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EDITORIAL

Sofosbuvir/velpatasvir: A promising combination

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Abstract

Hepatitis C virus (HCV) affects 3% of the world population. It represents the main cause of chronic liver disease and is responsible for extra-hepatic complications, such as type 2 diabetes and cardiovascular diseases. HCV includes 7 genotypes differing in the nucleotide sequence variability, the geographic distribution, the rates of viral clearance, the risk of progression to liver fibrosis and to hepatocellular carcinoma, and the response to therapy. Last years have seen remarkable advances in the field of HCV infection with the approval of direct antiviral agents (DAAs) targeting key viral proteins involved in the HCV replication. Several oral regimens combining DAAs from different families have been developed and these regimens showed increased and sustained virological response rates to above 90% reducing the treatment duration to 12 wk or less. In particular, sofosbuvir, a nucleotide analogue nonstructural (NS)5B polymerase inhibitor, and velpatasvir, a NS5A inhibitor, have been tested in two phase 3 trials, the ASTRAL-2 (against HCV genotype 2) and the ASTRAL-3 (against HCV genotype 3), demonstrating to be effective, safe, and well tolerated in patients who were 18 years of age or older and had at least a 6-mo history of HCV infection with a compensated liver disease.

Key words: Hepatitis C virus; Sofosbuvir; Velpatasvir; NS5A inhibitor; NS5B inhibitor

Core tip: Hepatitis C virus (HCV) spread all over the world. In the last years, new therapies with direct antiviral agents draw a great revolution thanks to several oral regimens combining different drugs of this class. The present editorial provides a brief overview on the association between two direct antiviral agents, sofozsbuvir and velpatasvir, and their implication in the treatment of HCV infection.

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INTRODUCTION

Hepatitis C virus (HCV) infects approximately 3% of the world population and leads to cirrhosis and hepatocellular carcinoma[1]. HCV is a member of the Flaviviridae family of RNA viruses and is classified into 7 genotypes; each genotype is different from the others in its nucleotide sequence. This remarkable genetic diversity represents a challenge for the development of new therapies[2]. HCV genotypes differ not only in the nucleotide sequence variability, but also in their geographic distribution, rates of viral clearance, risk of progression to liver fibrosis and to hepatocellular carcinoma, and response to therapy[3].

In last years, HCV treatment has undergone substantial advances. Direct antiviral agents (DAAs), when combined with pegylated interferon (PegIFN) and ribavirin (RVR), demonstrated increased rates of cure in chronic HCV infections when compared to PegIFN and RVR alone[4]. The first class of DAAs to be approved for treatment of HCV was that of protease inhibitors targeting the nonstructural protein (NS)3/4A serine protease, responsible for the processing of the nascent viral polyprotein[4]. The search for new key viral targets continued and compounds against two additional targets - NS5A replication scaffold and the NS5B RNA-dependent RNA polymerase (RdRp) - have been generated[5]. Drugs inhibiting NS5B include two subclasses: Nucleos(t)ide inhibitors (NIs) and non-nucleoside inhibitors (NNIs). NS5B is strictly required during the HCV replication both to copy the RNA genome and to transcribe messenger RNA: These steps are essential and the inhibition of NS5B is able to block viral propagation (Figure 1).

Many phase 2 trials have been conducted with promising results. Two phase 3 trials have done encouraging results with the combination of the nucleotide polymerase inhibitor sofosbuvir and the NS5A inhibitor velpatasvir in patients chronically infected with HCV genotype 2 and 3[6].

A BRIEF PRESENTATION OF SOFOSBUVIR AND VELPATASVIR

Sofosbuvir

Sofosbuvir (SOF) (formerly known as GS-7977; Gilead Sciences, Foster City, CA, United States) is a NS5B NI[7]. It is converted into a pharmacologically active form (GS-461203) within hepatocytes, inhibits RdRp activity by competing with uridine, and blocks RNA synthesis by acting as "chain terminator"[8]. Given the high conservation of the catalytic site of the NS5B protein, this drug is believed to have pangenotypic activity[8]. The combination of SOF and RVR achieved sustained virologic response (SVR) rates of 100% for genotype 2 infection and 91% for genotype 3 infection[9]. On December 6, 2013, the United States Food and Drug Administration (FDA) approved SOF (Sovaldi™) for the treatment of chronic HCV, genotypes 1, 2, 3 and 4, in combination with Peg-IFN and RVR or with RVR alone. SOF is also highly effective in HCV patients who are co-infected with human immunodeficiency virus (HIV). It should not be administered with potent inducers of intestinal P-glycoprotein, such as rifampin and Saint John's wort, resulting in reduced absorption[10]. Moreover, co-administration of SOF with select anticonvulsants, rifabutin, rifapentine, or tipranavir/ritonavir is not recommended[5]. SOF showed no clinically significant drug interactions with many of the common medications metabolized by CYP3A enzymes, such as tacrolimus, cyclosporine, and methadone, as well as with the common combination therapies for HIV[5].

Velpatasvir

Velpatasvir (VEL) (formerly GS-5816; Gilead Sciences; Foster City, CA, United States) is a new NS5A protein inhibitor with pan-genotypic activity *in vitro*[11]. In phase 2 trials, it demonstrated high rates of SVR in patients with HCV genotypes 2 and 3 in combination with sofosbuvir for a period of 12 wk of treatment[12,13]. Regimens including an NS5A inhibitor have demonstrated high tolerability and high antiviral efficacy in phase 3 studies[11].

SOF AND VEL IN PHASE 2 AND 3 STUDIES

The phase 2 studies

In phase 2 studies, the SOF/VEL combination administered for 12 wk showed high rates of SVR and was well tolerated in all HCV genotype infections[12,13].

In the phase 2 study by Everson *et al*[12], treatment-naïve non-cirrhotic patients achieved high rates of SVR at 12 wk with SOF/VEL, independently of HCV genotypes. Virologic failure was rare; the nonresponse of 1 patient with HCV genotype 3 infection and the relapse in a patient with HCV genotype 1 infection, both receiving 25 mg of VEL, may suggest that the 100 mg dose could have a clinical advantage over the 25 mg one. In general, the therapy with SOF/VEL with or without RVR was well tolerated. In the other phase 2 study by Pianko *et al*[13], SOF/VEL demonstrated to be safe, effective, and well-tolerated for treatment-experienced patients with HCV infection genotype 1 or 3, including those with compensated cirrhosis, which are the most common genotypes accounting for approximately 46% and 22% of all global infections, respectively[14]. In particular, the SVR at 12 wk in patients with genotype 3 HCV infection under VEL 100 mg compared favorably with those previously reported for other regimens[15-17].

The ASTRAL-2 and the ASTRAL-3 trials

The ASTRAL program aimed to evaluate the safety and the efficacy of the association SOF/VEL in patients with HCV genotype 1-6 infection including patients with decompensated liver disease. ASTRAL-2 and ASTRAL-3 trials are two randomized, controlled, phase 3 trials, in which a fixed-dose combination tablet of SOF/VEL for

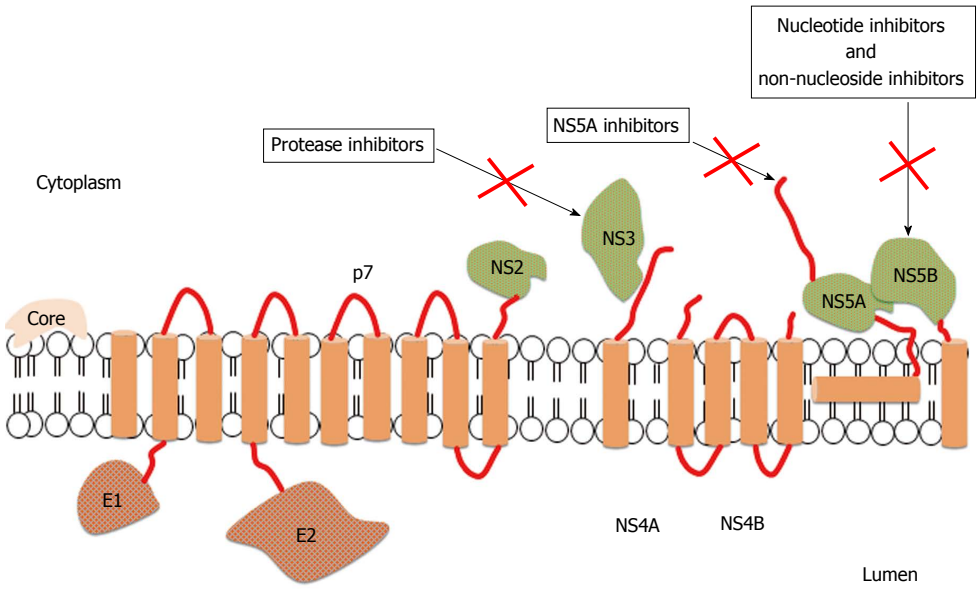

Figure 1 Structure of hepatitis C virus and mechanisms of action of direct antiviral agents[29]. The open reading frame of hepatitis C virus (HCV) encodes 11 proteins: 3 structural proteins (core, E1, and E2); 6 nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B); the p7 protein; and the frameshift (F) protein (not illustrated). The core protein has a role in viral capsid formation and can directly interact with many cellular proteins and pathways implicated in the HCV lifecycle. Envelope glycoproteins, E1 and E2, are fundamental components of the virion envelope and are essential for HCV entry and fusion; in particular, E2 has a central role in the early steps of infection because it functions as a host receptor binding protein and mediates the attachment to host cells. The F protein is probably produced during viral infection and could be involved in viral persistence, but the exact role has not been fully elucidated. The p7 protein is an integral membrane protein belonging to the viroporin family and maybe acts as a calcium ion channel, but further studies are needed to confirm its function. NS2 is a non-glycosylated transmembrane protein having a protease activity, which may interact with host cell proteins. NS3 is a multi-functional protein owning a serine protease domain and a helicase/nucleoside triphosphatase domain, while NS4A is a cofactor of NS3 protease activity. The 2 proteins can interact with host cell pathways and proteins involved in HCV lifecycle and for this reason they are an appealing viral target for anti-HCV therapies. NS4 is an integral membrane protein serving as a membrane anchor for the replication complex; moreover, it can inhibit cellular syntesis and modulate the HCV RdRp activity. NS5A is a zinc-metalloprotein playing a role in virus replication, cell growth replication, and in mediating interferon-resistance, even if some of these functions need to be still clarified. NS5B belongs to the class of tail-anchored proteins. Its crystal structure showed that the RdRp has a "fingers, palm and thumb" structure; interactions between the fingers and thumb subdomains create a fully surrounded catalytic site ensuring both HCV RNA strand synthesis. For this reason, the RdRp is an attractant target for new anti-HCV drugs.

12 wk was compared to standard treatment with SOF plus RVR for 12 or 24 wk in patients already treated for HCV genotype 2 and 3 infection and in those who had not received this treatment, including the ones with decompensated cirrhosis[6].

The two studies shared the same eligibility criteria, except for HCV genotype. Patients who were 18 years of age or older and with at least a 6-mo history of HCV infection could enter the study, whilst patients with clinical evidence of hepatic decompensation were excluded. In the two trials, patients with chronic HCV infection were randomly assigned to receive a fixed-dose combination tablet containing 400 mg of SOF and 100 mg of VEL once daily for 12 wk or 400 mg of SOF plus RVR for 12 wk (for patients with HCV genotype 2) or 24 wk (for patients with HCV genotype 3). RVR was administered orally twice daily, with body weight-determined doses. The primary endpoint was a SVR, defined as an HCV RNA level of less than 15 IU per milliliter at 12 wk after the end of treatment.

In the ASTRAL-2 trial, patients who had received SOF/VEL met the primary endpoint with no virologic failures. In the ASTRAL-3 trial, patients who were administered with SOF/VEL gained a SVR, but 4% of them showed virologic failure after the end of treatment. Overall, the rate of SVR was higher among patients without cirrhosis and who did not receive any previous treatment.

Safety

In both trials, rates of adverse events (AEs) were lower among patients who received SOF/VEL than those who received SOF/RVR. In both trials, only 1 patient receiving SOF/VEL stopped the treatment prematurely because of an AE (anxiety, headache, and difficulty in concentrating). Common AEs, such as headache, fatigue, and nausea have been reported in 10%-38% of SOF/VEL patients, together with insomnia, irritability, pruritus, nasopharyngitis, cough, and dyspesia[6]. Serious AEs in the SOF/VEL group of the ASTRAL-2 included enteritis, pneumonia, and abdominal pain, whereas in the ASTRAL-3 acute myocardial infarction, acute cholecystitis, food poisoning, hematochezia, intracranial aneurysm, and rupture of ovarian cyst have been described[6]. In both trials, only 1 patient receiving SOF/VEL stopped the treatment prematurely because of an AE (anxiety, headache, and difficulty of concentration). Death after treatment occurred in two patients in the ASTRAL-2 (1 from cardiac arrest and one from metastatic lung cancer complications); in the ASTRAL-3, two deaths occurred during treatment (1 from unknown cause and 1 from gunshot wounds) and one in the post-treatment period (from unknown cause).

Comparison between NIs and NNIs

NIs inhibit the RdRp by mimicking NS5B protein sub-

strate leading to the termination of the new viral RNA chain; they possess a high-resistance barrier, are highly effective, and own a pan-genotypic activity[18]. NNIs behave as allosteric inhibitors by binding to the RdRp blocking polymerase function through conformational change; this results in a lower barrier to resistance and lower anti-viral activity with respect to NIs[18].

SOF is the only NI approved and was associated with high SVR rates in all kind of patients. The addition of SOF to PegIFN and RVR demonstrated to be the most effective IFN-containing regimen in HCV patients with compensated cirrhosis. In the NEUTRINO and FISSION studies, patients receiving SOF had rapid and substantial decreases in serum HCV RNA levels. Moreover, AEs were uncommon among patients receiving SOF regimens as well as severe AEs were few in all study groups[19]. In IFN-free regimens, SOF was tested in combination with the first generation NS5A inhibitor ledispavir both in treatment-naïve and pre-treated patients and showed good response in terms of SVR[20,21]. Nonetheless, among all patients, the majority had at least one, mild-to-moderate AE (fatigue, headache, insomnia, and nausea), but also serious AEs occurred in 6%-8% of patients of the 24 wk-SOF regimens[20,21]. AEs were higher and more serious in the groups concomitantly treated with RVR. Finally, some mild modifications of the haemochrome were present, whilst the increase in bilirubin and transaminases were more frequently in the group treated with RVR.

Among NNI, dasabuvir is the only drug approved and is usually administered in combination with ritonavir/paritaprevir and ombitasvir. Dasabuvir is mainly effective against HCV genotype 1. When used in combination, these three drugs showed excellent SVR rates at 12 wk in patients with HCV-1-compensated cirrhosis. Mild AEs were recorded in nearly 80% of patients, especially when treated with RVR and included nausea, fatigue, pruritus, and headache. A modest decrease of hemoglobin was reported, sometimes reaching the lower limit of the normal range[22-26]. Serious AEs were rare. However, in the post-marketing surveillance many cirrhotic were found developing hepatic decompensation and/or liver failure. For this reason, the United States FDA issued a warning in which it was noted that this treatment could cause serious liver injury in cirrhotic patients[27].

CONCLUSION

The development of DAAs revolutionized the therapeutic weapons against HCV infection. These advances have been possible thanks to the increased knowledge of the structures of HCV protease and HCV polymerase, which permitted to design structure-based drug inhibiting key viral enzymes. Indeed, HCV drug development was fast for different reasons, such as a shorter treatment duration, no need for control arms, and the short period to evaluate the SVR (12-24 wk)[28].

Since 2014, many drugs were approved: The first one was SOF, followed by simeprevir and daclatasvir. In 2015, ledipasvir in combination with SOF, and paritaprevir in combination with ombitasvir and dasabuvir were approved in several countries. In 2016/2017, three other combinations could be approved, such as grazoprevir in combination with elbasvir, SOF with VEL, and ABT-493 plus ABT-530 combination therapy[28].

The combination SOF/VEL was extensively studied in phase 2 and 3 studies. In the ASTRAL-2 and ASTRAL-3 studies, SOF/VEL showed great efficacy against HCV infections genotype 2 and 3 (except for a virologic failure after the end of treatment for 11 patients in the ASTRAL-3) and a reasonable safety[6]. The treatment of patients with HCV genotype 3 is still a challenge, even in the era of DAAs and further researches are needed to increase the rate of SVR in this subtype of patients. As the same researchers of ASTRAL-2 and ASTRAL-3 state, the generalizability of the results are limited by the small number of black patients included in the trials, considering that genotype 2 is present in sub-Saharian Africa[14]. Moreover, the exclusion of patients with decompensated cirrhosis represents another pivotal point to cope with, given the huge importance of HCV in the natural history of cirrhosis and hepatocellular carcinoma, never forgiving the extrahepatic complications, such as type 2 diabetes mellitus and cardiovascular diseases.

Further trials evaluating new DAAs should focus on the prolongation of the SVR and the inclusion of patients such as those abovementioned, which can get a real benefit from these new therapies, if safe, effective, and well tolerated.

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Could there be light at the end of the tunnel? Mesocaval shunting for refractory esophageal varices in patients with contraindications to transjugular intrahepatic portosystemic shunt

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Abstract

Cirrhotic patients with recurrent variceal bleeds who have failed prior medical and endoscopic therapies and are not transjugular intrahepatic portosystemic shunt candidates face a grim prognosis with limited options. We propose that mesocaval shunting be offered to this group of patients as it has the potential to decrease portal pressures and thus decrease the risk of recurrent variceal bleeding. Mesocaval shunts are stent grafts placed by interventional radiologists between the mesenteric system, most often the superior mesenteric vein, and the inferior vena cava. This allows flow to bypass the congested hepatic system, reducing portal pressures. This technique avoids the general anesthesia and morbidity associated with surgical shunt placement and has been successful in several case reports. In this paper we review the technique, candidate selection, potential pitfalls and benefits of mesocaval shunt placement.

Key words: Portal hypertension; Surgical portacaval shunt; Gastrointestinal hemorrhage; Esophageal and gastric varices; Transjugular intrahepatic portasystemic shunt

Core tip: Cirrhotic patients who have recurrent variceal hemorrhage despite medical and endoscopic therapy have limited options if they are not transjugular intrahepatic portosystemic shunting candidates. One promising new method to decrease portal pressures while avoiding

surgical shunt placement is mesocaval shunt placement with fluoroscopic guidance. In this paper we review the technique, candidate selection, potential pitfalls and benefits of mesocaval shunt placement.

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INTRODUCTION

Patients with cirrhosis and recurrent variceal bleeding face a high mortality, 20% in the first year *vs* 5.4% for compensated patients[1]. Current standard of care for variceal bleeding includes three primary modalities: Medical therapy with beta blockade, endoscopic therapy with ligation of varices and shunt therapy with transjugular intrahepatic portosystemic shunting (TIPS). Each of these have been shown to improve rebleeding rates and mortality[2-4].

For an unfortunate cohort of patients with varices who fail medical and endoscopic therapy and are not TIPS candidates, there are limited options in the face of a grim prognosis. Historically, these patients have been offered surgical shunt approaches, however, mortality of surgical shunt placements is high - 20%-50% if emergent - and many patients may not be suitable surgical candidates[5]. First described in 1996 by Nyman *et al*[6], mesocaval shunting may provide an alternate route to alleviate portal hypertension in these challenging patients. This paper will review the technique, candidate selection, potential pitfalls and benefits of mesocaval shunting. While there are not enough data to comment on a mortality benefit, we believe that mesocaval shunting is a feasible procedure for the prevention of variceal bleeding. It will likely be most useful for patients whose anatomy prohibits TIPS to provide a bridge to transplant.

TECHNIQUE

Mesocaval shunting involves the creation of a shunt from the mesenteric vasculature, typically the superior mesenteric vein (SMV), into the inferior vena cava (IVC). Similar to TIPS, this provides relief of portal pressures by allowing blood to bypass the congested hepatic vasculature. Shunt placement is performed by interventional radiologists. There have been both femoral and transabdominal approaches reported (Table 1)[6-9].

Fluoroscopy from a recent case of refractory variceal bleeding in a patient with a portal vein thrombus (PVT) from our institution will be used to graphically illustrate the basic technique (Figure 1). Our patient had cirrhosis and prior medical and endoscopic attempts to control her varices were limited by significant chest pain attributed to her banding procedures that required inpatient admission. Her PVT prohibited TIPS placement and she consented to undergo endovascular mesocaval shunt placement.

In our patient, and, in general, first, a needle is directed, in our case transabdominally, through the SMV, or, in this instance, a portal vein remnant, at a target placed *via* internal jugular (IJ) access (Figure 2A). Then, a wire is threaded from this needle through the IVC and out the IJ access so that, when the needle is removed, the distal tip of the the wire is in the splenic vein and its proximal end functions as a guidewire exiting the IJ access (Figure 2B). Finally, a stent graft, in our case a covered VIATORR stent, is passed over the guidewire *via* IJ access using Seldinger technique and placement is confirmed with fluoroscopic guidance (Figure 2C).

