Hepatitis C virus production requires apolipoprotein A-I and affects its association with nascent low-density lipoproteins

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ABSTRACT

Background/aims The life cycle of hepatitis C virus (HCV) is intimately linked to the lipid metabolism of the host. In particular, HCV exploits the metabolic machinery of the lipoproteins in several steps of its life cycle such as circulation in the bloodstream, cell attachment and entry, assembly and release of viral particles. However, the details of how HCV interacts with and influences the metabolism of the host lipoproteins are not well understood. A study was undertaken to investigate whether HCV directly affects the protein composition of host circulating lipoproteins.

Methods A proteomic analysis of circulating very low-, low- and high-density lipoproteins (VLDL, LDL and HDL), isolated from either in-treatment naïve HCV-infected patients or healthy donors (HD), was performed using two-dimensional gel electrophoresis and tandem mass spectrometry (MALDI-TOF/TOF). The results obtained were further investigated using in vitro models of HCV infection and replication.

Results A decreased level of apolipoprotein A-I (apoA-I) was found in the LDL fractions of HCV-infected patients. This result was confirmed by western blot and ELISA analysis. HCV cellular models (JFH1 HCV cell culture system (HCVcc) and HCV subgenomic replicons) showed that the decreased apoA-I/LDL association originates from hepatic biogenesis rather than lipoprotein catabolism occurring in the circulation, and is not due to a downregulation of the apoA-I protein concentration. The sole non-structural viral proteins were sufficient to impair the apoA-I/LDL association. Functional evidence was obtained for involvement of apoA-I in the viral life cycle such as RNA replication and virion production. The specific siRNA-mediated downregulation of apoA-I led to a reduction in both HCV RNA and viral particle levels in culture.

Conclusions This study shows that HCV induces lipoprotein structural modification and that its replication and production are linked to the host lipoprotein metabolism, suggesting apoA-I as a new possible target for antiviral therapy.

Significance of this study

What is already known about this subject?

- Chronic HCV infection is associated with major modifications of the host lipid metabolism.
- The lipoprotein machinery is instrumental in several steps of the life cycle of HCV (ie, blood circulation, cell attachment and entry, viral particle assembly and release).
- Circulating very low-density and low-density lipoproteins (VLDL and LDL) of HCV-infected patients cause alterations in the lipid metabolism of macrophages.

What are the new findings?

- Chronic HCV infection causes an impaired association of apolipoprotein A-I (apoA-I) with the circulating LDL particles of patients.
- The impaired apoA-I/LDL association also occurs in hepatoma cells infected by HCV cell culture viral particles or expressing the sole viral non-structural proteins.
- Impairment of the apoA-I/LDL association occurs during lipoprotein generation and is caused by the viral replication stage.
- Downregulation of apoA-I induces significant impairment of HCV replication, showing that the replication stage of the viral life cycle also requires apolipoproteins.
- Downregulation of apoA-I induces a significant decrease in HCV particle production in cell culture.

How might it impact on clinical practice in the foreseeable future?

- This study provides new insight into how HCV and the host lipoprotein machinery are reciprocally influenced.
- This evidence may result in new interest in this research area which may allow innovative antiviral strategies to be defined.

INTRODUCTION

Hepatitis C virus (HCV) has a major impact on public health with an estimated 170 million infected individuals worldwide, and is the primary reason for liver transplantation in Western Europe and the USA. Its infection causes chronic liver disease eventually leading to cirrhosis and hepatocellular carcinoma.[1] Several abnormalities of lipid metabolism such as hypo-β-lipoproteinaemia and liver steatosis have also been associated with chronic HCV infection.[2 3]

Many experimental studies have shown that the life cycle of HCV is directly linked to lipoproteins. In the plasma of HCV-infected patients, the viral particles are associated with host lipids and apolipoproteins to form the so-called lipo-viro particles, mainly present in the range of very low-

to low-density fractions.[4] In particular, immunoprecipitation and western blot analysis have shown that lipo-viro particles could contain apolipoprotein (apo) B-100, apoB-48, apoE, apoC-I, apoC-II and apoC-III.[4–6] Notably, HCV infectious particles generated by the HCV cell culture (HCVcc) system, the only model which mimics the entire life cycle of the virus,[7] have sedimentation velocity and buoyant density profiles similar to those described for patients with HCV,[8] although their molecular composition has not been fully elucidated.

In addition to CD81, claudin-1 and occludin, the multistep process of viral cell entry is also mediated by components of lipoprotein uptake pathways such as glycosaminoglycans, low-density lipoprotein receptor and scavenger receptor B1.[9]

HCV cell entry, mediated by a pH- and clathrin-dependent endocytosis, is increased by apoC-I present in the lipo-viro particles, which promotes fusion between viral and endosomal membranes.[10 11]

Replication of HCV, like all positive-strand RNA viruses, occurs in association with cytoplasmic membrane vesicles.[12] Proteomic analysis of these membrane vesicles recently showed that they are enriched in several proteins involved in lipid metabolism and lipoprotein generation (ie, apoB-100, microsomal triglyceride transfer protein (MTP), apoE and Acyl-coA synthetase 3 (ACSL3)).[13] Moreover, viral RNA replication has been shown to be dependent on cholesterol, sphingomyelin and fatty acid synthetic pathways.[14–16]

