):6922–6925.
3. Castillo I, Bartolome J, Quiroga JA, et al. Long-term virological follow up of patients with occult hepatitis C virus infection. *Liver Int.* 2011;31(10):1519–1524.
4. Carreno V, Bartolome J, Castillo I, et al. New perspectives in occult hepatitis C virus infection. *World J Gastroenterol.* 2012;18(23):2887–2894.
5. Sepulveda-Crespo D, Resino S, et al. Hepatitis C virus vaccine design: focus on the humoral immune response. *J Biomed Sci.* 2020;27(1):78.
6. Austria A, Wu GY. Occult Hepatitis C Virus Infection: A Review. *J Clin Transl Hepatol.* 2018;6(2):155–160.
7. Lotfi AA, Mohamed AE, Shalaby NA, et al. Occult hepatitis C virus infection in patients with malignant lymphoproliferative disorders. *Int J Immunopathol Pharmacol.* 2020;34:2058738420961202.
8. Frias M, Rivero-Juarez A, Tellez F, et al. Evaluation of hepatitis C viral RNA persistence in HIV-infected patients with long-term sustained virological response by droplet digital PCR. *Sci Rep.* 2019;9(1):12507.
9. Martinez-Rodriguez ML, Uribe-Noguez LA, Arroyo-Anduiza CI, et al. Prevalence and risk factors of Occult Hepatitis C infections in blood donors from Mexico City. *PLoS One.* 2018;13(10):e0205659.
10. Sheikh M, Bokharaei-Salim F, Monavari SH, et al. Molecular diagnosis of occult hepatitis C virus infection in Iranian injection drug users. *Arch Virol.* 2019;164(2):349–357.
11. Mahmud S, Chemaiteily H, Al Kanaani Z, et al. Hepatitis C Virus Infection in Populations With Liver-Related Diseases in the Middle East and North Africa. *Hepatol Commun.* 2020;4(4):577–587.
12. Mekky MA, Sayed HI, Abdelmalek MO, et al. Prevalence and predictors of occult hepatitis C virus infection among Egyptian patients who achieved sustained virologic response to sofosbuvir/daclatasvir therapy: a multi-center study. *Infect Drug Resist.* 2019;12:273–279.
13. Yousif MM, Elsadek Fakhr A, Morad EA, et al. Prevalence of occult hepatitis C virus infection in patients who achieved sustained virologic response to direct-acting antiviral agents. *Infekz Med.* 2018;26(3):237–243.
14. Janjua NZ, Darvishian M, Wong S, et al. Effectiveness of Ledipasvir/Sofosbuvir and Sofosbuvir/Velpatasvir in People Who Inject Drugs and/or Those in Opioid Agonist Therapy. *Hepatol Commun.* 2019;3(4):478–492.
15. Elmasry S, Wadhwa S, Bang BR, et al. Detection of Occult Hepatitis C Virus Infection in Patients Who Achieved a Sustained Virologic Response to Direct-Acting Antiviral Agents for Recurrent Infection After Liver Transplantation. *Gastroenterology.* 2017;152(3):550–553. e8.
16. Mohamed AA, Eljaky AM, Abdelsamea EM, et al. Prevalence and effect of occult hepatitis C infection in patients with persistent liver enzyme elevation after achieving 24 weeks of sustained virological response. *Egypt J Intern Med.* 2019;31(3):288–291.
17. Yousef TM, Kamal El Din MMM, George TMA, et al. Detection of Occult Hepatitis C Virus Infection in Patients Who Achieved a Sustained Virologic Response to Direct-Acting Antiviral Agents. *QJM.* 2021;114(Suppl 1).
18. Silva E, Marques S, Salta S, et al. Occult hepatitis C infection detection in people who use drugs with or without direct-antiviral agents therapy. *International Liver Congress: J Hepatol.* 2022;77(Suppl 1).
19. Castillo I, Rodriguez-Inigo E, Bartolome J, et al. Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection. *Gut.* 2005;54(5):682–685.
20. Wroblewska A, Bielawski KP, Sikorska K. Occult Infection with Hepatitis C Virus: Looking for Clear-Cut Boundaries and Methodological Consensus. *J Clin Med.* 2021;10(24):5874.
21. Kato T, Date T, Miyamoto M, et al. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology.* 2003;125(6):1808–1817.
22. Lindenbach BD, Evans MJ, Syder AJ, et al. Complete replication of hepatitis C virus in cell culture. *Science.* 2005;309(5734):623–626.
23. Zhong J, Gastaminza P, Cheng G, et al. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA.* 2005;102(26):9294–9299.
24. Kawamoto M, Yamaji T, Saito K, et al. Identification of Characteristic Genomic Markers in Human Hepatoma HuH-7 and Huh7.5.1-8 Cell Lines. *Front Genet.* 2020;11:546106.
25. Blight KJ, McKeating JA, Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol.* 2002;76(24):13001–13014.
26. Germi R, Crance JM, Garin D, et al. Mosquito cells bind and replicate hepatitis C virus. *J Med Virol.* 2001;64(1):6–12.
27. Guo J, Yan R, Xu G, et al. Construction of the Vero cell culturesystem that can produce infectious HCV particles. *Mol Biol Rep.* 2009;36(1):111–120.
28. Silva E, Osorio H, Thompson G. Hepatitis C-like viruses are produced in cells from rabbit and hare DNA. *Sci Rep.* 2015;5:14535.
29. Costa-Matos L, Batista P, Monteiro N, et al. Hfe mutations and iron overload in patients with alcoholic liver disease. *Arg Gastroenterol.* 2013;50(1):35–41.
30. Zein NN. Clinical significance of hepatitis C virus genotypes. *Clin Microbiol Rev.* 2000;13(2):223–235.
31. Silva E, Marques S, Osorio H, et al. Endogenous hepatitis C virus homolog fragments in European rabbit and hare genomes replicate in cell culture. *PLoS One.* 2012;7(11):e49820.

32. Rivero-Juarez A, Caruz A, Real LM, et al. Longitudinal evaluation of hepatitis C viral persistence in HIV-infected patients with spontaneous hepatitis C clearance. *Eur J Clin Microbiol Infect Dis.* 2015;34(11):2171–2175.
33. Osorio H, Silva C, Ferreira M, et al. Proteomics Analysis of Gastric Cancer Patients with Diabetes Mellitus. *J Clin Med.* 2021;10(3):407.
34. Bartolome J, Lopez-Alcorocho JM, Castillo I, et al. Ultracentrifugation of serum samples allows detection of hepatitis C virus RNA in patients with occult hepatitis C. *J Virol.* 2007;81(14):7710–7715.
35. De Marco L, Manzini P, Trevisan M, et al. Prevalence and follow-up of occult HCV infection in an Italian population free of clinically detectable infectious liver disease. *PLoS One.* 2012;7(8):e43541.
36. Xiao F, Fofana I, Heydmann L, et al. Hepatitis C virus cell-cell transmission and resistance to direct-acting antiviral agents. *PLoS Pathog.* 2014;10(5):e1004128.
37. Dustin LB, Bartolini B, Capobianchi MR, et al. Hepatitis C virus: life cycle in cells, infection and host response, and analysis of molecular markers influencing the outcome of infection and response to therapy. *Clin Microbiol Infect.* 2016;22(10):826–832.
38. Jacobs JM, Diamond DL, Chan EY, et al. Proteome analysis of liver cells expressing a full-length hepatitis C virus (HCV) replicon and biopsy specimens of posttransplantation liver from HCV-infected patients. *J Virol.* 2005;79(12):7558–7569.