In the initial case report[6], contrast-enhanced computed tomography (CT) was first performed to define cross-sectional anatomy. The patient underwent bowel preparation pre-procedurally and was given prophylactic antibiotics as a transcolonic approach was anticipated. Using CT and fluoroscopic guidance, a needle was inserted through and through the transverse colon and SMV into the IVC to a retrieval basket. The retrieval basket had been placed in the IVC *via* the right internal jugular vein. A guide wire was then passed from the abdominal access through the SMV to IVC and jugular access. A stent was placed under angiographic guidance from the internal jugular access across the IVC to SMV and the wire was removed.

Another case report, by Moriarty *et al*[9], used similar methodology but opted for a transgastric rather than transcolonic approach to reduce the risk of infection. Interestingly, the case reported by Moriarty *et al*[9] required a cardiac transseptal needle to puncture the IVC as attempts made with a Rosch-Uchida TIPS needle were unsuccessful. The final published percutaneous approach to date was remarkable for the ability to avoid luminal puncture; Bercu *et al*[8] were able to approach transabdominally without perforating the bowel and relied on fluoroscopic rather than CT-guidance for visualization of the patient's anatomy during the procedure.

Hong *et al*[7] reported an interesting series of three cases in which they were able to place mesocaval shunts but avoid a transabdominal approach. Using techniques similar to direct intra-hepatic portosystemic shunt (DIPS) placement, they describe a series of cases in which they relied on intravascular ultrasound to avoid transabdominal puncture to access the SMV. The stent itself is extra-hepatic (and thus distinct from DIPS) and possible in patients who are not candidates for TIPS or DIPS given portal vein thrombi. In short, sheaths were placed both femorally and in the internal jugular vein. A guide wire was used to couple the jugular and femoral sheaths. Following guide wire placement, a longitudinal side-firing intravascular ultrasound (IVUS), akin to those used in placement of DIPS, was introduced through the femoral sheath so that the SMV could be cannulated using a needle introduced at the jugular access. In this

Table 1 Summary of published mesocaval shunt placements

Ref.	Case history	Details and outcomes	
Nyman *et al*[6] 1996	37-year-old male with history of recurrent massive variceal bleeds attributed to congenital PVT and failed prior surgical shunt attempt	Visualization	CT angiography
		Approach	Transcolonic
		Duration of follow-up	5, 12 and 14 mo
		Thrombosis	Yes[1]
		Recurrent bleeding	No
		Hepatic encephalopathy	NR
Moriarty *et al*[9] 2012	57-year-old male with history of metastatic CRC and extrahepatic PVT who failed prior TIPs and was thought not to be surgical candidate	Visualization	CT and fluoroscopy
		Approach	Transgastric
		Duration of follow-up	3 mo
		Thrombosis	Yes[2]
		Recurrent bleeding	Yes[2]
		Hepatic encephalopathy	NR
Bercu *et al*[8] 2015	58-year-old female with history of HCV cirrhosis, PVT with recurrent ascites who failed prior TIPs attempt and was a poor surgical candidate	Visualization	Fluoroscopy
		Approach	Transabdominal
		Duration of follow-up	3 and 6 mo
		Thrombosis	No
		Recurrent bleeding	No
		Hepatic encephalopathy	Yes[3]
Hong *et al*[7] 2012	16-year-old female with history of chronic PVT and hematemesis who was felt to have high surgical risk	Visualization	Fluoroscopy and IVUS
		Approach	Endovascular
		Duration of follow-up	1 mo
		Thrombosis	No
		Recurrent bleeding	No
		Hepatic encephalopathy	NR
Hong *et al*[7] 2012	60-year-old female with history of HBV, HCV, HCC with thrombus obliterating PV	Visualization	Fluoroscopy and IVUS
		Approach	Endovascular
		Duration of follow-up	2 and 10 mo
		Thrombosis	No
		Recurrent bleeding	No
		Hepatic encephalopathy	NR
Hong *et al*[7] 2012	53-year-old male with history of pancreatic teratoma treated with Whipple with clot at SMV and splenic veins	Visualization	Fluoroscopy and IVUS
		Approach	Endovascular
		Duration of follow-up	1 and 3 mo
		Thrombosis	No
		Recurrent bleeding	No
		Hepatic encephalopathy	NR

[1]The shunt was found to be thrombosed on POD #1 so the patient underwent ballooning of his stent and directed thrombolysis and was started on a therapeutic heparin. His hematocrit began to fall on the heparin but stabilized when the anticoagulation was held; [2]The shunt was found to be thrombosed on POD #2 and on POD #3 the patient had a recurrent upper gastrointestinal hemorrhage. A new shunt was placed and the patient had no further bleeding; [3]The patient had no encephalopathy at a 3-mo follow-up visit but was noted by an outside hospital to have encephalopathy 6 mo after shunt placement when the patient was hospitalized for concern for partial small bowel obstruction. The patient's home lactulose and rifaximin had been held; when these medicines were resumed her encephalopathy resolved. NR: Not reported; PVT: Portal vein thrombus; CRC: Colorectal cancer; TIPs: Transjugular intrahepatic portosystemic shunting; HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; SMV: Superior mesenteric vein; PV: Portal vein; CT: Computed tomography.

way, they were able to avoid a percutaneous transabdominal approach altogether. It should be noted that the third patient included in this series was not a cirrhotic patient but rather had portal and SMV clots due to a pancreatic tumor; we chose to include this patient in our review to demonstrate the feasibility of the procedure but appreciate that his underlying pathophysiology may be different from the others presented.

SELECTION OF CANDIDATES

Candidates likely to benefit from mesocaval shunting include those with recurrent variceal bleeds who have failed prior medical and endoscopic therapies. Traditionally, TIPS has been employed in these patients to alleviate portal pressures. We propose that mesocaval shunting be offered to patients who are not TIPS candidates, particularly the group awaiting transplant, as there are not yet mortality data for mesocaval shunting and the mortality benefit of other portosystemic shunts, including TIPs, has been questioned[10].

As in our illustrative case, PVT, for example, are known to make TIPS more difficult and result in lower success rates, ranging from 40%-75%[11]. In some cases, when PVT is chronic, TIPS is not only difficult but actually technically impossible as in order to re-establish flow, there must be functional vessels surrounding the planned recanalized clot segment. This intact vasculature is often absent in those with chronic PVT as intrahepatic vessels may have atrophied while extrahepatic vessels form collaterals at high risk of bleeding[6]. Given the relatively high prevalence of PVT in cirrhotics, up to 5% to 16% of patients at the time of liver transplantation, mesocaval shunting has the potential to offer a therapy

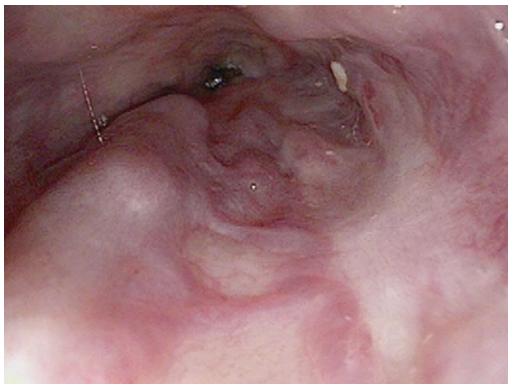

Figure 1 Grade Ⅲ varices in distal esophagus on pre-procedure esophagogastroduodenoscopy.

to a large group of patients who were previously thought to be without options, particularly in those patients whose PVT prohibits them from receiving a liver transplant[12].

Prior to endovascular placement of mesocaval shunts, the other option for patients in this scenario was surgical shunt placement. Historically, surgically placed portosystemic shunts have had high mortality[5]. While experienced centers are reporting improved operative mortality[13,14], the ability to replicate these lower mortality rates at smaller, less experienced centers remains to be seen. Furthermore, several of the patients in published percutaneous mesocaval shunt cases to date were thought to be poor surgical shunt candidates due to a history of prior abdominal surgeries and/or anatomy of their PVT[6,8,9].

If a patient is felt to be appropriate for consideration of mesocaval shunt placement, assessment of cross-sectional anatomy should be undertaken with computed tomography or magnetic resonance imaging of the abdomen to assist in procedural planning. For successful shunt placement, the IVC and SMV should be aligned and proximal in an anatomic window without any significant vasculature or viscera interposed between the two vessels[8]. The IVC and SMV (or a large collateral) must be patent for shunt placement.

POTENTIAL PITFALLS

There are a few potential pitfalls we consider with placement of a mesocaval shunt. First, similar to the surgical expertise required for safe surgical shunt placement, institutional interventional radiologic expertise will be required to safely recommend this procedure and this may not be available at all centers. This procedure, unlike TIPS, has not been reported to be performed in the setting of active variceal bleeding and thus there are no data to support its safety in that setting.

The most serious risk is that of procedure-related hemorrhage due to puncture of proximal vasculature. As noted by Hong *et al*[7] both the SMV and infrahepatic IVC lack any surrounding solid organs that could provide tamponade during shunt placement, creating a risk of major hemorrhage. Of the published cases to date, one noted intraabdominal hemorrhage-multiple small bowel hematomas - which was successfully treated conservatively with intravenous fluid, transfusion support and discontinuation of anticoagulation[6]. If the cannulated vesels require predilation prior to shunt placement, this risk of bleeding is likely increased[7]. Finally, while they did not experience intrabdominal hemorrhage despite use of an uncovered stent, Moriarty *et al*[9] note that use of an uncovered stent certainly increases risk of bleeding and recommend using covered stents and/or balloons to minimize this risk. In addition to procedural technique, we anticipate that, similar to other invasive procedures in cirrhotic patients, platelet counts influence the risk of hemorrhage. The cases reviewed here unfortunately do not provide patient platelet counts or other measures of clotting function.

In addition to the potential for vessel perforation, depending on each individual patient's anatomy, there are risks of perforation of different structures. If an intestinal perforation is created, risk of sepsis, hemorrhage and/or abscess formation will certainly be increased[8]. In two reported cases, the transabdominal approach necessitated intestinal puncture[6,9]. In one case the track was transcolonic while the other approach was transgastric. Bowel preparation and antibiotic prophylaxis were administered in the transcolonic case and neither case resulted in sepsis. Although there were no reported infectious complications in the cases we reviewed, this risk should be underscored as it is likely not negligible in cirrhotic patients with impaired immunity. In addition to the risk of intestinal perforation, other nearby viscera are at risk of puncture as well. If the pancreas is punctured, both hemorrhage and pancreatitis are potential risks[8]. One published case to date notes pancreatic bisection and reports that serum amylase levels were within the normal range for at least five days post-operatively[7].

In the six cases reviewed, two cases reported subsequent shunt occlusion and need for further revision, a rate of 33% in our, appreciably, small series. Shunt thrombosis is an important outcome as it presumably puts the patient at risk for further portal hypertension, variceal formation and variceal bleeding. In the first of the two cases complicated by occlusion, lack of flow through the shunt was noted on both Doppler and CT on POD #2[6]. The patient underwent repeat angiography and had ballooning and directed thrombolysis of his stent with subsequent patency on 5 mo follow-up angiogram. The stent was again noted to be occluded on 12 mo follow-up angiography but he had no further gastrointestinal bleeding and no attempt to revise the shunt further was made. In the second case complicated by shunt occlusion, lack of flow through the shunt was noted on POD #2 on CT[9]. This was attributed to the severe angle of the initial shunt placement and its proximity to the wall of the IVC. This patient experienced upper gastrointestinal bleeding on POD #3 and underwent repeat angiography with stent replacement and had a patent stent and no further bleeding at 3 mo follow-up.

Finally, as with any form of portosystemic shunting, we anticipate that these patients will have higher rates

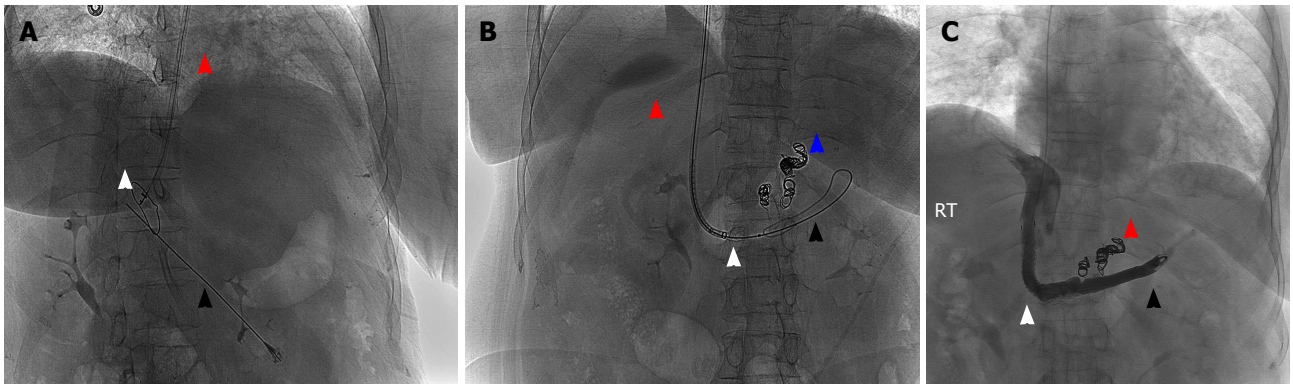

Figure 2 Intra-procedure steep oblique fluoroscopy of upper abdomen during mesocaval shunt placement. First, a needle is inserted percutaneously (black arrowhead) and directed through the portal vein remnant at a target snare placed in the inferior vena cava (white arrowhead) *via* internal jugular (IJ) access sheath (red arrowhead) (A). The wire is then threaded from its original percutaneous entry *via* the needle through the IJ sheath (red arrowhead) so that it extends from the IJ and is seen coiling in the splenic vein (black arrowhead) (B). The unexpanded stent graft (white arrowhead) is passed over the wire using Seldinger technique with fluoroscopic guidance (B). Coils are placed in varices (blue arrowhead) (B). Shuntogram with contrast 22 mo post-procedure shows functioning mesocaval shunt (white arrowhead) with tip in the splenic vein (black arrowhead) and absent varices (C). Previous coils in the varices are still visible (red arrowhead).

of hepatic encephalopathy (HE) than patients without portosystemic shunting. Given the limited numbers of patients who have undergone percutaneous or endovascular mesocaval shunt placement, there are no data to evaluate rates of HE with these shunts *vs* TIPS or surgical shunt creation but presumably the rate is similar, around 30%[15,16]. In the cases reviewed above, only one case reported on the presence or absence of encephalopathy. In that case, the patient was noted to have no encephalopathy during index hospitalization or at 3 mo follow-up but was noted to be encephalopathic 6 mo post-operatively when her lactulose and rifaximin were held at an outside hospital for partial small bowel obstruction[8]. Her encephalopathy reportedly resolved with resumption of these medications.

BENEFITS

As above, mesocaval shunting offers a treatment for bleeding varices for patients who otherwise face a high mortality with virtually no options. It can be offered to patients with PVT who cannot undergo TIPS and may be best utilized as a bridge to transplant. Furthermore, if an IVUS is utilized, vessels are directly visualized, avoiding the blind puncture method used in TIPS[7]. As with other similar endovascular procedures, we anticipate a lower mortality with this less invasive approach *vs* surgical shunt placement. Regardless, the majority of the published patients to date were not felt to be surgical candidates[6,7,9].

In the six adult cases published to date, two stents thrombosed within two days post-operatively while the remaining four remained patent[6,9]. Of the two patients with shunt thrombosis, one had a recurrent upper gastrointestinal bleed. In this case, it was postulated that the severe angle of the initial stent placement may have contributed to turbulence and subsequent thrombosis[9]. In both cases, subsequent shunt revision was performed and the revised shunts remained open during five and three months follow-up respectively. One shunt ultimately lacked patency at 12 mo follow-up but the patient had no further bleeding up to 14 mo follow-up. In summary, all reported cases have follow-up ranging from 1 to 14 mo in which, with the exception of the post-operative day 2 bleed noted above, there were no further variceal bleeding episodes. These are promising results in light of the known 60% 1-year risk of rebleeding and 33% 1-year mortality in patients who survive an episode of variceal hemorrhage[17].

In addition to offering a rescue therapy for a group of patients with minimal options, mesocaval shunting has an advantage compared to local variceal therapy, it will result in lower portal pressures and thus will reduce recurrent ascites as well reducing the risk of variceal bleeding. As noted by Garcia-Tsao and Bosch in a recent review, judgment of treatment success of varices should include mindfulness about the impact of variceal treatment on other complications of portal hypertension-ascites, jaundice, encephalopathy - rather than artificially isolating the treatment's impact on variceal bleeding alone[17]. Finally, given that the presence of portal vein thrombosis is no longer thought to be an absolute contraindication to transplant[18], for those patients that are transplant eligible, placement of a mesocaval shunt may enable survival to the operating room table for transplant, a pressing concern given that our most recent national statistics are dire. In 2014, 1821 patients died while awaiting transplant and an additional 1290 were removed from the waiting list as they were felt to be "too sick" for transplant[19].

CONCLUSION

As reviewed above, endovascular mesocaval shunting is a feasible procedure that offers a promising intervention to a patient population with few options and one-year mortality as high as 20%[1]. TIPS has been shown to be an effective intervention to prevent recurrent variceal

bleeding[2] and mesocaval shunting provides similar physiologic relief of portal pressure in patients who are not TIPS candidates. Like TIPS, mesocaval shunting avoids major surgery and may require less anesthesia than a surgical shunt approach. Furthermore, it can be offered to patients who are not surgical candidates. Mesocaval shunting alleviates portal hypertension, a key component of reducing the rate of variceal bleeding, and one that will potentially reduce recurrent ascites as well. The patient who stands to gain the most from this procedure has recurrent variceal bleeds, has failed endoscopic and medical therapies, cannot undergo TIPS due to anatomy and needs a bridge to transplant to minimize the chance of further decompensating while awaiting an organ. In order to have successful shunt placement, these patients must have alignment between IVC and SMV or SMV collaterals. Potential procedural complications include perforation of nearby vessels or viscera which could result in hemorrhage, sepsis, pancreatitis or abscess formation as well as stent thrombosis. Placement of a portosystemic shunt will also increase the risk of hepatic encephalopathy although there is little data to compare mesocaval shunts to surgical shunts or TIPS. To date, several approaches and imaging techniques have been utilized by reporting groups, notably including one approach that avoids transabdominal puncture[7]. In the cases reported, all have prevented rebleeding for the post-procedural monitoring period after initial shunt or initial shunt revision[6,8,9]. Further research should be performed to better assess outcomes - variceal bleeding, hepatic encephalopathy rates and mortality - in these patients compared to standard-of-care controls so that the benefits of this promising technique may be maximized.

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Authors Jessica Davis, Albert K Chun and Marie L Borum have all contributed to this manuscript and endorse the data and conclusions within.

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ORIGINAL ARTICLE

Basic Study

Assembly and release of infectious hepatitis C virus involving unusual organization of the secretory pathway

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Author contributions: Triyatni M designed and performed experiments and analyzed the data; Berger EA provided facilities and resources for research, analyzed the data, and contributed to editing the manuscript; Saunier B designed most and performed some experiments, analyzed the data and wrote the manuscript.

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Conflict-of-interest statement: Saunier B, Triyatni M and Berger EA are co-inventors on NIH-owned patent #US 9052321 B2 on the HCV particle production system.

Data sharing statement: No additional data are available.

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Abstract

AIM: To determine if calnexin (CANX), RAB1 and alpha-tubulin were involved in the production of hepatitis C virus (HCV) particles by baby hamster kidney-West Nile virus (BHK-WNV) cells.

METHODS: Using a siRNA-based approach complemented with immuno-fluorescence confocal microscope and Western blot studies, we examined the roles of CANX, RAB1 and alpha-tubulin in the production of HCV particles by permissive BHK-WNV cells expressing HCV structural proteins or the full-length genome of HCV genotype 1a. Immuno-fluorescence studies in producer cells were performed with monoclonal antibodies against HCV structural proteins, as well as immunoglobulin from the serum of a patient recently cured from an HCV infection of same genotype. The cellular compartment stained by the serum immunoglobulin was also observed

in thin section transmission electron microscopy. These findings were compared with the JFH-1 strain/Huh-7.5 cell model.

RESULTS: We found that CANX was necessary for the production of HCV particles by BHK-WNV cells. This process involved the recruitment of a subset of HCV proteins, detected by immunoglobulin of an HCV-cured patient, in a compartment of rearranged membranes bypassing the endoplasmic reticulum-Golgi intermediary compartment and surrounded by mitochondria. It also involved the maturation of N-linked glycans on HCV envelope proteins, which was required for assembly and/or secretion of HCV particles. The formation of this specialized compartment required RAB1; upon expression of HCV structural genes, this compartment developed large vesicles with viral particles. RAB1 and alpha-tubulin were required for the release of HCV particles. These cellular factors were also involved in the production of HCVcc in the JFH-1 strain/Huh-7.5 cell system, which involves HCV RNA replication. The secretion of HCV particles by BHK-WNV cells presents similarities with a pathway involving caspase-1; a caspase-1 inhibitor was found to suppress the production of HCV particles from a full-length genome.

CONCLUSION: Prior activity of the WNV subgenomic replicon in BHK-21 cells promoted re-wiring of host factors for the assembly and release of infectious HCV in a caspase-1-dependent mechanism.

Key words: Membrane rearrangements; Hepatitis C virus; Flavivirus replicon; Virus assembly and secretion; Host cellular factors

Core tip: Our system for production of authentic infectious hepatitis C virus (HCV) in non-humanized, non-hepatic cells involves the rearrangement of inner cellular membranes triggered by the replication of flaviviruses. The present results suggest that this feature relies on the re-wiring of host factors that usually contribute to the secretion of glycoproteins to generate an unusual secretory pathway. This model offers a new way to study the properties of free HCV particles, *i.e.*, independently from lipoproteins.

Triyatni M, Berger EA, Saunier B. Assembly and release of infectious hepatitis C virus involving unusual organization of the secretory pathway. *World J Hepatol* 2016; 8(19): 796-814 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i19/796.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i19.796

INTRODUCTION

Hepatitis C virus (HCV) genotype 1 has accounted for up to 70% of HCV infections worldwide and is more often resistant than the other genotypes to the combination of pegylated interferon plus ribavirin, the standard of care until recently[1]. New antiviral drugs specifically target either the viral protease NS3, the protein NS5A or the RNA polymerase NS5B and are able to clear HCV infections in a higher number of individuals with a shorter duration of treatment than the standard of care[2]. However, a significant proportion of patients requiring treatment still cannot be cured[3], does not have access to treatment[4] and/or will need the development of a vaccine[5]. Experimental models are being developed[6], but may not cover all aspects of the pathogenesis of hepatitis C, such as the mechanism by which antibodies prevent the spread of infection[7].

In an effort to develop alternative systems, we have established a model in which infectious HCV production is independent from its replicon-mediated RNA replication[8], hence circumventing limitations inherent to existing cell culture models[9]. For instance, the replication of HCV genotype 1 isolates in hepatocellular carcinoma cell lines is inefficient or generates adaptive mutations interfering with viral fitness *in vivo*. Briefly, our system relies on the amplification provided by a dual bacteriophage RNA polymerase plasmid system (referred to as P2B) that generates large amounts of HCV RNA transcripts from a T7 promoter-driven plasmid in the cytoplasm of baby hamster kidney (BHK)-21 cells conditioned by a lineage Ⅱ West Nile virus (WNV) subgenomic replicon[8]. We observed that the WNV replicon in this cell line created an environment permissive for the assembly and release of infectious HCV of various genotypes, including virions of strains H77 (genotype 1a)[10] or Con1 (genotype 1b)[11]; these virions infected human liver slices *ex vivo*[8].

BHK-WNV cells produce infectious HCV particles independently from lipoprotein biosynthesis. The fact that these particles retain the possibility to interact with lipoproteins *in vitro*[8] is in line with previous results[12-14] and supports the view that HCV particles may interact with lipoproteins in a second step (*e.g.*,[15,16]) and not necessarily co-assemble with them (*e.g.*,[17]). Potential mechanisms for WNV-conditioned BHK cells producing highly infectious HCV virions could relate to common genomic features between the *Flavivirus* and *Hepacivirus genera* within the *Flaviviridæ* family[18]. In addition, several flaviviruses infect hepatocytes[19,20] and may use similar host factors as HCV for their production[21-25].

Our previous results showed that after curing BHK cells from the WNV subgenomic replicon, the production and release of infectious HCV particles were still observed for a while[8]. In addition, although recombinant expression of HCV structural genes in cultured cells, including in human hepatocytes[26], usually leads to their retention in the endoplasmic reticulum (ER)[27], BHK-WNV cells released infectious HCV particles even in the absence of HCV non-structural genes[8]. These findings suggested that, while the viral replication machineries played no direct role in the secretion process, the reorganization of intracellular membranes induced by the WNV subgenomic replicon contributed to the permissiveness

of BHK cells.

In mammalian cells, conventional protein traffic from the ER to the Golgi complex passes through the membrane clusters of the ER-Golgi intermediate compartment (ERGIC), the marker of which is the lectin ERGIC-protein of 53 kDa (ERGIC-53). ER-derived cargo is first shuttled to the ERGIC in a coat protein (COP) Ⅱ-dependent step and subsequently to the Golgi apparatus in a second vesicular transport step involving COP Ⅰ-coated vesicles, RAB and ARF GTPases, as well as cytoskeletal networks; incoming vesicles can also be recycled to the ER in a COP Ⅰ-mediated process[28]. The ERGIC contributes to the concentration, folding, and quality control of newly synthesized proteins and is required for the production of several viral pathogens[29]. N-linked glycosyl antenna are matured by Golgi-resident enzymes along with glycoproteins' progression from the proximal to the distal Golgi *cisternæ*, then across the plasma membrane for their secretion, *via* the *trans*-Golgi/endosomal network.

In the present work, we studied the potential involvement of components of this secretory machinery in the production of HCV particles by BHK-WNV cells and compared this model with the JFH-1 strain/Huh-7.5 cells model. We show that, upon expression of an HCV genome of genotype 1a in these cells, a subpopulation of HCV proteins were recruited through calnexin (CANX) to a cytoplasmic compartment of rearranged membranes. The small GTPase RAB1 was involved in the formation of this compartment. The secretion of HCV particles produced from a full-length genome required also the N-linked glycosylation of HCV envelope glycoproteins and alpha-tubulin (α-TUB), a component of microtubules and, surprisingly, the activity of cysteine protease caspase-1. As our understanding of the HCV virus life cycle has recently widened to alternative routes of transmission, elucidating mechanisms at work in BHK-WNV cells could shed some light on the production of HCV *in vivo*.

MATERIALS AND METHODS

Cell cultures

BHK-21 cells were grown in E-MEM supplemented with 10% fetal bovine serum (FBS; HyClone, United States), Glutamax-I (Gibco, Life Technologies, United States); BHK cells harboring WNV lineage Ⅱ SG-replicon encoding *Renilla* luciferase[30], herein called BHK-WNV cells, were propagated in D-MEM supplemented with 10% FBS, Glutamax-I and 5 μg/mL blasticidin. Huh-7.5 cells were maintained in D-MEM supplemented with 10% FBS, Glutamax-I, non-essential amino acid mix (Gibco, Life Technologies, United States).

Plasmid constructs

A previously described system of two plasmids (P2B = dual phage RNA polymerases plasmid system for generation of T7 RNA polymerase in the cytoplasm) was used to amplify the cytoplasmic transcription of a plasmid encoding HCV bicistronic particles (HCVbp) under the control of bacteriophage T7 DNA-dependent RNA polymerase's cognate promoter[8]; a sequence encoding an HDV antigenomic ribozyme[31] was added at its C termini; as a consequence, HCV transcripts were uncapped and have correct 5'- and 3'-ends. p90 HCVcon-FLlongpU[10] and pH-SGNeo (L + I) encoding a SG-replicon of the same strain with cell-culture adaptive mutations[32] were used as templates to construct HCVbp-coding plasmids. pHCV STp7 is a pcDNA3.1(+)-based plasmid (Life Technologies, United States) encoding the structural genes (*core*, *E1*, *E2*) plus *p7* of HCV genotype 1a linked to the human cytomegalovirus (CMV) immediate early promoter. HCVbp-4cys are HCVbp particles with a sequence encoding a tetracysteine tag[33] inserted in the part of their genome encoding NS5A[8].

Antibodies and cellular markers

Anti-E2 (ALP98 and AP33)[34], and anti-E1 (A4) monoclonal antibodies were used for Western blot (WB) analysis. Conformational AP33 monoclonal antibody, human serum from an HCV patient (genotype 1a) that recognizes conformational HCV core and E2 protein subspecies by WB and anti-HCV core peptides 9-21 (C1) or 7-50 (Thermo Scientific, United States) monoclonal antibodies were used for confocal microscopy analysis. Neutralizing monoclonal antibody E16 recognizes WNV E by WB[35]. Antibodies against various cellular proteins are as followed: ERGIC-53 (Alexis Biochemicals, United States), GDI (Life Technologies, United States); RAB1 and atlastin (Santa Cruz Biotechnology, United States); CANX and GM130 (Abcam, United States); and calreticulin (Cell Signaling Technology, United States). For immunofluorescence analysis, the secondary antibodies used were Alexa Fluor 488-, 594- or 635-conjugated goat anti-mouse and anti-human antibodies, and Alexa Fluor 594-, 635- or -680 conjugated goat anti-rabbit antibodies from Molecular Probes (Life Technologies, United States). Mito Tracker Orange CMTMRos, TC-ReASH Ⅱ In-Cell Tetracysteine Tag detection kit and Paclitaxel (Taxol) Oregon Green® 488 Conjugate were obtained from Molecular Probes (Life Technologies, United States). Nuclei were counterstained with DAPI (blue). The cells were observed with a laser-scanning confocal microscope and the pictures were deconvoluted. Bar scales = 5 μm.

Production of HCV particles in mammalian cells

One day before transfection, BHK-WNV cells were seeded at a density of 6×10^6 cells per 162 cm^2 flask. Plasmids encoding the HCV sequence under the control of the CMV early promoter or the bacteriophage T7 promoter were transfected using Lipofectamine® LTX with Plus™ Reagent according to the manufacturer's protocol (Life Technologies, United States). Culture medium after transfection was D-MEM supplemented with 10% FBS, non-essential amino acid mix, Glutamax-I, 25 mmol/L Hepes and 3.7 g/L sodium bicarbonate. Cells were incubated at 37 ℃ for 3 d in an incubator with a 95% air/5% CO_2 atmosphere saturated in humidity. Culture media were harvested, centrifuged at 30000 × g for 30

min at 4 ℃ to remove cell debris; then clarified supernatants (SN) were filtered with 0.45 μm PVDF membrane (Millipore, United States) and centrifuged at 100000 × g for 3 h at 4 ℃. Pellets were suspended in ice-cold Tris-buffered saline solution (TBS; Quality Biologicals, United States) containing protease inhibitor cocktail (Roche, United States). HCVcc (Huh-7.5-produced JFH-1) was obtained by electroporating IVT RNA into Huh-7.5 cells as described[36]; the SN was concentrated, and aliquots of the virus stock were stored at -80 ℃.

Gene knockdown in BHK-WNV cells using siRNAs

CANX, melanocortin 5 receptor (MC5R), α-TUB were analyzed for their effects on HCV release. BHK-WNV cells were treated with the corresponding siRNA (2 siRNAs per target gene; Dharmacon, United States) for 2 d, re-seeded and transfected the next day with HCV-coding plasmid. Cells and supernatants were harvested 48 h later, and analyzed by WB. To verify the effects of these genes on HCV release, CANX and α-TUB cDNAs were synthesized from BHK-WNV mRNA, and cloned into pTracer-CMV/Bsd (Invitrogen, United States). The corresponding pTracer plasmid was co-transfected with HCV-coding plasmid in siRNA-treated BHK-WNV cells to show the specificity of their knockdown.

Effect of RAB1 on HCV production by BHK-WNV cells

To study co-localization of HCV and RAB1 in the producer cells, BHK and BHK-WNV cells were transfected with HCVbp-coding plasmid, and the following day, re-seeded on 8-well chambered coverglass (5×10^3 cells/well). Two days later, cells were fixed and permeabilized as above, then incubated with serum from an HCV-infected patient (HCV genotype 1a) and anti-RAB1 antibodies. BHK-WNV cells were then treated with siRNA against RAB1 (2 siRNA per target gene; Dharmacon, United States) for 2 d, re-seeded, and the following day were transfected with HCVbp-coding plasmid. Cells and SN were harvested 3 d after transfection; cell lysates and particles released into SN were analyzed by WB.

Electron microscopy

BHK-WNV cells seeded in a 6-well plate (2.5×10^5 cells) were transfected with HCVbp-coding plasmid. Three days later, cells were fixed in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate for 1 h at RT, then at 4 ℃, overnight. Cells were subsequently processed for transmission electron microscopy (TEM) as described[37].

Effects of siRNAs on HCVcc production and release in Huh-7.5 cells

Cells were treated with the siRNAs (CANX, α-TUB, RAB1A, RAB1B, or non-target) for 48 h; the same number of cells was reseeded on 24-well plates, and the next day was inoculated with HCVcc at MOI 0.5. Cells were harvested daily, total RNA was extracted using lysis buffer of TaqMan Gene Expression Cells-to-ct kit (Life Technologies, United States) and HCV RNA was analyzed by RT-TaqMan™ PCR with a StepOne Plus thermocycler (Applied Biosystems, United States).

IF experiments

For live staining after inoculation with HCVbp-4cys, Huh-7.5 cells were incubated with two cell-permeant reagents: The arsenical ReASH-ethane dithiol (Life Technologies) that fluoresces upon binding a tetra-cysteine tag[33], and Oregon Green 488 paclitaxel bis-acetate (TubulinTracker Green Reagent; Life Technologies, United States) that binds to polymerized alpha tubulin. Immuno-fluorescence studies on fixed cells were performed on stacks of images (a dozen cells per IF condition); co-localization was analyzed using Coloc 2 Plugin (http://imagej.net/Coloc_2) with ImageJ software (https://imagej.nih.gov/ij/).

Experimental reproducibility

Unless specified otherwise, all shown results are representative of at least three independent experiments.

RESULTS

Identification of a compartment possibly linked to the assembly of HCV particles in the cytoplasm of BHK-WNV cells

To gain insight regarding the mechanisms by which BHK-WNV cells produce HCV particles, we transfected the cells with either of three different plasmids based on HCV genotype 1a (HCV1a): (1) HCV structural and *p7* genes[38] linked to a CMV promoter (Str + p7); (2) HCV subgenomic replicon (SG repl); or (3) full-length HCV genome (FL) linked to the bacteriophage T7 promoter; for (2) and (3), the cells were also co-transfected with the P2B (dual phage RNA polymerases) plasmid system for generation of T7 RNA polymerase in the cytoplasm. The cells were then permeabilized and incubated with monoclonal antibodies (mAbs) against HCV1a core (C1), E1 (A4) and E2 (AP33) or a control mAb. Like in hepatic cells expressing a HCV genome[22], we observed that the three HCV1a mAbs stained a large and heterogeneous area in the cytoplasm of BHK-WNV cells expressing the structural genes, while the control mAb did not stain these cells (Figure 1A). AP33 is a neutralizing mAb that targets a linear epitope within a flexible region of E2[34] and, as expected, did not stain cells not expressing HCV1a structural genes (Figure 1A). In contrast, a neutralizing IgG from the serum of a patient (HCV1a sIgG) who had been cured from hepatitis C of this genotype[8] stained a more limited and heterogeneously shaped compartment of the cytoplasm of BHK-WNV cells expressing a full-length genome of HCV1a, which was also stained by AP33 but not by control human sIgG (Figure 1A); of note, HCV1a sIgG did not stain BHK-WNV cells expressing a HCV1a subgenomic replicon. A mitochondrion-specific staining surrounded this compartment (Figure 2A). By WB, beside non-specific bands (NS), HCV1a sIgG specifically recognized bands with apparent molecular weights of 21 kDa and 75 kDa (Figure 1B) whose sizes coincided with those reported for HCV core and E2, both known to

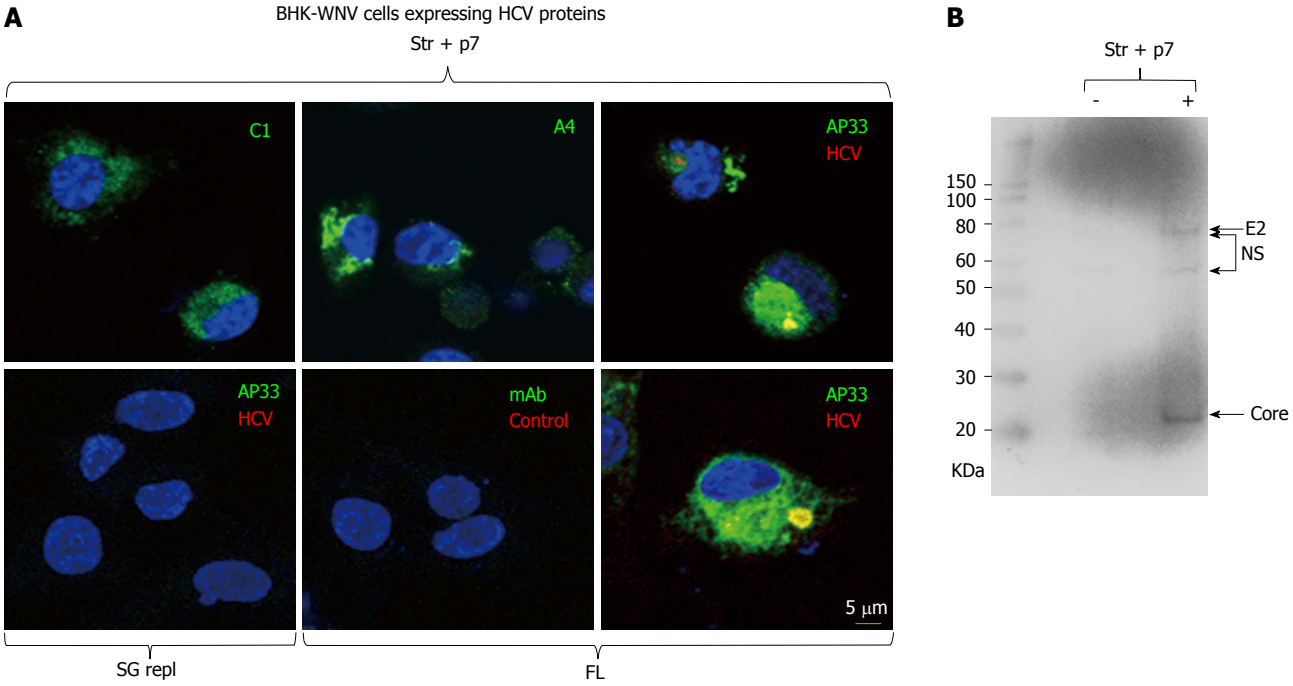

Figure 1 Immunodetection of hepatitis C virus proteins of genotype 1a expressed in baby hamster kidney-West Nile virus cells. A: A plasmid encoding Str + p7 of HCV strain H77 (genotype 1a) from an early human cytomegalovirus promoter or a system of plasmids (P2B) expressing a subgenomic replicon or genome (FL) of same genotype in the cytoplasm (8) were transfected in BHK-WNV cells; after 2 d, IF study was performed with monoclonal antibodies (green) targeting core 9-21 (C1), envelope E1 (A4) and E2 (AP33) (28) glycoproteins of strain H77 (all IgG1a), or anti-rabbit IgG mouse Ab (mAb) of same isotype; and with human serum IgG (red) obtained from a patient recently cured from an infection of same genotype (HCV) or uninfected (control). Nuclei were counterstained with DAPI (blue) and cells were observed with a laser-scanning confocal microscope; B: BHK-WNV cells were either mock transfected (-) or transfected with a system of plasmids expressing the genome of H77 strain (+); after 3 d, cell lysates were prepared and human anti-HCV IgG tested in (a) were used as a Western blot probe. The scale on the left shows molecular weight markers. HCV: Hepatitis C virus; BHK-WNV: Baby hamster kidney-West Nile virus; Str + p7: Structural and *p7* genes.

display most HCV epitopes recognized by human B cell (http://www.iedb.org/).

To further characterize the cytoplasmic compartment stained by HCV1a sIgG, thin sections of BHK-WNV cells expressing HCV genome of genotype 1a were observed by TEM. The sub-compartment was easily identified due to the presence of mitochondria in its periphery (Figure 2B)[39]. As previously, it was comprised of large vesicles containing viral-like particles - not observed in cells expressing an HCV1a subgenomic replicon, *i.e.*, without structural genes - as well as membranous web with vesicle packets, both typical of membrane rearrangements triggered by WNV replication[8]. This time, we also observed a mix of rearranged cellular organelles (ER, Golgi *cisternæ*) with a few additional viral-like particles (Figure 2B). Altogether, these results support the view that HCV particles assembled in BHK-WNV cells were specifically recognized by sIgG from a cured patient.

CANX recruits a subset of HCV proteins into a compartment of BHK-WNV cells for the release of HCV particles

Classically, the secretion of glycoproteins involves lectins of the ER, CANX and calreticulin (CRT), microtubule-dependent ER-to-Golgi vesicular traffic[40,41], maturation of their carbohydrates in the Golgi apparatus and the secretion machinery of the trans-Golgi network[42]. We had previously observed that brefeldin A prevented the secretion of HCV by BHK-WNV cells, while it did not affect that of exosomes[8]. Brefeldin A is an inhibitor of GBF1 that activates small-sized GTPase ARF1 and, in turn, COPI-dependent traffic[43]. The effect of brefeldin A was consistent with HCV particles being released *via* a classical secretion path. Two components of the classical secretion path, CANX and microtubules, have been previously implicated in HCV life cycle in other systems[27,44,45]. Since what appeared to be the assembly compartment displayed major membrane rearrangements, we explored whether a classical secretion path was involved in the production of HCV particles by BHK-WNV cells.

In BHK-WNV cells expressing an HCV1a genome, most CANX was unexpectedly detected in the same compartment as HCV proteins stained by HCV1a sIgG (Figure 3A, left panels). After a treatment of BHK-WNV cells by CANX siRNA, which decreased the level of CANX (Figure 3A, right panels, and Figure 3C, top panels), HCV1a sIgG staining was no longer localized in this compartment but, instead, displayed a scattered pattern in the cytoplasm (Figure 3A, right panels). The effect of knocking down CANX and α-TUB expression on HCV production was analyzed. Figure 3B shows WB analysis of E2 glycoprotein in particles purified from the supernatants and cell lysates as a marker for HCV production. Treatment of BHK-WNV cells by control or

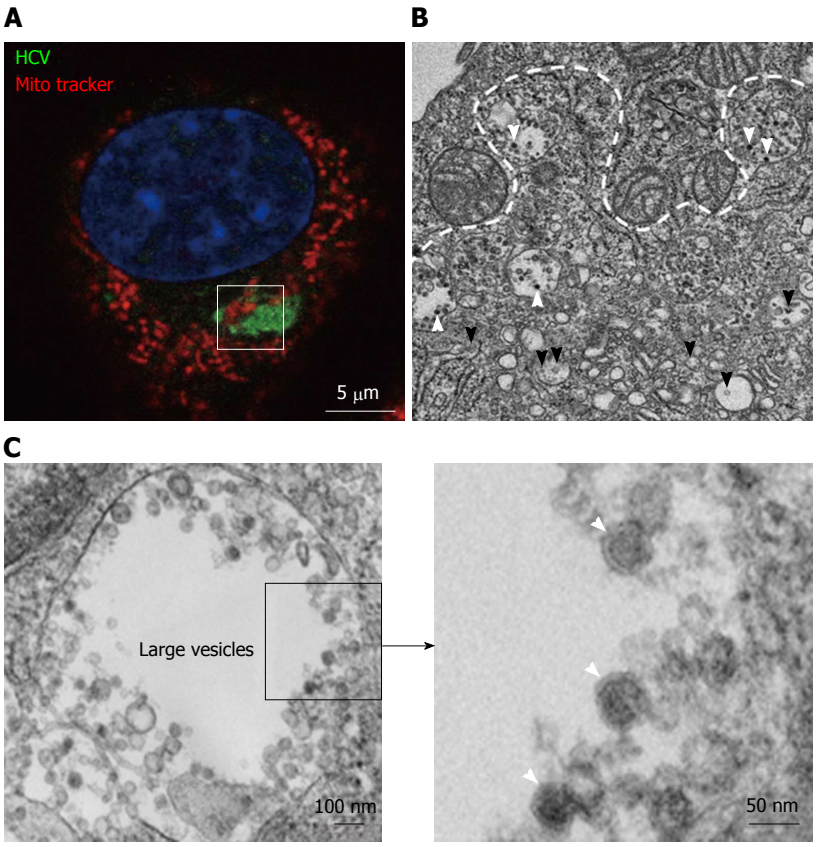

Figure 2 Transmission electron microscopy view of a hepatitis C virus compartment forming in baby hamster kidney-West Nile virus cells cells. BHK-WNV cells were transfected with a mix of HCVbp-expressing and P2B plasmids (8). A: IF analysis showing a compartment recognized by human serum HCV IgG (green) surrounded by mitochondria (red); nucleus is counterstained with DAPI (blue). The white square delimits an area similar to that displayed in the next panel; B: Thin section observed by transmission electron microscopy: White arrowheads: Electron-dense viral particles in large vesicles; black arrowheads: Nascent viral particles in traffic vesicles; white dotted line: Limit of mitochondria surrounding the compartment containing viral particles (cf. area within white square in previous panel); C: Left panel: Example of large vesicle that develops in the cytoplasm of permissive BHK-WNV cells upon expression of full-length HCV genome; Right panel: Magnification of viral particles. HCV: Hepatitis C virus; BHK-WNV: Baby hamster kidney-West Nile virus.

MC5R siRNA did not alter the production or release of HCV (Figure 3B). In contrast, siRNA-mediated down-regulation of CANX or α-TUB significantly reduced the secretion of HCV (Figure 3B). Albeit incomplete, the siRNA effect on CANX expression was sufficient to abolish BHK-WNV permissiveness for HCV production (Figure 3C, top panels).

Hsp70 or Hsp90 were chosen as controls since they associate with exosomes[46,47], which are particulate materials secreted by BHK cells yet distinct from HCV particles[8]. After a treatment of BHK-WNV cells with α-TUB siRNA, the releases of Hsp70- and HSp90-containing particles in the supernatant decreased (Figure 3B and C, bottom panels), in accordance with the requirement for a functional cellular traffic to release exosomes and consistent with an accumulation of Hsp70 in the producer cells. In contrast, HCV E2 did not accumulate in BHK-WNV cells (Figure 3B).

To evaluate whether inhibition of HCV release could result from an off-target effect, CANX and α-TUB cDNAs cloned from BHK-WNV cells were inserted into mammalian expression plasmids. In BHK-WNV cells treated by CANX siRNA, expression of recombinant *CANX* gene partially restored both CANX levels (Figure 4) and HCV secretion (Figure 3C, middle panels). A similar result was observed with α-TUB cDNA expressed in α-TUB siRNA-treated BHK-WNV cells (Figure 3C, middle panels). The release of WNV particles upon expression of WNV structural genes was minimally affected by either siRNA treatment (Figure 3C, bottom panels). These results show that HCV production in BHK-WNV cells specifically involved CANX and α-TUB.

N-glycans of HCV envelope proteins are required for the non-classical secretion of HCV particles by BHK-WNV cells

Together with CANX, CRT usually ensures the proper conformation of nascent glycoproteins in the ER lumen. However, CRT expression was lower in BHK-WNV cells than in parental cells (Figure 5A) and did not co-localize with HCV proteins detected by the immune sIgG (Figure 5B). An additional C-type lectin, ERGIC-53, transports properly folded glycoproteins from the ER to the Golgi apparatus. Its expression was also slightly lower in BHK-WNV cells (Figure 5A) and did not co-localize with this pool of HCV proteins (Figure 5B).

Treatment of producer cells with deoxynojirimycin (DNJ), which prevents the binding of nascent glyco-

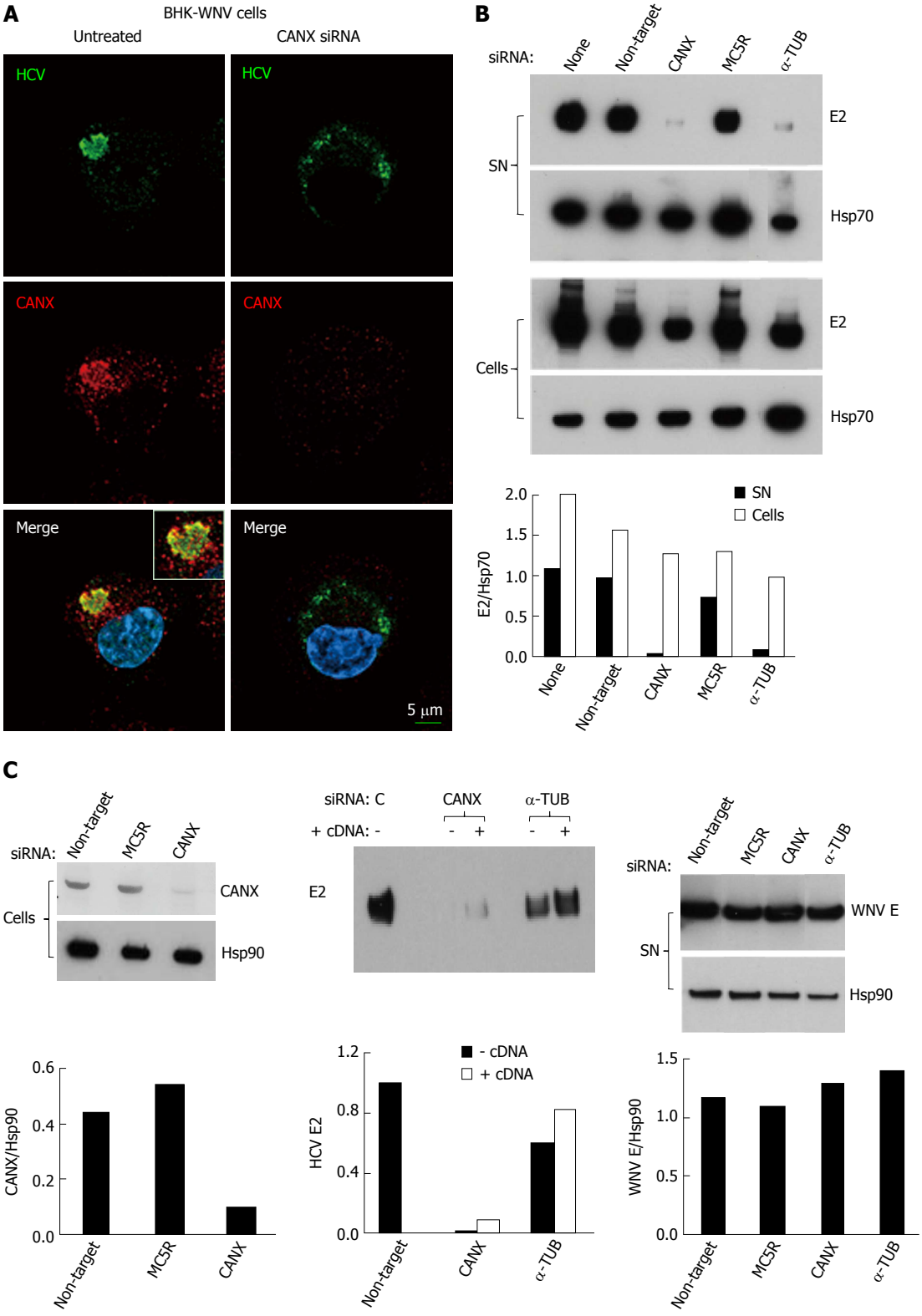

Figure 3 Involvement of calnexin and alpha-tubulin in the release of hepatitis C virus particles by baby hamster kidney-West Nile virus cells. A: BHK-WNV cells were treated, or not, with CANX siRNA and, three days later, transfected with the HCV-coding and P2B plasmids. Two days later, IF was performed with anti-HCV serum of Figure 1 (green) and anti-CANX antibody (red) followed by confocal microcopy analysis; nucleus were counterstained with DAPI (blue); B: Top panels: BHK-WNV cells were treated with the indicated siRNA for 2 d, then transfected with a plasmid encoding full length HCV (HCVbp) in the cytoplasm. Contents in E2 envelope protein of both supernatant (SN) and cell lysate (Cells) were analyzed 2 d later by Western blot (WB); Bottom panel: Densitometry analysis; C: Left panels: BHK-WNV cells were treated with the indicated siRNA for 2 d and content in CANX was analyzed by WB; Hsp90 was used as a control; Middle panel: BHK-WNV cells were treated with siRNA targeting CANX or a-TUB transcript for 2 d; cells were then reseeded and transfected the next day with the HCV-coding plasmid together with a control plasmid (-) or one expressing the cDNA of the knocked-down transcript (+). Two days later, HCV materials released in SN were analyzed by WB; Right panels: BHK-WNV treated as in (B) were transfected with a plasmid encoding West Nile virus (WNV) structural genes (core, prM and E). Two days later, materials released in the SN were analyzed by WB with an antibody recognizing WNV E (29); Hsp90 was used as a control; Bottom panels: Densitometry analyses. CANX: Calnexin; α-TUB: Alpha-tubulin; HCV: Hepatitis C virus; BHK-WNV: Baby hamster kidney-West Nile virus; C: Control siRNA.

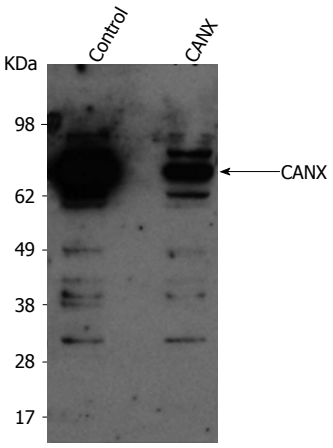

Figure 4 Baby hamster kidney-West Nile virus cells treated with the indicated siRNA (on top). After 2 d, cells from both conditions were reseeded and transfected the next day with either a control plasmid or plasmid expressing CANX cDNA, respectively in control or CANX siRNA-treated BHK-WNV cells. The following day, content in CANX was analyzed by Western blot in cell lysates. CANX: Calnexin; BHK-WNV: Baby hamster kidney-West Nile virus.

proteins to CANX[42], modified the glycosylation and stability of HCV envelope proteins in BHK-WNV cells (Figure 5C, bottom panel), which was accompanied by a lower amount of HCV particles released (Figure 5C, top panels). As expected, DNJ treatment resulted in decreased resistance of carbohydrates on E1 to a digestion by endo-β-N-acetylglucosaminidase H (Endo-H). In contrast, O-glycosylation[48] inhibitors PAG and ALL did not display any effect on HCV production or E1 glycosylation (Figure 5C, middle panels). A similar pattern was observed with BHK cells bearing a Dengue 2 subgenomic replicon (Figure 6). Therefore, the decreased production of HCV in the presence of DNJ resulted from a lack of maturation of N-linked glycosyl antenna on HCV envelope proteins, consistent with the effect of CANX siRNA observed.

RAB1 and conformational HCV protein subspecies co-localize within a compartment of reorganized ER and Golgi components

We looked for additional cellular factors that could contribute to the secretion of HCV particles. RAB1 exerts a key control on the ER-to-Golgi traffic. In parental BHK-21 cells, RAB1 was spread in the cytoplasm, but co-localized with HCV proteins expressed after transfection (Figure 7A). The WNV subgenomic replicon enhanced RAB1 staining, coalescing into a few membranous-like spots within which most HCV proteins detected by HCV1a sIgG were localized (Figure 7B). Treatment of BHK-WNV cells with RAB1 siRNA greatly reduced HCV protein expression (Figure 7D); concomitantly, fewer HCV particles were released (Figure 7D).

Although maturation of HCV envelope glycoproteins did not involve a classical secretory pathway, some carbohydrate residues at the surface of released HCV particles became resistant to a treatment by Endo-H[42]. This indicates that the ER-to-Golgi machinery as well as the Golgi apparatus contributed to HCV production in BHK-WNV cells. Interestingly, in these cells, the expression of RAB1 GDP dissociation inhibitor (GDI)[49] was enhanced and co-localized with HCV proteins in a pattern reminiscent of RAB1's (Figure 8). In addition, the expression of Atlastin 1, a high molecular weight GTPase that is involved in maintenance of the ER compartment[50] and fusion of ER tubules[51], as well as in ER-to-Golgi trafficking[52], was up-regulated in BHK-WNV cells compared to parental BHK-21 cells (Figure 9). We detected its presence within the CANX-enriched compartment formed in BHK-WNV cells (Figure 10), as well as that of p115/USO1[53], required for membrane fusion of ERGIC vesicles with the Golgi apparatus. We also detected the presence of GM130/GOLGA2 (Figure 11), which contributes to the progression of glycoproteins between Golgi cisternæ[41], which in turn correlates with the maturation of their carbohydrate residues[54].

Similarities between HCV productions in BHK-WNV and Huh-7.5 cells

We next tested whether host factors assisting HCV production in BHK-WNV cells also play a role in the JFH-1 strain of genotype 2a/Huh-7.5 hepatocarcinoma cell paradigm. Mock transfection and non-target siRNA slightly decreased HCV production in Huh-7.5 cells infected with HCVcc. All other siRNAs tested further decreased the amount of HCV RNA, the inhibition ranging between 1 and 3 logs; HCV particle release in the supernatant was diminished as well (Figure 12A).

The human HCV serum used in BHK-WNV cells was of a different genotype than JFH-1; hence, for our IF studies we used a different antibody recognizing HCV core 7-50 peptide. There was not obvious co-localization of the staining it elicited in Huh-7.5 cells inoculated with HCVcc and that of the studied cellular factors. It has been reported that the localization of HCV proteins changed with time[55]. Therefore, we examined Huh-7.5 cells infected with the JFH-1 strain for over two weeks. HCV core staining was more widely spread within the cytoplasm and several hot spots were observed, similar to HCV proteins detected with the immune human serum in BHK-WNV cells. Core preferentially co-localized with CANX and RAB1 (Figure 12B); it did less so with tubulin.

Huh-7.5 cells were then inoculated with HCVbp-4cys particles (encoding a tetra-cysteine tag on NS5A) of genotype 1a produced in BHK-WNV cells (Figure 12C). In a live-cell immunofluorescence study, the pattern of NS5A-4cys was reminiscent of that observed with the same construct expressed in BHK-WNV cells[8]. However, this pattern was different from the staining of NS5A (without a tag) detected with an in-house antibody against a 48 aa-long peptide[8], suggesting that distinct NS5A protein species were observed with two detection methods; each species could differentially affect HCV particle assembly and secretion. Interestingly, in Huh-7.5 cells, NS5A-4cys closely associated with microtubules (Figure 12C)[56].

With minor differences, these results suggest that cellular factors involved in permissiveness of BHK-WNV cells are also required for long term HCV production in Huh-7.5 cells.

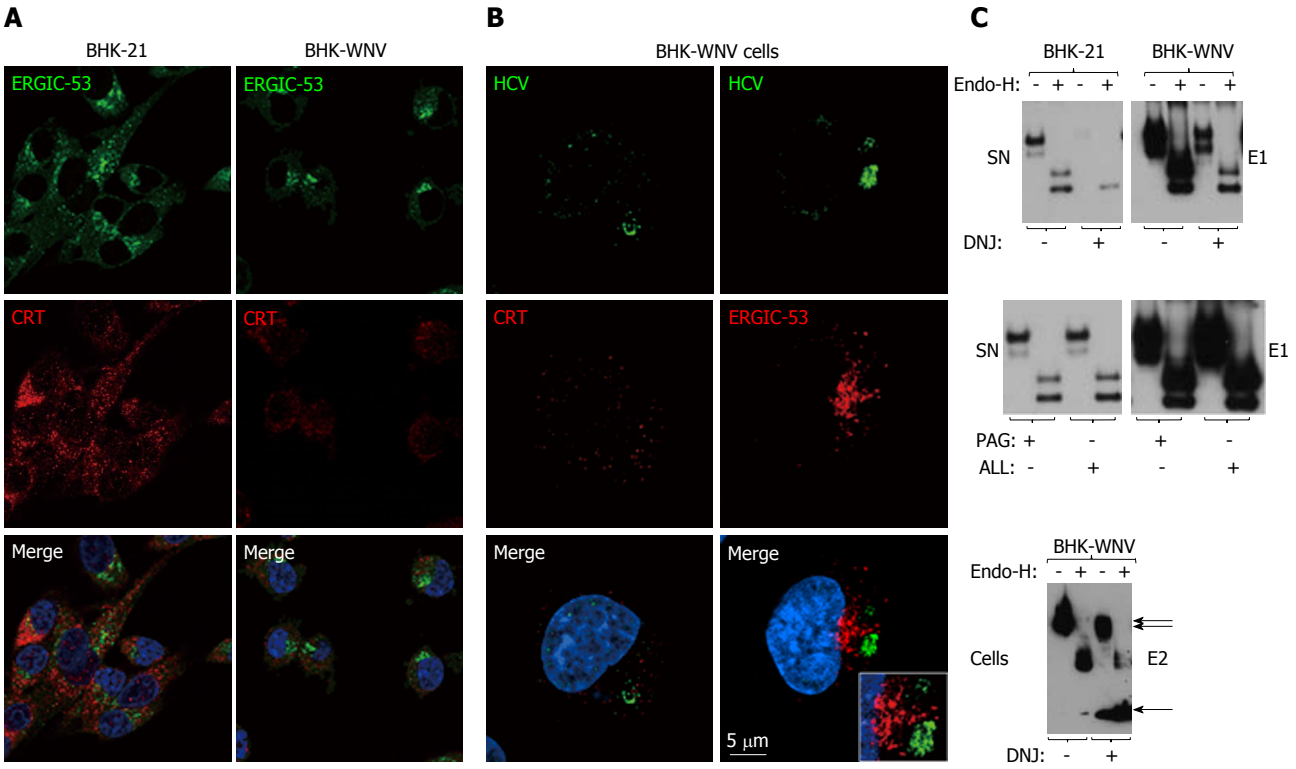

Figure 5 Calnexin and N-linked glycosylation are involved in the release of hepatitis C virus particles *via* a non-classical secretion path in baby hamster kidney-West Nile virus cells. A: BHK-21 and BHK-WNV cells were transfected with the HCV expression plasmid system. IF was performed three days later with anti-ERGIC-53 (green) and anti-CRT (red) antibodies, followed by confocal microscopy analysis; B: Same protocol as in (A) with BHK-WNV cells transfected with the HCV-coding plasmids; C: Twelve hours after transfection with the HCV expression plasmid mix, parental BHK cells or BHK-WNV cells were treated, or not, with N- (DNJ) or O- (PAG, ALL) glycosylation inhibitors; materials released in SN (top and middle panels) or cell lysates (bottom panel) were collected, incubated with or without Endo-H and analyzed by Western blot. HCV: Hepatitis C virus; BHK-WNV: Baby hamster kidney-West Nile virus; ERGIC-53: Endoplasmic reticulum-Golgi intermediate compartment-protein of 53 kDa; CRT: Calreticulin; SN: Supernatants; DNJ: Deoxynojirimycin; Endo-H: Endo-β-N-acetylglucosaminidase H.

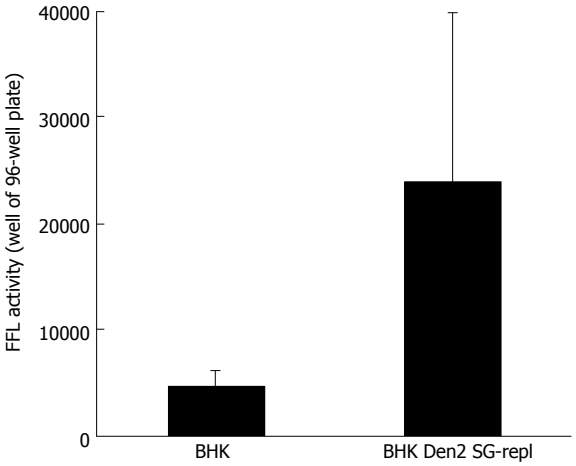

Figure 6 Huh-7.5 cells were incubated with hepatitis C virus reporter particles particles produced either in parental baby hamster kidney cells or in baby hamster kidney cells chronically replicating a dengue 2 subgenomic replicon (similar to the West Nile virus's). Infectivity was measured in target cells with a Firefly luciferase (FFL)-based reporter system, as previously decribed[8]. Error bars represent the SD in a representative experiment. BHK: Baby hamster kidney.

Caspase-1 is required for the release of HCV particles via interplay with viral non-structural genes

Caspase-1 is a cysteine protease and the common denominator of some twenty NLR/ALR inflammasome complexes identified so far and that contribute to host defenses against infections[57]. Cleavage sites for caspase-1 are present on HCV E2, NS2 and NS3 (http://web.expasy.org/peptide_cutter/). They are well conserved between genotypes, but those on E2 and NS2 are generally missing in genotypes 6 and 2, respectively. Additional sites on E1, NS3, NS4B or NS5A are found only in particular HCV strains/isolates. Overall, an average of 3 (2 to 5) cleavage sites are found in HCV polyproteins.

The HCV strain H77 polyprotein displays cleavage sites for caspase-1 at aspartate residues 728 (E2), 989 (NS2) and 1302 (NS3), but none for other caspases. This prompted us to test whether this protease could interfere with the production of HCV particles by BHK-WNV cells. We tested the effect of an inhibitor preferentially targeting caspase-1 (ZYVAD-FMK) on the production of this strain of HCV by BHK-WNV cells. While neither production of envelope E1 protein nor its release by parental BHK cells changed with the inhibitor, the release of HCVbp particles by BHK-WNV cells was dramatically reduced (Figure 13A). This was associated with a different processing of the core protein, with a predominant size of 21 kDa in treated *vs* 23 kDa in untreated cells (Figure 13A). This observation is reminiscent of the shorter core protein in HCVcc released by Huh7.5.1 cells, compared to that of HCV progenies produced in human liver slices or primary

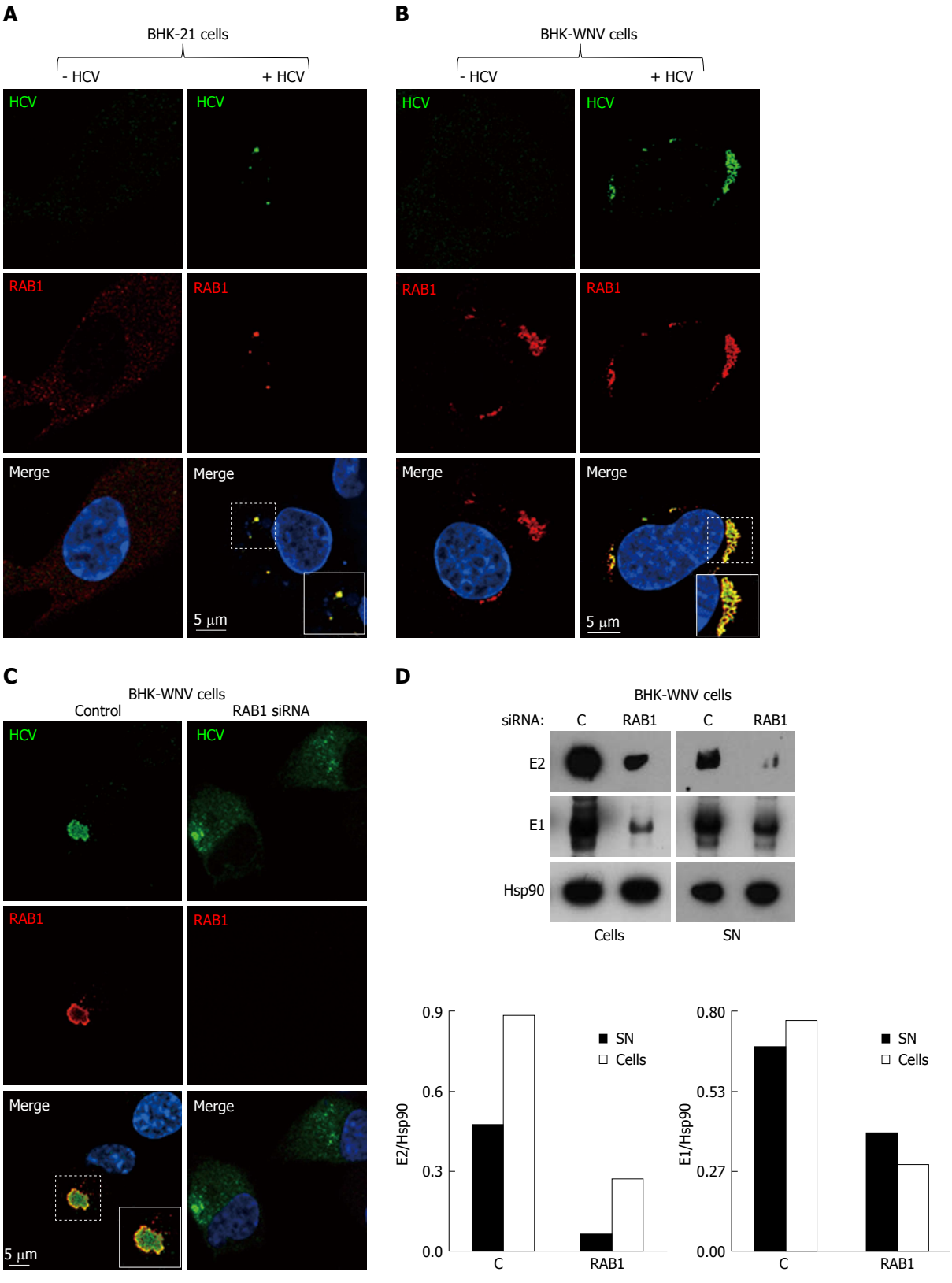

Figure 7 Release of hepatitis C virus particles by baby hamster kidney-West Nile virus cells requires RAB1 in a cytoplasmic subcompartment. Three days after transfection with the HCV-coding and P2B plasmids, or not, IF of BHK-21 (A) or BHK-WNV (B) cells was performed with anti-HCV serum (green) and anti-RAB1 antibody (red), followed by confocal microscopy analysis; C: BHK-WNV cells treated (right panels) or not (left panels) with RAB1 siRNA were transfected with the HCV expression plasmid system; IF was performed as in (B); A-C: Nuclei were counterstained with DAPI (blue); D: BHK-WNV cells treated with RAB1 siRNA were transfected with the HCV expression plasmid system. Cells and SN were harvested 3 d later, and analyzed by Western blot; Hsp90 = control; bottom: Densitometry analysis. HCV: Hepatitis C virus; BHK-WNV: Baby hamster kidney-West Nile virus; SN: Supernatants.

hepatocytes[58]. In contrast, no clear effect of the inhibitor was observed on HCV production with BHK-WNV cells

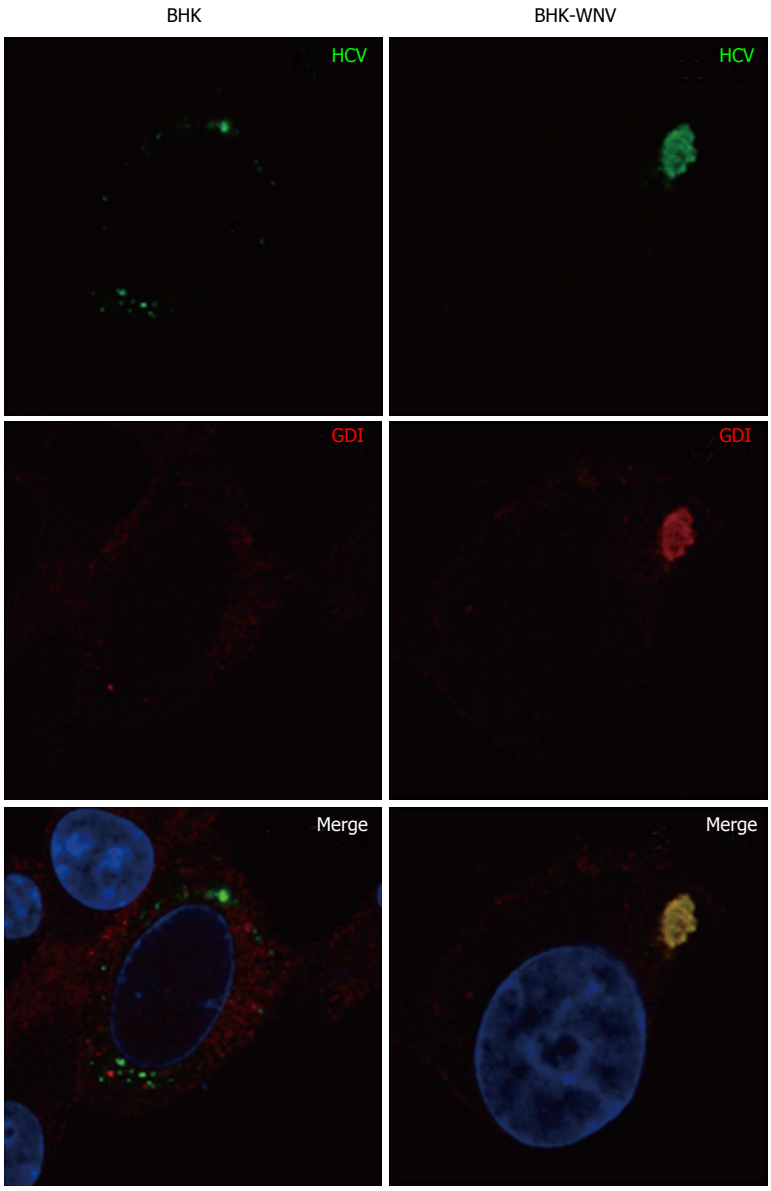

Figure 8 Parental or baby hamster kidney-West Nile virus cells were transfected to express hepatitis C virus genome in the cytoplasm and IF experiment was performed and analyzed as described throughout the manuscript. HCV: Hepatitis C virus; BHK-WNV: Baby hamster kidney-West Nile virus; GDI: GDP dissociation inhibitor.

expressing only HCV structural genes, with or without p7 (Figure 13B).

DISCUSSION

A classical secretion pathway has been implicated in the release of HCV virions[59] that are associated with apolipoprotein E (apoE) in Huh-7.5 cells[60] or other cell types[61]. In spite of a fully functional secretion pathway, parental BHK-21 cells, which do not express apoE, released only trace amounts of HCV particles[8]. Lack of apoE was overcome by the prior reorganization of intracellular trafficking by the replication of WNV (or dengue virus) for the production of infectious HCV particles (Figure 14). Epitopes detected by IgG from a patient cured of an infection of same genotype as the HCV strain used in this study (H77), are likely to be amongst those displayed on native HCV virions. This pool of HCV protein isoforms/conformers represented only a fraction of the HCV structural proteins stained by monoclonal antibodies. It overlapped with a compartment of rearranged membranes. The presence of virus particles in this compartment supports the hypothesis that it could be the HCV assembly site in permissive BHK-WNV cells.

Some arguments are consistent with the involvement of a conventional secretion pathway in the production of HCV particles by BHK-WNV cells; other arguments suggest otherwise. On one hand, an involvement of the ERGIC implies a requirement for RAB1, which was the case for the formation of the assembly compartment, as well as for the production of HCV particles. In accordance, RAB1 and p115/USO1 were co-enriched in this compartment[43,62]. GBF1, an additional component of

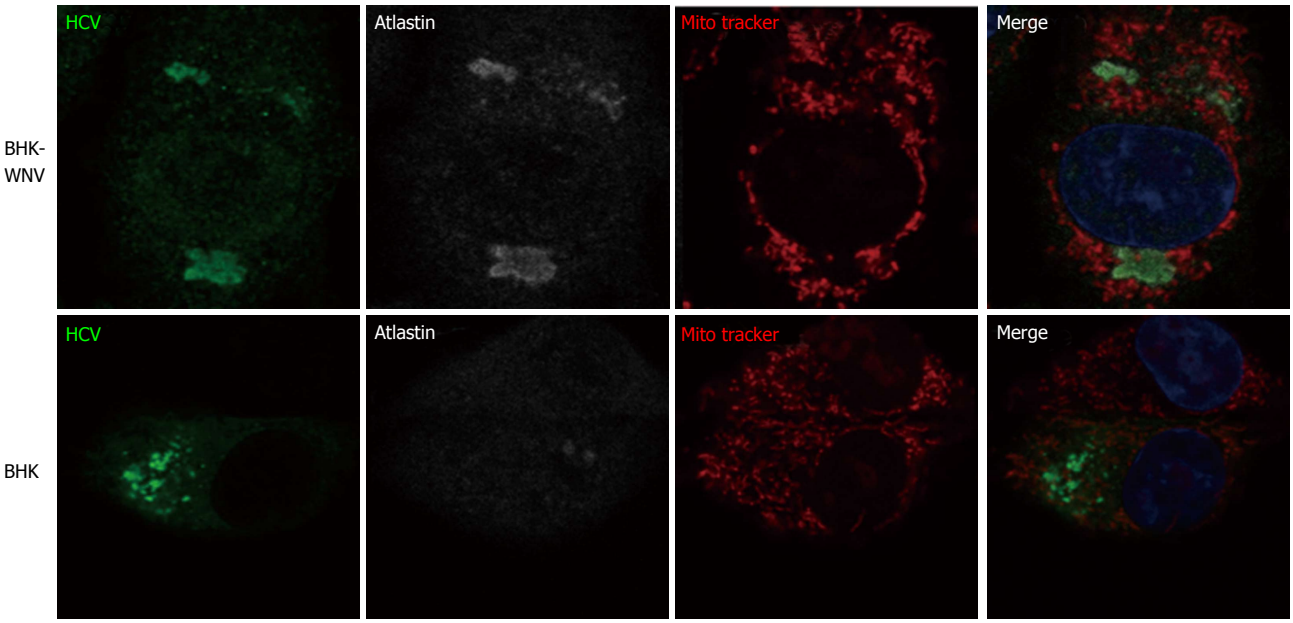

Figure 9 Parental or baby hamster kidney-West Nile virus cells were transfected to express hepatitis C virus genome in the cytoplasm and IF experiment was performed and analyzed as described throughout the manuscript. HCV: Hepatitis C virus; BHK-WNV: Baby hamster kidney-West Nile virus.

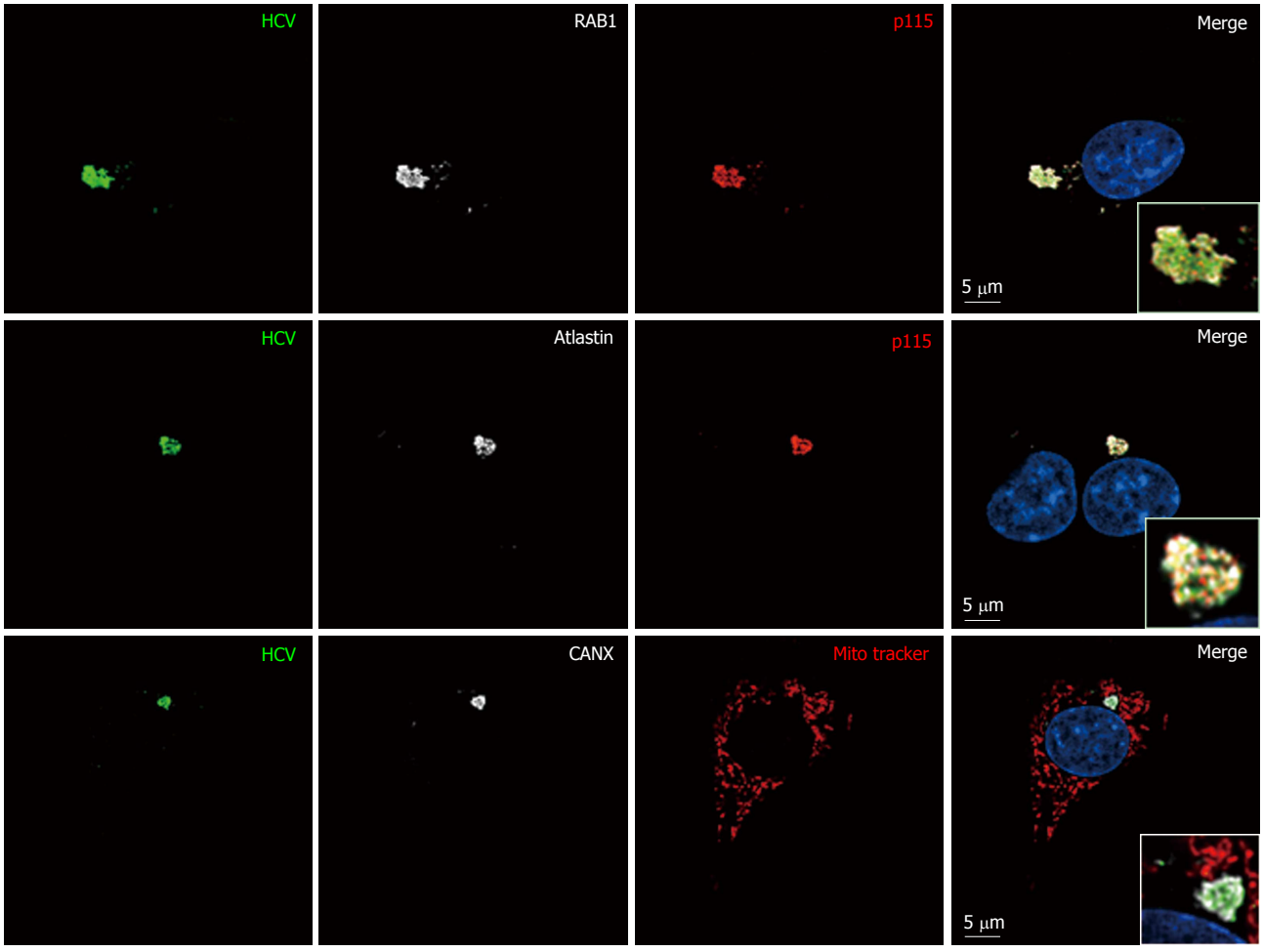

Figure 10 Endoplasmic reticulum-Golgi membrane remodelers (RAB1, p115 and atlastin) are recruited into the hepatitis C virus assembly compartment in baby hamster kidney-West Nile virus cells. Three days after transfection of BHK-WNV cells with the HCV expression plasmid system, IF was performed with anti-HCV serum (green) and anti-p115 (red), RAB1, Atlastin-1 or CANX (white) antibodies. Mitochondria were labeled with Mito-Tracker-Orange-CMTMRos (red). HCV: Hepatitis C virus; BHK-WNV: Baby hamster kidney-West Nile virus; CANX: Calnexin.

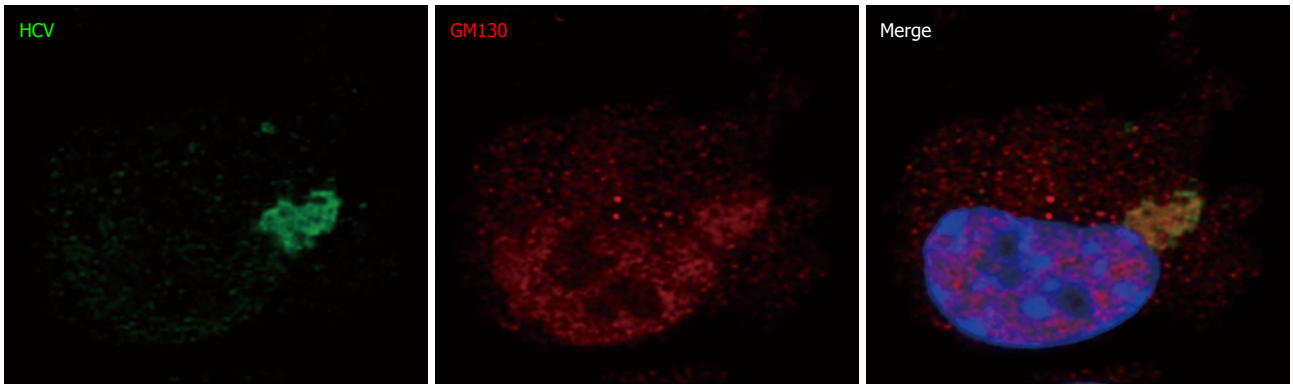

Figure 11 This experiment was performed with baby hamster kidney-West Nile virus cells transfected to express hepatitis C virus genome in the cytoplasm and IF experiment was performed and analyzed as described throughout the manuscript (anti-GM130 monoclonal antibodies have been reported to cross-react with an unidentified protein of lower molecular weight). HCV: Hepatitis C virus.

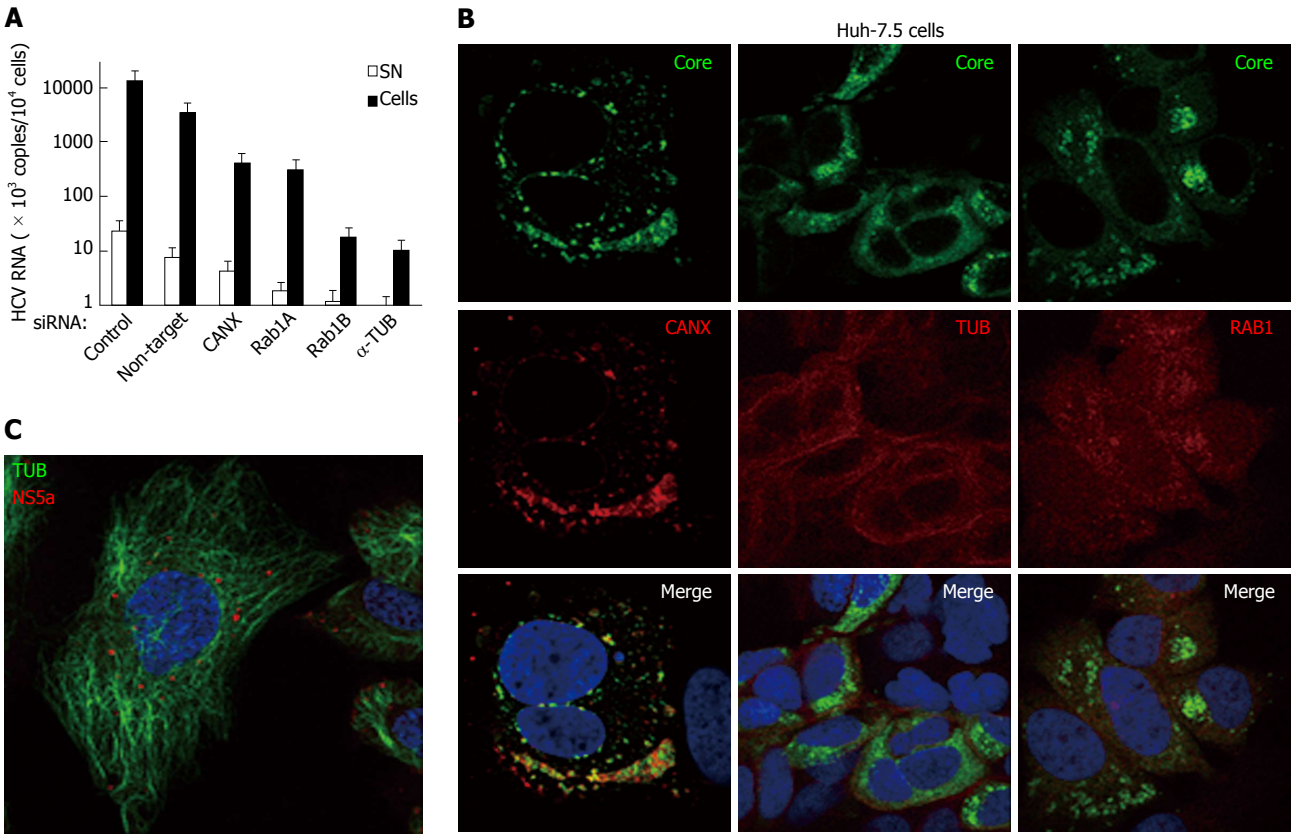

Figure 12 Hepatitis C virus produced in cultured human hepatic cells involves same cellular factors as those enhancing hepatitis C virus production in baby hamster kidney-West Nile virus cells. A: Huh-7.5 cells were seeded on 24-well plates and the next day were transfected, or not, with siRNA, as indicated. After 2 d, cells were reseeded into 24-well plates and, the next day, incubated at a MOI = 0.5 with HCVcc produced with the JFH-1 strain in Huh-7.5 cells. At 3 dpi, HCV RNA contents were determined in the cells (closed bars) and SN (open bars) by RT-qPCR (TaqMan). Results are plotted on a log-scale; errors bars represent maximum variations observed in this assay; B: Huh-7.5 cells were electroporated with *in vitro*-transcribed genome of the JFH-1 strain and passaged for two weeks, then were seeded onto coverslips. Two days later, IF was performed with anti-HCV core 7-50 (green) and anti-human CANX, tubulin (TUB) or RAB1 antibodies (red); C: Huh-7.5 cells were inoculated with HCVbp-4cys produced in permissive BHK-WNV cells (8); 2 d later, the cells were incubated with both ReASH (red) and Taxol fluorophore conjugate (green); result representative of two independent experiments; B and C: Nuclei were counterstained with DAPI and cells were observed by confocal microscopy. CANX: Calnexin; HCV: Hepatitis C virus; BHK-WNV: Baby hamster kidney-West Nile virus; SN: Supernatants.

the ERGIC that activates small-sized GTPase ARF1 and, in turn, COPI-dependent traffic[43], was also involved in the production of HCV particles[8]. On the other hand, another feature of the ERGIC, the lectin ERGIC-53, was excluded from the assembly compartment. As observed in TEM, the latter directly brought together membranes from the ER and the Golgi apparatus, which was consistent with an enrichment of this compartment in, respectively, Atlastin 1[50-52] and GM130/GOLGA2[41], both reported to reorganize and/or maintain membranes of their organelle. In addition, an enrichment in both RAB1 and its GDI, like what was observed in the assembly

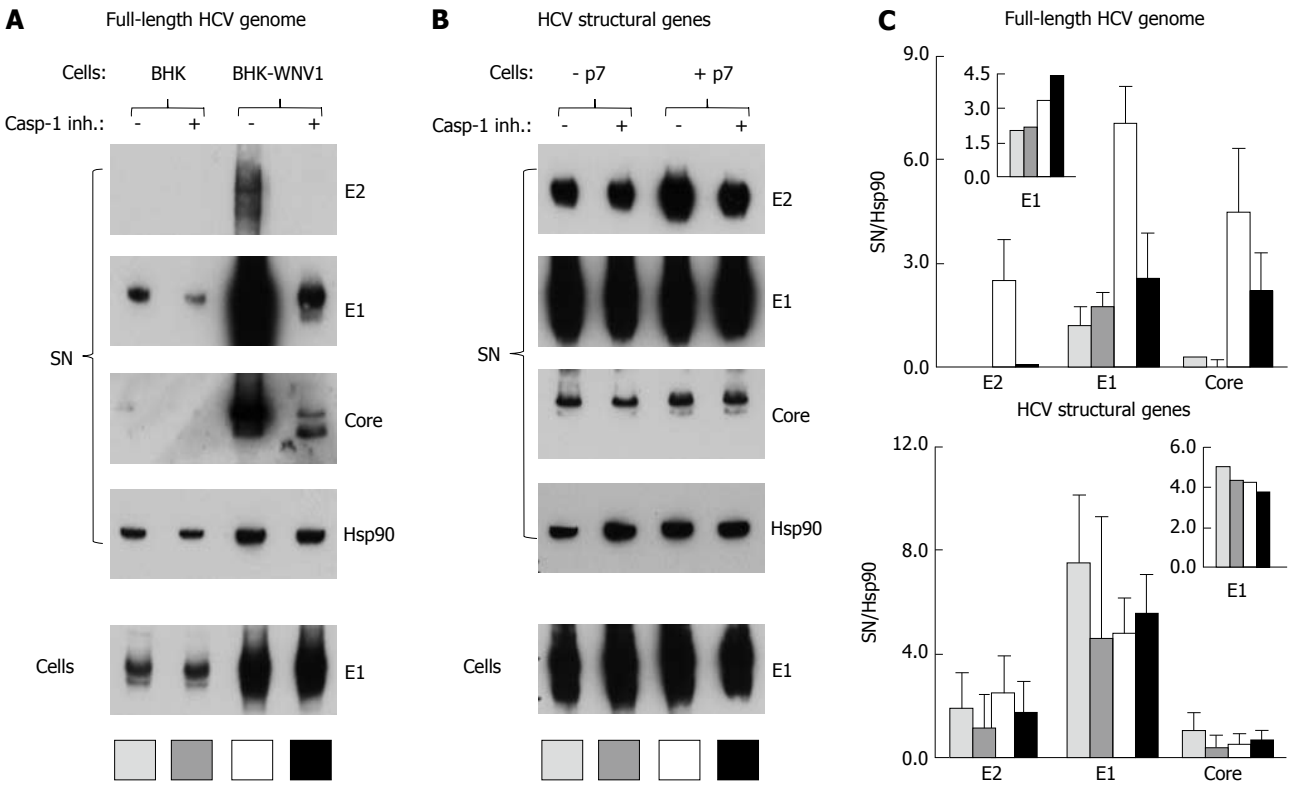

Figure 13 Caspase-1 inhibitor conditionally inhibits the secretion of hepatitis C virus particles by baby hamster kidney-West Nile virus cells. A: BHK-21 and BHK-WNV cells were transfected with a mix of HCVbp-coding and P2B plasmids; the next day, a caspase-1 inhibitor was added in the culture medium and the cells were incubated for 2 more days; cell lysates and HCV particles were harvested and analyzed by Western blot (WB); B: BHK-WNV cells were transfected with a plasmid coding for the structural (*core*, *E1*, *E2*) genes of HCV H77 strain, plus (+) or minus (-) p7, then were analyzed as in (A); C: Quantification of WBs: Top panels for (A) and bottom panels for (B); bar inside patterns are displayed underneath corresponding data; error bars represent standard deviations; inserts = results in cells. HCV: Hepatitis C virus; BHK-WNV: Baby hamster kidney-West Nile virus.

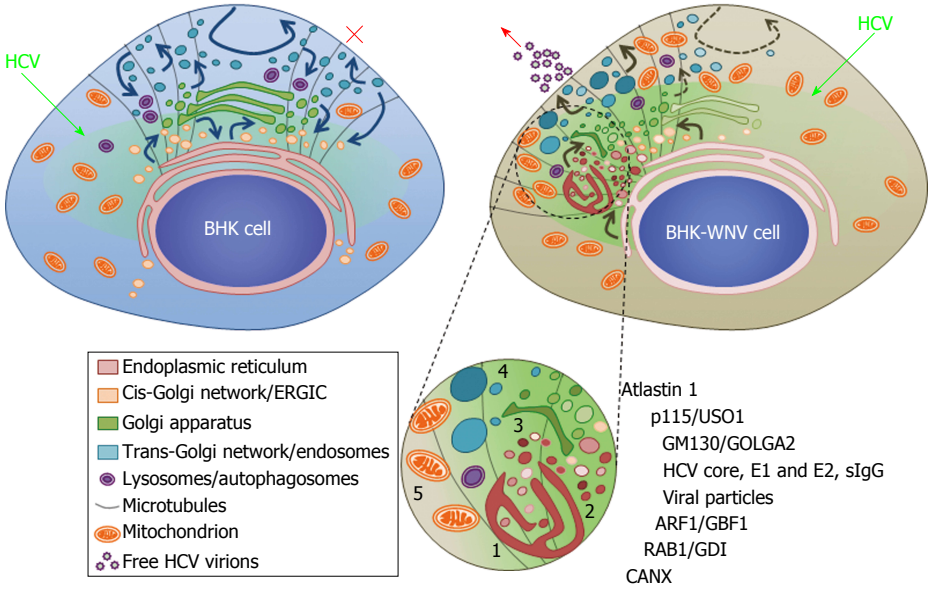

Figure 14 Model of a hepatitis C virus assembly compartment in baby hamster kidney-West Nile virus cells. Schematic organization of cell traffic in parental BHK-21 (top left) and BHK-WNV (top right) cells; curved arrows represent cell traffic; HCV genome is produced by the P2B plasmid system (green arrows) and expressed (greenish areas) in the cytoplasm of BHK cells. Bottom, sketches' legend and close up of a HCV assembly site (cf. also Figure 2B): (1) convoluted membranes; (2) vesicular packets; (3) Golgi cisternæ; (4) large vesicles filled with viral particles; and (5) mitochondrion; host and viral factors identified within this compartment. SIgG: Antibodies from the serum of a cured HCV patient; CANX: Calnexin; HCV: Hepatitis C virus; BHK-WNV: Baby hamster kidney-West Nile virus; ERGIC: Endoplasmic reticulum-Golgi intermediate compartment; GDI: GDP dissociation inhibitor.

compartment, has been reported to initiate a cascade of events involving GBF1, ARF1 and phosphatidylinositol 4-kinase Ⅲ alpha (PI4KⅢα)[43], which may increase the local proportion of phosphatidylinositol 4-phosphate[63]

and subvert endocytic trafficking[64]. In Huh-7.5 cells, this role is normally devoted to HCV NS5A that brings together PI4KⅢα and TBC1D20, an activator of RAB1[65]. Therefore, replication of the genomic RNA of flaviviruses in BHK-21 cells may pre-position components - or their equivalent - HCV establishes by itself in the cells in which it usually replicates. Although excluding ERGIC as we know it in the classical secretion pathway, such re-organization of the secretion machinery in BHK-21 cells (Figure 14) could explain why a BFA treatment inhibited HCV particle production.

Finally, if CANX was required for the production of HCV particles, most of it shuttled a pool of HCV protein subspecies to the assembly compartment of BHK-WNV cells, instead of co-localizing with CRT for the proper folding of glycoproteins. How CANX really assists these cells to produce HCV virions is not fully understood. CANX still probably requires binding N-linked glycans on the HCV envelope proteins. The reported slow dissociation rate between CANX and HCV envelope proteins[27] could contribute to unusual involvements of CANX, such as what is observed with proteins targeted to mitochondria-ER associated membranes (MAMs) for the formation of inflammasome and autophagy compartments[66]. HCV has previously been shown to target MAMs in hepatocytes[67]. However, mitochondria were excluded from the assembly compartment in BHK-WNV cells. And, upon expression of HCV structural genes, this coincided with the appearance within the assembly compartment of large vesicles containing particles[8].

Oxidative stress, inflammation and/or infection can lead to the appearance in the cytoplasm of large vesicles, often indiscriminately referred to as multivesicular bodies, releasing extracellular particles of very different compositions depending on their origin. They are formed by the fusion of late endosomal vesicles with membranes from lysosomal and/or autophagosomal compartments, or originating from the periphery of the ER-Golgi complex assembling - independently from a COPⅡ- and COPⅠ-mediated membrane transport - into a compartment of unconventional protein secretion[68]. Unconventional pathways are used by intracellular pathogens reorganizing the vesicular traffic to secrete proteins (*e.g.*,[69]). The reorganization of the secretion machinery required for the production of HCV particles by BHK-WNV cells displayed similar features.

Knocking down α-TUB expression not only resulted in a lesser release of HCV particles, but also of particulate materials containing Hsp70 and Hsp90, a feature of exosomes. Such extracellular vesicles have been shown to participate in the transfer of viral materials[46]. Recently, exosomes have also been implicated in the propagation/dissemination of HCV genome, proteins and replication complexes, although in the former case with a weaker effectiveness than lipoprotein-associated virions[70,71]. However, the production of HCV particles by BHK-WNV cells cannot be reduced to the secretion of exosomes. We had previously shown that, albeit overlapping, the peaks of particles containing HCV structural and heat-shock proteins released by BHK-WNV cells did not coincide after ultracentrifugation on a density gradient[8]. In addition, while the secretion of HCV particles was blocked, the release of heat-shock proteins was insensitive to a treatment by brefeldin A (BFA), an inhibitor of GBF1[8]. In this work, the secretions of Hsp70 and Hsp90-containing particles and HCV particles displayed different patterns. Therefore, the dual effect of α-TUB siRNA on the secretions of exosomes and HCV particles probably reflects separate needs for microtubules taking place at different steps. For exosomes, it could relate to effects on the BFA-insensitive *trans*-Golgi network, which is consistent with an accumulation of hsp70 inside producer cells following knockdown of α-TUB expression. Conversely, the production of HCV particles could be inhibited at the level of a GBF1/ARF1-dependent traffic, within the assembly compartment, or later during their secretion.

Treatment of BHK-WNV cells with a caspase-1 inhibitor abolished the release of HCV particles. Caspase-1 has been shown to activate IL-1β and IL-18 proinflammatory cytokine precursors[57]. It has long remained unknown how their matured species translocate across the plasma membrane. Recent results support the idea that their secretion involves a cytosolic compartment containing vesicles and their exocytosis[72]. It could be the mechanism by which a caspase-1-dependent secretory pathway contributed to the production of HCV particles by BHK-WNV cells. However, inhibition of HCV production with the caspase inhibitor occurred only in the context of a full-length genome, suggesting the existence of interaction(s) between caspase-1 and HCV non-structural genes, here NS2 and/or NS3. Although the catalytic activity of NS2 is dispensable, this viral cysteine protease is required for the production of HCV particles[73], as is the helicase/serine protease NS3[74]. Therefore, the cleavage(s) of NS2/3 by caspase-1, in addition, could prevent the maturation of non-structural proteins required for RNA replication and/or remove interactions, either of them or both being detrimental to the formation and secretion of HCV particles.

At variance with the JFH-1/Huh-7.5 cell model[59,75], infectious HCV particles released by BHK-WNV cells did not require lipoproteins or exosomes. It is still unclear whether discrepancies between models[9] could not also relate to active HCV replication in a non-physiological environment[76], differences between viral genotypes[12] and/or differential processing of some HCV proteins[58]. Nevertheless, host factors involved in BHK-WNV cells were also required in Huh-7.5 cells that, over time, developed a cytoplasmic compartment[26] enriched in HCV core, CANX and RAB1. Infectious HCV particles are assembled in the cytoplasm of Huh-7.5 cells[13] before their association to lipoproteins[8,77] or exosomes[75,78]. The associations to lipoprotein particles and extracellular particles have been proposed as mechanisms to regulate

vertebrate Hedgehog dispersion during development[79]. In addition, ApoE[25,80] and extracellular particles[47] are suspected to contribute also to the pathogenesis of viruses unrelated to HCV. Therefore, rather than having evolved a unique mechanism of viral propagation, HCV may have instead subverted existing cellular processes. It could involve intrinsic properties of its envelope proteins, since HCV E1 interacts with apoE[81] and possibly lipids[82], which is believed to help the virus escape from immune recognition[83].

The release of HCV particles by BHK-WNV cells, instead, involved an unusual, if not unconventional secretion pathway. The present data suggest that their mechanism of assembly and egress could be a mean by which HCV circumvents intracellular defenses. These infectious particles are not those usually observed as such in infected patients. Therefore, as for other HCV particles produced *in vitro*[9], we are not sure of their relationship to the normal viral cycle. Are they merely a precursor for the main HCV species, which will eventually associate to lipoproteins, or do they represent different viral species of higher buoyant density? In patients, the proportion of circulating HCV particles associated to lipoproteins varies between individuals and during the course of infection. Denser viral species can amount up to half of the circulating HCV genome[84]; they are usually opsonized by IgG in the serum of infected patients, making the study of free virions difficult. At least during the assembly process in BHK-WNV cells, HCV particles exposed epitopes that were recognized by neutralizing sIgG from a patient previously chronically infected by HCV of same genotype. We propose that the BHK-WNV cell model could be useful to study the structure of free HCV virions and their immunological properties.

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COMMENTS

Background

The production of infectious hepatitis C virus (HCV) particles by model human hepatocellular carcinoma Huh-7.5 cells is believed to involve a classical secretion pathway; it has also been reported to involve lipoproteins and exosomes. The authors previously demonstrated that the intracellular environment generated by a West-Nile virus (WNV) subgenomic replicon rendered non-human, non-hepatic mammalian cells [here referred to as baby hamster kidney-WNV (BHK-WNV) cells] permissive for assembly and release of infectious HCV particles of various genotypes wherein the HCV genome is generated in the cytoplasm independently of its replication machinery. HCV production did not require ongoing activity of the WNV replicon, but instead was associated with persisting replicon-induced changes in the cellular environment. Since secretion of HCV particles by BHK-WNV cells neither involved lipoproteins nor exosomes, could it still follow a conventional pathway? Activity of the small GTPase ARF1 and the maturation of carbohydrates on envelope proteins of released HCV particles were both required for the production of infectious HCV by permissive BHK-WNV cells, suggesting that, indeed, it followed a classical secretion pathway. The authors, therefore, examined the possibility that the endoplamic reticulum (ER)-bound lectin calnexin (CANX), the small GTPase RAB1 and microtubule-associated alpha-tubulin, which all contribute to the secretion of several glycoproteins by BHK-21 cells, were involved in the production of HCV particles by BHK-WNV cells. Surprisingly, the results show that secretion of HCV particles went through a re-organized and re-wired pathway bypassing the conventional ER-to-Golgi intermediary compartment and involving components of the inflammasome.

Research frontiers

The structure of HCV virions/progenies remains elusive. The fact that infectious HCV particles are produced independently from lipoprotein biosynthesis, yet retain the possibility to interact with lipoproteins *in vitro* supports the view that, *in vivo*, HCV particles may interact with lipoproteins in a second step and not necessarily co-assemble with them. This has probably important consequences regarding the way HCV interacts with producer cells and the immune system.

Innovations and breakthroughs

The authors' results suggest that the HCV production made possible by the prior WNV replication in BHK cells could be related to that observed in human hepatocytes. It is unclear whether this reflects an organization of HCV production more complex than expected in hepatocytes or the existence of additional route(s) of HCV secretion.

Applications

Although no immediate application of these findings is foreseen, the production of free HCV virions could pave the way for identifying the most important epitopes for HCV neutralization and the development of an effective vaccine.

Terminology

Subgenomic replicon: Fragment of a viral RNA genome encoding the non structural genes required for its own replication in the cytoplasm, but not the structural genes required for the formation of virions.

Peer-review

In this manuscript, the authors aimed to examine whether the cellular factors including CANX, RAB1 and alpha-tubulin were involved in the production of HCV particles by BHK-WNV cells. Moreover, it brings an interesting contribution to the current literature.

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ORIGINAL ARTICLE

Prospective Study

Is neutrophil gelatinase associated lipocalin useful in hepatitis C virus infection?

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Abstract

AIM: To evaluate neutrophil gelatinase associated lipocalin (NGAL) in patients infected by hepatitis C virus (HCV) before and during treatment with directly acting antivirals (DAAs).

METHODS: NGAL was measured in a group of patients with chronic HCV infection ranked, at baseline, by age, gender, anti-hypertensive therapy, HCV viral load, liver fibrosis stage and, either at baseline or after 1 year, estimated glomerular filtration rate (eGFR). Then, NGAL and eGFR evolutions were monitored in a subgroup of patients who started antiviral therapy with DAAs. Differences of median NGAL levels were evaluated through Wilcoxon-Mann-Whitney test for non-parametric data. Differences in dichotomous variables were evaluated through χ^2 test. At baseline, a univariate regression analysis was conducted to verify if NGAL values correlated with other quantitative variables [age, fibrosis four (FIB-4), AST to platelet ratio index (APRI), and eGFR].

RESULTS: Overall, 48 patients were enrolled, 8 of them starting HCV treatment. At baseline, statistically significant differences were found in median NGAL values only between patients with eGFR < 60 mL/min *vs* patients with eGFR ⩾ 90 mL/min. Differences in NGAL were not significant among patients ranked by HCV viral load, FIB-4 score and APRI, when patients with NGAL > 118.11 ng/dL were compared with those of NGAL ⩽ 118.11 ng/dL, not statistically significant differences were present for age, gender, chronic kidney disease classification and liver fibrosis (P > 0.05). Linear correlation was found between NGAL and both age (P = 0.0475) and eGFR (P = 0.0282) values. Not statistically significant predictions of NGAL at baseline were demonstrated for eGFR evolution 1 year later. Interestingly, in the 8 patients treated with DAAs, median NGAL significantly increased at week 12 compared to baseline (P = 0.0239).

CONCLUSION: Our results suggest that NGAL should be further evaluated as an adjunct marker of kidney function in these patients.

Key words: Directly acting antivirals; Hepatitis C virus; Inflammation; Neutrophil gelatinase lipocalin; Tubular impairment

Core tip: For the first time, we evaluated the evolution of neutrophil gelatinase associated lipocalin (NGAL), a novel biomarker of renal impairment, in patients infected by hepatitis C virus before and during treatment with directly acting antivirals (DAAs). In our study, we documented a significant increase of NGAL during the first 12 wk of therapy with DAAs and a correlation of NGAL with both age and estimated glomerular filtration rate before starting treatment. In a context of paucity of information about safety of the new DAAs, we believe that these data are both informative and novel, provoking urgent investigations.

Strazzulla A, Coppolino G, Di Fatta C, Giancotti F, D'Onofrio G, Postorino MC, Mazzitelli M, Mammone SV, Gentile I, Rivoli L, Palella E, Gravina T, Costa C, Pisani V, De Maria V, Barreca GS, Marascio N, Focà A, Fuiano G, Gulletta E, Torti C. Is neutrophil gelatinase associated lipocalin a useful marker in hepatitis C virus infection? *World J Hepatol* 2016; 8(19): 815-824 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i19/815.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i19.815

INTRODUCTION

Estimated prevalence of renal insufficiency is increased by 40% in patients infected by hepatitis C virus (HCV) compared to the HCV negative population[1]. Renal disease, ranging from mild to end-stage renal disease (ESRD), often complicates prognosis and treatment of HCV infection with haemodialysis being required in some cases. Moreover, in the context of liver disease, kidney function is one of the key predictors of death and serum creatinine, is a component of both King's college criteria and model for end-stage liver disease scoring systems that are used for prognostic stratification in patients with acute and chronic liver failure[2].

Virus related kidney diseases mainly show up as a glomerular impairment, predominantly a membrano-proliferative glomerulonephritis with type 2 crioglobulinaemia and sub-endothelial or intra luminal deposits of IgG, IgM and complement components[3]. Moreover, HCV core proteins were isolated in both glomerular and tubular tissues, suggesting the presence of a parallel tubular-interstitial damage[4].

Treatment of HCV infection in patients with renal impairment has always been challenging due to the side effects of the past treatment used as standard, an association of pegylated-interferon (PEG-IFN) and ribavirin (RBV), which was more difficult to manage in patients with renal impairment than in patients with normal renal function. In 2014, with the advent of first generation directly acting antivirals (DAAs), efficacy of anti HCV treatment was substantially improved. However, the first generation DAAs, boceprevir and telaprevir (TVR), were not recommended in patients with severe renal impairment or ESRD because these patients were excluded from registrational trials[5]. Moreover, in real-life patients without renal impairment, an increase of serum creatinine was observed during treatment with TVR, even if it was not associated with more severe renal impairment[6]. In March 2015, second generation DAAs were available in Italy for the treatment of HCV. Even if second generation DAAs are better tolerated, there is a paucity of information about the possible impact of these

drugs on renal function[7]. Therefore, the evidence of kidney impairment may make clinicians less comfortable to begin an antiviral treatment.

Estimated glomerular filtration rate (eGFR) can be calculated by creatinine values, however in circumstances such as toxic drug dosage, kidney disease improving global outcome (KDIGO) guidelines suggest to add at least another biomarker. In fact, GFR estimating equation alone is biased in many situations, such as acute kidney disease (AKI), high GFR, non-GFR determinants of serum creatinine and interferences with creatinine assays[8]. Additionally, creatinine-based measures are even more limited in cirrhotic patients due to a decrease in hepatic synthesis of creatinine, reduced skeletal muscle mass and increased tubular secretion[9].

Among novel kidney biomarkers, one of the most promising is neutrophil gelatinase associated lipocalin (NGAL) either in urine and serum (or plasma)[10]. This is a small glycoprotein in three different forms: A monomer (25 kDa), a homodimer (45 kDa) and a heterodimer (135 kDa). It is secreted by many human cells, such as epithelial cells (liver, kidney, lungs) and blood cells (neutrophils, monocytes and macrophages), filtered in the glomerulus and reabsorbed by the proximal tubules[11,12]. It is removed by hemodyalisis[13]. In general, NGAL urinary levels increase after a tubular injury and reveal a kidney damage earlier than the increased levels of creatinine[14]. On the other hand, plasmatic or serum NGAL are more extensively adopted in AKI contexts, because their measurement is less limited by availability of samples when patients are anuric[15]. Remarkably, an increase of plasmatic NGAL is considered to be an early predictor of AKI in various critical settings such as cardiac surgery, septic shock, contrast induced nephropathy, renal and liver transplantation[16-22]. However, NGAL is increased also in case of epithelial damage or inflammation outside the kidney[10].

In cirrhotic patients, NGAL is a marker of AKI and urinary NGAL that can help distinguish among different causes of renal impairment[10]. Recently, a significant difference in plasmatic NGAL levels amongst HCV positive patients with cirrhosis and eGFR < 60 mL/min *vs* ⩾ 60 mL/min has been demonstrated[23]. Also, recent data showed that NGAL is a good marker of renal damage due to drug toxicity. For example, urinary NGAL is a good predictor of tacrolimus induced AKI in liver transplanted patients and nonsteroidal anti-inflammatory drug (NSAID) associated AKI in cirrhotic patients[24,25]. To our best knowledge, no published data on NGAL during HCV treatment with DAAs are available so far.

The objectives of this study were to explore: (1) whether there is a difference in plasmatic NGAL between HCV positive patients and HCV negative people; (2) whether there is a difference in plasmatic NGAL among HCV positive patients ranked by age, gender, viral load, eGFR and liver fibrosis stage; (3) whether NGAL levels at baseline correlate with modification of eGFR after 1 year; and (4) the evolution of renal function in patients treated with DAA including regimens.

MATERIALS AND METHODS

Recruitment of patients and data collection

A prospective study was conducted. Patients with chronic hepatitis C who attended the Outpatient Service of the Infectious Diseases Unit and the Hepatology Unit of the "Mater Domini" Teaching Hospital in Catanzaro (Italy) from February 1, 2014 to April 30, 2014 were included in this study. Exclusion criteria included: Leukocytosis (leukocyte count higher than 12000 cells/μL), variceal bleeding, primary kidney diseases (glomerular nephropathy), KDIGO classification of chronic kidney disease (CKD) ⩾ G4 (eGFR < 30 mL/min), ongoing HCV therapy (with or without interferon or DAA). Approval from local ethical committee was obtained. All enrolled patients signed an informed consent.

All patients underwent physical examination and history taking at baseline. The following blood tests were collected: AST, ALT, total and fractioned bilirubin, albumin, γGT, alkaline phosphatase, prothrombin time, total blood cell count (including neutrophil and platelet count) and urea. Serum creatinine levels were measured at baseline and after 1 year. For patients who started anti-viral therapy, serum creatinine levels and GFR were studied at week 4 and week 12 after baseline.

Glomerular filtration rate was estimated through Chronic Kidney Disease Epidemiology Collaboration formula (CKD-EPI, 2009) since CKD-EPI is less biased and more accurate for eGFR ⩾ 60 mL/min than MDRD (Modification of Diet in Renal Disease) and Cockcroft-Gault formulas[7]. The following formula was used: $141 \times \min(SCr/k, 1)^{a} \times \max(SCr/k, 1)^{-1.209} \times 0.993^{Age} \times$ (1.018 if female or 1.159 if black), where SCr is serum creatinine (in mg/dL), k is 0.7 for females and 0.9 for males, a is 0.329 for females and 0.411 for males, min is the minimum of SCr/k or 1, and max is the maximum of SCr/k or 1.

Liver fibrosis was estimated at baseline by either fibrosis four (FIB-4) score or AST to platelet ratio index (APRI) which are the most used formulas for estimating stage of liver disease. FIB-4 has a negative predictive value of 94.7% to exclude severe fibrosis with a sensitivity of 74.3% when < 1.45 and a positive predictive value to confirm the existence of a significant fibrosis (F3-F4) of 82.1% with a specificity of 98.2% when ⩾ 3.25. The following formula was used: Age × AST/(platelets × √ALT) where AST and ALT were measured as IU/L, platelets were measured as number × 10^6/μL and age was measured in years[26]. An APRI value ⩽ 0.5 rules out significant fibrosis and cirrhosis while values ⩾ 1.5 indicates significant fibrosis[27]. More specifically, when APRI score is greater than 1.0 it has a sensitivity of 76% and a specificity of 72% for predicting cirrhosis[28]. The following formula was used: [(AST/AST upper normal limit)/platelets] × 100 where AST was measured as IU/L, platelets were measured as number

$\times 10^6/\mu L$ and AST upper normal limit was fixed at 35 IU/L.

NGAL measurement

Peripheral venous blood samples were taken from each patient at baseline and then processed for NGAL measurement. Plasmatic NGAL was measured in all patients at baseline. For patients who started anti-viral therapy, NGAL was measured at different endpoints (baseline, week 4 and 12). NGAL assay was performed using human NGAL Rapid Elisa Kit (BioPorto Diagnostic). A 96-well microtiter plate coated with purified anti-human NGAL monoclonal antibody was used. In each well 50 μL of each sample, diluted 1:100 with sample diluting buffer, undiluted calibrators and controls were added; then 50 μL of horseradish peroxidase -conjugated NGAL antibodies were added. During this first step of incubation at room temperature for 30 min, NGAL bind either the specific antibody adsorbed to microwells or the second antibody; an aspiration-washing step (3 times with 300 μL of wash solution) was performed to remove excess and unbounded reagents; 100 μL of tetramethyl-benzidine solution were added to each well and a second step of incubation performed at room temperature for 15 min; at end of incubation 100 μL of stop solution was added and the color developed in each well was measured at 450 nm. The values of samples were determined on the basis of a standard curve. The methodology was performed using a Triturus automatic analyzer. The commercial kit, that is validated by Pedersen *et al*[29] is used in our study. The reference range (41.19-118.11 ng/mL) had been previously validated in our laboratory on a group of healthy volunteers.

Groups of patients

Differences of NGAL values were evaluated in patients ranked by age (< 65 years *vs* ⩾ 65 years), gender (female *vs* male), anti-hypertensive therapy (present *vs* absent), HCV viral load (< 1000000 copies/mL *vs* ⩾ 1000000 copies/mL), FIB-4 score (⩽ 1.45 *vs* 1.45 to 3.25 *vs* ⩾ 3.25), APRI (⩽ 0.5 *vs* 0.5 to 1.5 *vs* ⩾ 1.5), and eGFR (⩾ 90 mL/min *vs* ⩾ 60 mL/min to < 90 mL/min *vs* < 60 mL/min). Patients were ranked by eGFR worsening *vs* stable/improved with respect to baseline values after 1 year. Any reduction of eGFR was considered as worsening. Viral load cut-off was set at 1000000 HCV RNA copies/mL because this value is commonly considered to be high[30]. Cut-off for age was set at 65 years because that is the threshold discriminating adulthood and elderly life in many western countries[31]. We analyzed eGFR as a continuous measure and did not consider any specific-cut off for change of eGFR.

Differences in dichotomous variables (gender, FIB-4 ⩾ 3.25, APRI ⩾ 1.5, KDIGO CKD classification ⩾ G3a, KDIGO CKD classification ⩾ G2, age ⩾ 65 year) were evaluated in patients ranked at baseline by NGAL values (> 118.11 ng/dL *vs* ⩽ 118.11 ng/dL).

Evolution of NGAL at different time points (baseline, week 4 and week 12) was evaluated separately in patients who started antiviral treatment with DAA containing regimens during the first twelve weeks of therapy.

Statistical analysis

Differences of distribution NGAL levels from baseline to one year in the overall population and differences of NGAL and eGFR distribution in patients prescribed HCV therapy (evaluated at baseline, week 4 and week 12) were evaluated through Wilcoxon-Mann-Whitney test for non parametric data. Also, at baseline, a Spearman correlation analysis was conducted to verify if NGAL values correlated with other quantitative variables (age, FIB-4, APRI, and eGFR). Differences in dichotomous variables (age, gender, CKD classification and liver fibrosis) in patients with NGAL > 118.11 ng/dL or NGAL ⩽ 118.11 ng/dL were evaluated through χ^2 test. Statistical analysis was performed using Graphpad Prism 6.01 (GraphPad Software, La Jolla, CA, United States). Nominal statistical significance was set at $P < 0.05$.

RESULTS

Patient characteristics at baseline

Forty-eight HCV RNA positive patients were enrolled with median NGAL of 68.5 ng/dL (range: 136-27). Main characteristics of the population are summarized in Table 1. Seventeen (35%) patients were < 65 years old, 25 (52%) patients were females, 10 (21%) patients suffered from blood hypertension. Median NGAL was: 63 ng/dL (111-27) for patients < 65 years old and 77 ng/dL (136-28) for patients ⩾ 65 years old ($P = 0.1353$); 70 ng/dL (132-27) for males and 63 ng/dL (111-27) for females ($P = 0.7822$); 72.5 ng/dL (102-28) for patients with hypertension and 66.5 ng/dL (136-27) for patients with normal blood pressure ($P = 0.7756$).

Differences in NGAL were not significant among patients ranked by HCV viral load, FIB-4 score and APRI. Quantitative HCV RNA was available in 36 patients, 19 (53%) of them having HCV RNA < 1000000 copies/mL (*i.e.,* low HCV RNA group). Median NGAL at baseline was 70 ng/dL (range: 132-27) *vs* 63 ng/dL (121-28), respectively. For FIB-4, 8 (17%) patients with FIB-4 ⩽ 1.45 had median NGAL of 60.5 ng/dL (111-27), 16 (33%) with FIB-4 from 1.45 to 3.25 had median NGAL of 82 ng/dL (136-36) and 24 (50%) with FIB-4 ⩾ 3.25 had median NGAL of 74.5 ng/dL (132-28). Regarding APRI, 10 (21%) patients were ⩽ 0.5, 22 (46%) were between 0.5 and 1.5, and 16 (33%) were ⩾ 1.5. Median NGAL values were 60.5 ng/dL (range: 136-27), 74.5 ng/dL (124-36) ng/dL and 80 ng/dL (132-28) in the three APRI groups, respectively.

According to eGFR ranking, 25 (52%) patients had eGFR ⩾ 90 mL/min, 19 (40%) had Egfr ⩾ 60 but less than 90 mL/min and 4 (8%) had eGFR < 60 mL/min. Median NGAL was 63 ng/dL (range: 136-36), 70 ng/dL (124-27) and 102.5 ng/dL (132-88) in the three groups, respectively. Statistical analysis showed significant

Table 1 Characteristics of the population at baseline *n* (%)

Patients' characteristics	Overall population (*n* = 48)	Patients treated with DAAs (*n* = 8)
Qualitative variables		
Gender		
Female	25 (52)	1 (12)
Male	23 (48)	7 (88)
HCV RNA genotype		
1a	3 (6)	0 (0)
1b	29 (61)	8 (100)
2a/2c	4 (8)	0 (0)
3	1 (2)	0 (0)
4	2 (4)	0 (0)
Not available	9 (19)	0 (0)
Quantitative variables, median (range)		
Age (yr)	67.0 (36.0-84.0)	63.5 (51.0-69.0)
eGFR (mL/min)	90.0 (30.0-111.7)	93.0 (77.0-109.0)
FIB-4	3.0 (0.5-18.3)	3.7 (1.5-9.0)
AST (IU/L)	44.0 (17.0-180.0)	70.0 (28.0-103.0)
ALT (IU/L)	45.0 (12.0-268.0)	53.5 (35.0-171.0)
Albumin (mg/dL)	4.2 (3.0-4.8)	4.3 (3.3-4.6)
Total bilirubin (mg/dL)	0.8 (0.3-2.2)	0.6 (0.3-1.0)
PLT (n/μL)	141000 (13500-312000)	132000 (71000-229000)
WBC (cells/μL)	5560 (2220-11500)	6245 (2790-9200)
HCV-RNA (copies/mL)	776500 (90100-19500000)	2330000 (793000-3250000)

HCV: Hepatitis C virus; eGFR: Estimated glomerular filtration rate; FIB-4: Fibrosis four index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PLT: Platelet; WBC: White blood cell; DAAs: Directly acting antivirals.

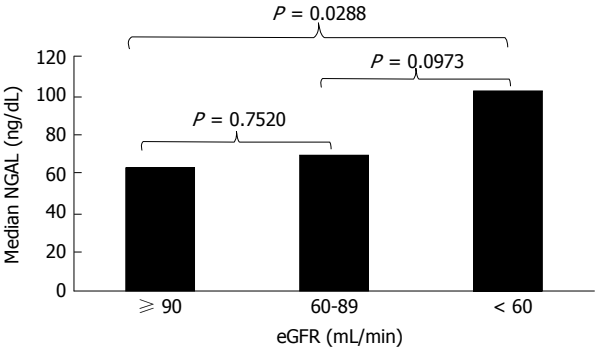

Figure 1 Plasmatic neutrophil gelatinase associated lipocalin at baseline according to estimated glomerular filtration rate ranking. eGFR: Estimated glomerular filtration rate; NGAL: Neutrophil gelatinase associated lipocalin.

differences in median NGAL values only in patients with eGFR < 60 mL/min *vs* patients with eGFR ≥ 90 mL/min (Figure 1; $P = 0.0288$).

Spearman correlation analysis showed statistically significant positive correlation between NGAL and age (Spearman $r = 0.341$; 95%CI: 0.05450-0.5759; two-tailed $P = 0.0088$) and a negative correlation between NGAL and eGFR values (Spearman $r = -0.257$; 95%CI: -0.5164 to 0.04421; two-tailed $P = 0.0419$) (Figure 2). Not statistically significant results were obtained when NGAL was correlated FIB-4 score ($P = 0.413$) or APRI ($P = 0.7430$).

In 6 (12.5%) patients, NGAL exceeded the upper limit of the reference interval (NGAL > 118.11 ng/mL). When patients with NGAL > 118.11 ng/dL were compared with patients with NGAL ≤ 118.11 ng/dL, no statistically significant differences were present for age, gender, CKD classification and liver fibrosis ($P > 0.05$). In fact: 6/6 (100%) patients with NGAL > 118.11 ng/mL *vs* 25/42 (60%) patients with NGAL ≤ 118.11 ng/mL were ≥ 65 years old; 3/6 (50%) patients with NGAL > 118.11 ng/mL *vs* 20/42 (48%) patients with NGAL ≤ 118.11 ng/mL were males; 1/6 (17%) patients with NGAL > 118.11 ng/mL *vs* 7/42 (17%) patients with NGAL ≤ 118.11 ng/mL had a KDIGO CKD ≥ G3a; 4/6 (67%) patients with NGAL > 118.11 ng/mL *vs* 26/42 (62%) patients with NGAL ≤ 118.11 ng/mL had a KDIGO CKD classification ≥ G2; 4/6 (67%) patients with NGAL > 118.11 ng/mL *vs* 19/42 (45%) patients with NGAL ≤ 118.11 ng/mL had a FIB-4 ≥ 3.25; 3/6 (50%) patients with NGAL > 118.11 ng/mL *vs* 13/42 (31%) patients with NGAL ≤ 118.11 ng/mL had an APRI ≥ 1.5.

Evaluation of renal parameters after one year

Serum creatinine and eGFR were collected after 1 year for 40 patients, while the remaining 8 were analyzed separately because they started HCV treatment in the meanwhile. No statistically significant differences were demonstrated in median NGAL values at baseline between patients with worsening eGFR *vs* patients with stable/improved eGFR at year 1. Indeed, median NGAL at baseline was 71.5 ng/dL (range: 136-36) in patients with worsening eGFR after 1 year, while it was 73.5 ng/dL (132-27) in patients with stable/improved eGFR after 1 year ($P = 0.4898$).

Evolution of renal parameters during HCV treatment

A separate prospective analysis was conducted in the 8 patients who started with antiviral therapy. Baseline

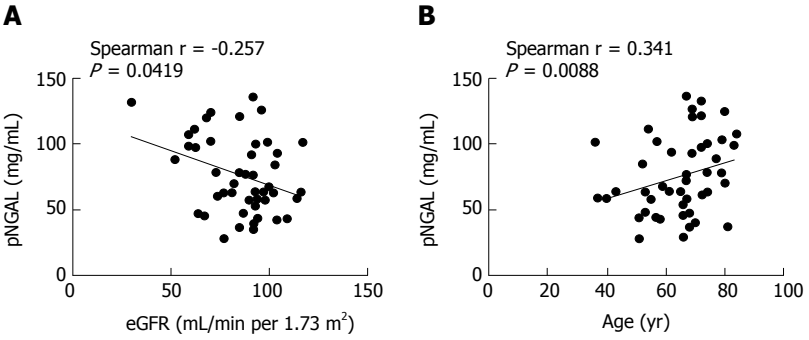

Figure 2 Univariate linear correlations between plasmatic neutrophil gelatinase associated lipocalin (NGAL) at baseline and estimated glomerular filtration rate (A) and NGAL at baseline and age (B). Data are represented as a scatter plot in which each point represents a patient. Correlation coefficient, Pearson *r* values, and statistical significance are indicated. *n* = 48 patients. eGFR: Estimated glomerular filtration rate; NGAL: Neutrophil gelatinase associated lipocalin.

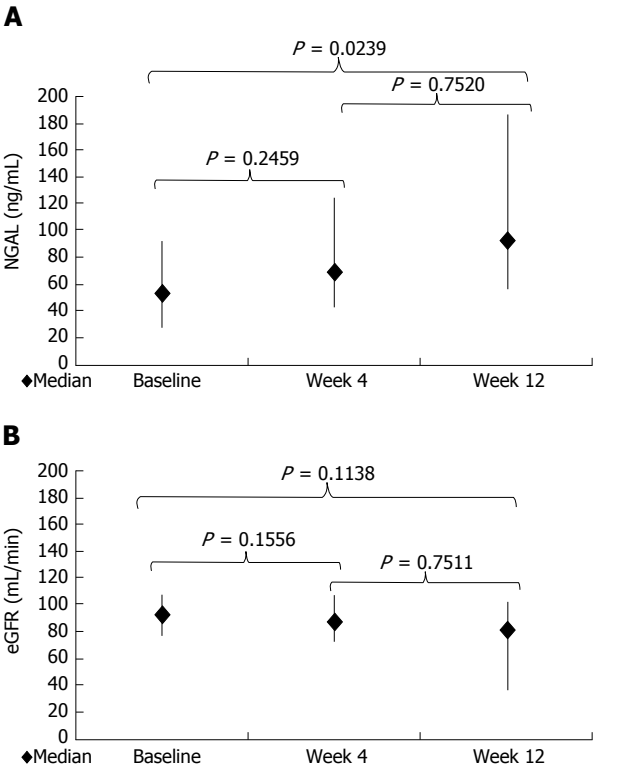

Figure 3 Evolution of plasmatic neutrophil gelatinase associated lipocalin (A) and estimated glomerular filtration rate (B) during the first 12 wk of hepatitis C virus therapy with directly acting antiviral containing regimens. eGFR: Estimated glomerular filtration rate; NGAL: Neutrophil gelatinase associated lipocalin.

characteristics of these patients are summarized in Table 1. Two patients started simeprevir (SMV) + daclatasvir (DCV) for 24 wk, while 6 patients started PEG-IFN + RBV + TVR for 12 wk followed by PEG-IFN + RBV for other 24 wk (36 wk overall). Three patients were relapser to previous treatments with PEG-IFN + RBV, while 5 were partial responders. At week 4, 2 patients had negative but detectable HCV RNA (⩽ 15 UI/mL) while 6 had undetectable HCV RNA. At week 12, HCV RNA was undetectable in all patients. Median NGAL was 53 ng/dL (range: 92-28) at baseline, 69 ng/dL (125-43) at week 4, and 93 ng/dL (186-56) at week 12 (P = 0.0239 compared with baseline value) (Figures 3A and 4A). Median eGFR was 93 mg/dL (109-77) at baseline, 86.5 mg/dL (108-72) at week 4, and 82.5 mg/dL (103-72) at week 12; no statistically significant differences were found for eGFR along the time points of the study (Figures 3B and 4B).

DISCUSSION

This is the first study which investigated NGAL, both before and during the first twelve weeks of therapy with DAA including regimens. Until now, other investigators evaluated NGAL but only in HCV positive patients with cirrhosis and before treatment with DAA containing regimens[23].

We found that, in patients not exposed to DAA, eGFR was below normality around 50%, while NGAL was in the range of normality in most individuals. Moreover, among the six patients with increased NGAL, two cases had eGFR normal. These results suggest that a discordance between the two methods exists when interpretation is "categorical". Despite these findings, in the overall population plasmatic NGAL was statistically correlated with eGFR. Particularly, NGAL was significantly higher in patients with eGFR < 60 mL/min than those with eGFR ⩾ 90 mL/min. It is difficult to explain apparent discrepancies with the current literature data because Alhaddad *et al*[23] previously demonstrated that HCV positive cirrhotic patients with eGFR < 60 mL/min had a significantly lower plasmatic NGAL than HCV positive cirrhotic patients with eGFR ⩾ 60 mL/min. Inclusion of both cirrhotic and non-cirrhotic patients in our study could be an explanation. In conclusion, we think that further studies should evaluate the rate of concordance between the two methods in diverse stages of liver disease. Also, it has to be seen whether these markers provide useful insights on the glomerular (especially detected by e-GFR) or tubular (especially detected by NGAL) damage in these conditions.

We were not able to find any correlations explaining the variability in NGAL values apart from age and incontrast with Bolignano *et al*[11] who, however, selected patients with non-terminal CKD of various etiologies. The correlation found in our study (increasing NGAL with increase in age) was consistent with the inverse

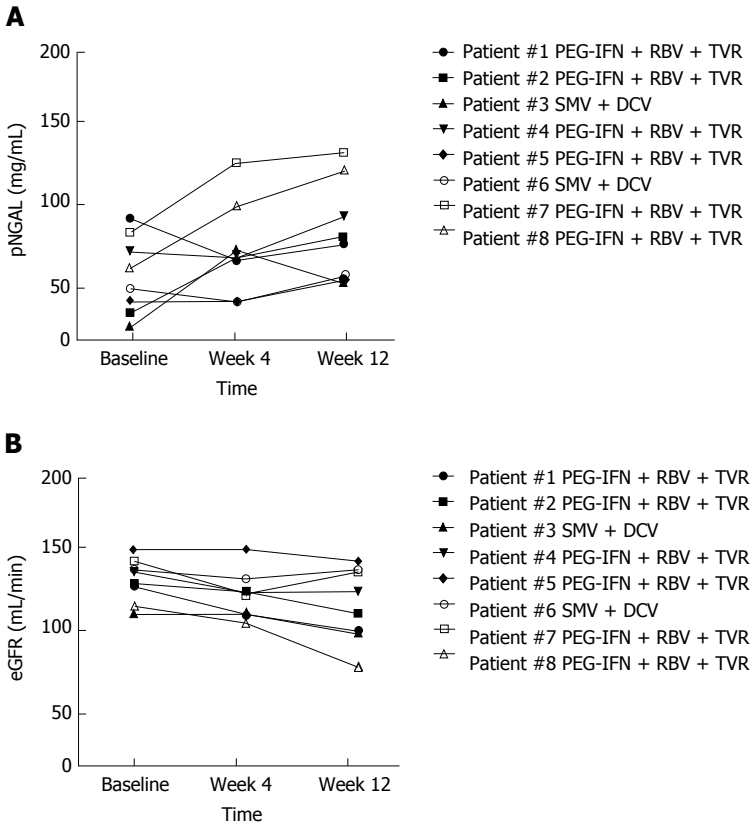

Figure 4 Evolution of neutrophil gelatinase associated lipocalin (A) and estimated glomerular filtration rate (B) in 8 patients during the first 12 week of hepatitis C virus therapy with directly acting antiviral containing regimens. Number of patients and respective treatment for hepatitis C virus infection are specified. PEG-IFN: Pegylated-interferon; RBV: Ribavirin; TVR: Telaprevir, SMV: Simeprevir; DCV: Daclatasvir; eGFR: Estimated glomerular filtration rate; NGAL: Neutrophil gelatinase associated lipocalin.

and expected correlation between eGFR and age. Importantly, NGAL was not correlated with stage of liver disease or burden of HCV RNA, suggesting that further mechanisms are implicated. Similarly, in their study, Gungor *et al*[22] did not find differences between cirrhotic patients and healthy controls but, at the same time, they found that a high plasmatic NGAL was associated with a higher risk of mortality in cirrhotic patients. Our data support the fact that NGAL is not influenced by HCV RNA, suggesting that glomerular and tubular damages have different pathogenic and clinical significances.

We did not find any signals pointing to a predictive role of NGAL for eGFR evolution during the follow-up. Indeed, contrary to this hypothesis, patients with a reduction of eGFR at 1 year had lower NGAL than patients with an improvement of eGFR at 1 year. Bolignano *et al*[11] found that patients with non-advanced CKD who progressed in their kidney disease during the follow-up period (median 18.5 mo, range 1-20) had a significantly higher urinary and serum NGAL levels at baseline than patients with non-advanced CKD who did not progress. Our patients did not suffer from significant CKD and renal function did not decrease significantly during the follow-up. These considerations, together with the small sample size and limited length of follow-up may have limited our possibility to demonstrate a significant prediction in this study.

We measured NGAL for the first time during therapy with DAA. Importantly, NGAL increased significantly from baseline to week 12, whereas eGFR did not change significantly, suggesting that tubular damage induced by drugs is not accompanied by glomerular impairment. An alternative hypothesis could be that NGAL increase is induced by a pro-inflammatory status, so this increase in NGAL would be a consequence, at least in part, of inflammation due to activity of antiviral drugs and consequent reduction of HCV RNA. Until now, data about relationship of HCV RNA and inflammation have been controversial. Indeed, some studies pointed to a pro-inflammatory role of HCV RNA, highlighting the enhanced activity of tumor necrosis factor-alpha and the consequent increase of some pro-inflammatory interleukins (ILs), such as IL-10, in presence of detectable HCV RNA[32,33]. Instead, other studies showed that a higher HCV RNA load was correlated with a lower value of C-reactive protein (CRP) and that levels of other ILs, such as IL-6, were correlated with liver fibrosis rather than with HCV RNA load[34]. Lastly, successful IFN-free regimens resulted in improved functional responses by natural killer cells (such as degranulation and TRAIL expression) to *in vitro* stimulation with IFNα[35]. Further studies are necessary to understand evolutions of immunologic or inflammatory markers (including NGAL) after DAA treatment and the underlying mechanisms.

According to this hypothesis that increase of NGAL during HCV treatment is due to drug toxicity, it is likely that our results were scarcely affected by the use of PEG-IFN and RBV. In fact, these two molecules did not demonstrate significant nephrotoxicity, while the reverse may be true because renal impairment reduces excretion of both PEG-IFN and RBV, leading to an increased risk of side effects, such as hemolytic anaemia due to intra-erythrocyte accumulation of RBV[5,36].

All regimens analyzed in our study included a NS3/4A protease inhibitor (*i.e.*, TVR or SMV). Currently, far more data are available about renal safety of TVR than renal safety of SMV or other DAAs. The available data showed that TVR causes a significant but reversible reduction of eGFR which may be associated with an increase of side effect related to the drug[37,38]. Our study showed that, aside reduction of eGFR, there was also an increase of NGAL which could be a consequence of tubular damage. Currently, there is a paucity of data about renal safety of interferon free regimens including DAAs different from TVR. Particularly, it is unknown if second or third generation DAAs have a direct nephrotoxic effect. In our study, both patients treated with SMV + DCV experienced an increase of NGAL with a concomitant eGFR decrease during the first 12 week of treatment. Our results need to be confirmed by further and larger studies, however.

Several limitations may affect the study conclusions: (1) sample size was small and length of follow-up was limited; (2) correlations of NGAL with other factors which could have impaired renal function, such as diabetes, cryoglobulinaemia, treatment with non NSAIDs or diuretics were not evaluated; and (3) we did not measure urinary NGAL or inflammatory molecules such as CRP and ILs which could confirm or disprove the hypotheses above. Notwithstanding these limitations, we feel that our results are important and should provoke further investigations. In particular, we hypothesize that NGAL reveals kidney damage earlier than eGFR during DAA containing regimens.

However, many questions still remain to be answered. Indeed, it has to be established whether a clinical cut-off of NGAL may guide clinical decisions (*e.g.*, dosage modification or stopping of the offending drug). Also, it has to be evaluated whether NGAL could predict AKI during HCV treatment, especially in most-at-risk patients such as those with advanced cirrhosis and a high risk of renal complications (*e.g.*, hepato-renal syndrome). Lastly, cost-effectiveness studies need to be conducted to verify the hypothesis that NGAL should be routinely used to monitor kidney function during HCV treatment instead of (or in addition to) creatinine.

COMMENTS

Background

Neutrophil gelatinase associated lipocalin (NGAL) is a novel biomarker of renal impairment but also a marker of inflammation. For the first time, the authors evaluated the evolution of NGAL in patients infected by hepatitis C virus (HCV) before and during treatment with directly acting antivirals (DAAs).

Research frontiers

Kidney toxicity of new DAAs has not been established until now. The authors' results suggest that NGAL could provide information complementary to estimated glomerular filtration rate in monitoring the kidney toxicity of DAAs.

Innovations and breakthroughs

The literature suggests that NGAL is a good marker of acute kidney injury but no data are available about NGAL in HCV positive patients. This article adds to literature data about evolution of NGAL before and during HCV treatment with DAA containing regimens.

Applications

This study serves as an additional evidence supporting the fact that NGAL can reveal the presence of a kidney impairment before creatinine.

Terminology

NGAL is a small glycoprotein secreted by multiple human cells, such as epithelial cells (liver, kidney, lungs) and blood cells (neutrophils, monocytes and macrophages), filtered in the glomerulus and reabsorbed by the proximal tubules. An increase of plasmatic NGAL is considered to be an early predictor of acute kidney disease in various critical settings.

Peer-review

This study examined the measures of a novel biomarker for kidney function, NGAL, before and after HCV treatment with direct acting antivirals among 48 patients. The authors have performed a good study, the manuscript is interesting.

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LETTERS TO THE EDITOR

Lot to give, got to live - the restless minds of the "Liver on Tour" project

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Abstract

The Liver on Tour was a special project devoted to increase the public awareness on Liver Health and Liver Diseases that the Portuguese Association for the Study of Liver Diseases launched throughout the country in 2010.

Key words: Liver disease; Hepatology; Hepatitis; Public health

Core tip: The Liver on Tour was a special Project devoted to increase the public awareness on Liver Health and Liver Diseases that the Portuguese Association for the Study of Liver Diseases launched throughout the country, between mid-April and mid-June 2010, and consisted of a road show, travelling through and reaching all 18 district capitals of Portugal. It was mainly focused on giving simple, reliable and practical information about liver problems with emphasis in messages trying to raise public attention on Liver Health. In a ten million people country where there is evidence of more than 10% being affected with some form of liver diseases, all efforts need to be made to enroll everyone in this never ending battle. High priority to education, gathering on board committed doctors with expertise in mass communication and engage reluctant policymakers (pressured by well-informed voters): A formula that could be a brighter way to raise the standards in prevention, detection and management of liver diseases.

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TO THE EDITOR

The Liver on Tour (LOT) was a special Project devoted to increase the public awareness on Liver Health and Liver Diseases that the Portuguese Association for The Study of Liver Diseases (APEF) launched throughout the country, between mid-April and mid-June 2010, and consisted of a road show, travelling through and reaching all 18 district capitals of Portugal. It was mainly focused on giving simple, reliable and practical information about liver problems with emphasis in messages trying to raise public attention on Liver Health. "Meet the liver", "Care for your Liver", "Mind the Liver", were repeatedly announced sound bites, and had a professional media support so that liver problems reached the level of widespread lay public interest and, of course, politicians rule makers, while members of the APEF Board were literally on the road, claiming for help and protection for the liver.

Although pointing out the need for understanding and for taking care of the Liver, the deep goal was directed to the knowledge of the social and individual threats of Hepatitis, Alcoholic Liver Disease and The Obesity Epidemics. Hepatitis B and Hepatitis C were major players in these topics, and all efforts were made to promote public to decide to look (individually) for serological markers of infection to, give referral and guidance to liver specialists, creating an environment of highly awareness and concern on liver problems.

We believe that policy makers, in these times of funding constraints, will only be sensible to these matters if the public itself realizes how deep and broad impact liver diseases may cause either individually or collectively.

The scientific responsibility of all the pedagogical messages and contents of this Road Show (which included small conferences and media interviews) was from the Board of Direction of APEF.

It was one of our tasks to show how liver diseases may lead to social exclusion and how social exclusion can promote liver diseases. The stigmata and inadequate understanding and misperception often associated with liver diseases are truly cumbersome and there is an urgent need to overcome these misconceptions: Liver diseases were shown not to be caused only by self-inflicted deviated behaviors, previous drugs use and alcohol abuse, but instead that there is a wide spectrum of causes, chances and risk factors, that may affect each and any one of us, regardless social, cultural or, economic status.

Our itinerant road show, located in central areas of the main Portuguese towns, addressed precisely these topics, stressed the fact why liver diseases are generally referred as "silent killers", how the liver itself copes with inflammation and regeneration, which ubiquitous factors challenge our livers. The hepatotropic viruses of course, alcohol consumption, excess weight and medical conditions as diabetes that cooperate to damage liver cells, were object of description, thorough but simple, clear enough to raise the public curiosity and awareness without frightening but with plenty of hope and need for global commitment.

Several thousands of people, from teens to elderly, walked through the exhibition displayed in the truck, reading carefully all the texts and pictures, from historical trivia to the most advanced virtuosities of liver technology. A small questionnaire was distributed, trying to promote the understanding of eventual individual risk factors for liver diseases and to evaluate a possible previous perception of the exposed topics.

The media came along and had different approaches depending on the places and towns we visited. Local radios interviewed liver specialists, national TVs broadcasted several messages regarding liver health. Underage drinking and alcohol marketing were actively challenged. Our presence in several students' parties, the recruitment of local doctors to get themselves to be available for questioning and even for some counselling, were extremely important to bring everyone together. We were able to gather thousands of in loco measurements of body mass index, and thousands of leaflets with simple knowledge on viral hepatitis were widely distributed.

In a ten million people country where there is evidence of more than 10% being affected with some form of liver diseases[1], all efforts need to be made to enroll everyone in this never ending battle. High priority to education, gathering on board committed doctors with expertise in mass communication and engage reluctant policymakers (pressured by well-informed voters): A formula that could be a brighter way to raise the standards in prevention, detection and management of liver diseases.

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