Finally, proteins required for apoB-100-containing lipoprotein assembly such as apoB-100, apoE, MTP and ACSL3 have been shown to be necessary for HCV virion production and infectivity.[13 17–22]

In view of these findings, it is not surprising that HCV affects more than one aspect of lipoprotein metabolism. For instance, a direct correlation has been observed between the virus and inhibition of apoB-100 secretion and MTP activity.[23 24] Moreover, we recently found that the circulating lipoproteins of HCV-infected patients induce a change in the cellular lipid metabolism of human monocyte-derived macrophages. In these cells the very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) isolated from HCV-infected patients induce a decrease in the production of cholesterol ester and triglycerides (TG) and an increase in TG production, respectively.[25] One possible explanation for this observation is that HCV may directly affect the molecular composition of host circulating lipoproteins.

In this study we attempted to verify this hypothesis by performing a proteomic analysis of circulating VLDL, LDL and high-density lipoprotein (HDL) fractions derived from either HCV-infected patients or healthy subjects using a two-dimensional gel electrophoresis (2-DE) tandem mass spectrometry (MALDI-TOF/TOF) approach. We found a specific variation in the LDL fraction of HCV-infected patients which had a lower apoA-I content. By using HCV cellular models (HCV subgenomic replicons and HCVcc system), we demonstrated that the sole viral replication is sufficient to affect the association of apoA-I with apoB-100 present in the low-density fraction. Finally, using a siRNA-based approach, we collected evidence for a functional role of apoA-I in different steps of the life cycle of HCV such as RNA replication and viral production in culture.

MATERIALS AND METHODS

Patients

Thirty-one HCV-infected patients were recruited from the National Institute for Infectious Diseases L Spallanzani. All participants were in-treatment naïve HCV mono-infected with either genotype 1 or genotype 3 viruses.

Lipoprotein isolation

Blood samples in EDTA-containing tubes (BD Biosciences, San Jose, California USA) were obtained from donors after overnight fasting. Plasma was obtained by centrifugation (10 min, 1300×g) at room temperature. Lipoproteins were isolated according to the method of Schumaker and Puppione.[26] Briefly, plasma was ultracentrifuged in a Ti 70 rotor (Beckman) at 40×10^3 rpm for 18 h at 8°C. The heavily light-scattering layer of the VLDL fraction on the top of the tube was collected by glass pipette. Samples were then brought to a density of 1.063 g/ml with solid potassium bromide (KBr), ultracentrifuged as above and the LDL fraction collected. For collection of HDL, samples were brought to a density of 1.21 g/ml with solid KBr and ultracentrifuged as above.

For proteomic analysis, a second density gradient ultracentrifugation step was introduced to improve lipoprotein enrichment and purity. KBr was added to the isolated lipoprotein fractions to give a final density of 1.21 g/ml (VLDL and LDL) or 1.31 g/ml (HDL). These solutions were gently layered with two sodium bromide solutions (density 1.063 g/ml and 1.006 g/ml for VLDL; density 1.21 g/ml and 1.063 g/ml for LDL; density 1.24 g/ml and 1.21 g/ml for HDL, respectively) and ultracentrifuged as described above. Lipoprotein fractions were collected from the top of the tube.

Lipoprotein pools used for the first proteomic analysis (shown in figure 1) were obtained by pooling plasma samples and isolated as described above. For the second proteomic analysis (shown in figure 2), lipoproteins were first isolated from a single donor, then half of the lipoprotein fractions were pooled and further ultracentrifuged at the appropriate density gradients.

Cell, virus and lipoprotein isolation

Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Sigma-Aldrich).

Rep60 cells harbouring the HCV genotype 1b (Con1) subgenomic replicon,[27] the NNeo/C-5B cell line derived from the genotype 1b HCV-N[28] and the HCV cell culture infection system HCVcc based on the HCV JFH-1 molecular clone[29] have been described previously. RepBlast cells were derived from Huh7 cells transfected with the same subgenomic replicon or Rep60 cells in which the Neomicin gene has been substituted with a blasticidine resistance gene.

For lipoprotein production, 6×10^6 cells were plated in 15 cm dishes in 20 ml of medium. Huh7.5.1 were infected with JFH1-derived virus (MOI=0.1) 2 days before plating. After 3 days of cultivation the culture media were collected and the lipoproteins were isolated by the KBr method as described above.

Proteomic analysis, densitometric analysis, western blotting, ELISA assay, immunoprecipitation, RNA interference, real-time PCR and HCV RNA copy number quantification are reported in the online supplement.

RESULTS

ApoA-I levels are decreased in the LDL particles of HCV-infected patients

The aim of this study was to test whether the circulating lipoproteins of HCV-infected patients have a different protein composition from that of healthy donors (HD). Initially we focused our attention on in-treatment naïve patients infected with the viral genotype 1. Highly enriched VLDL, LDL and HDL fractions were obtained from plasma pools (10 HCV-infected patients and 9 HD) by a sequential two-step ultracentrifugation procedure using KBr density gradients. After delipidation,

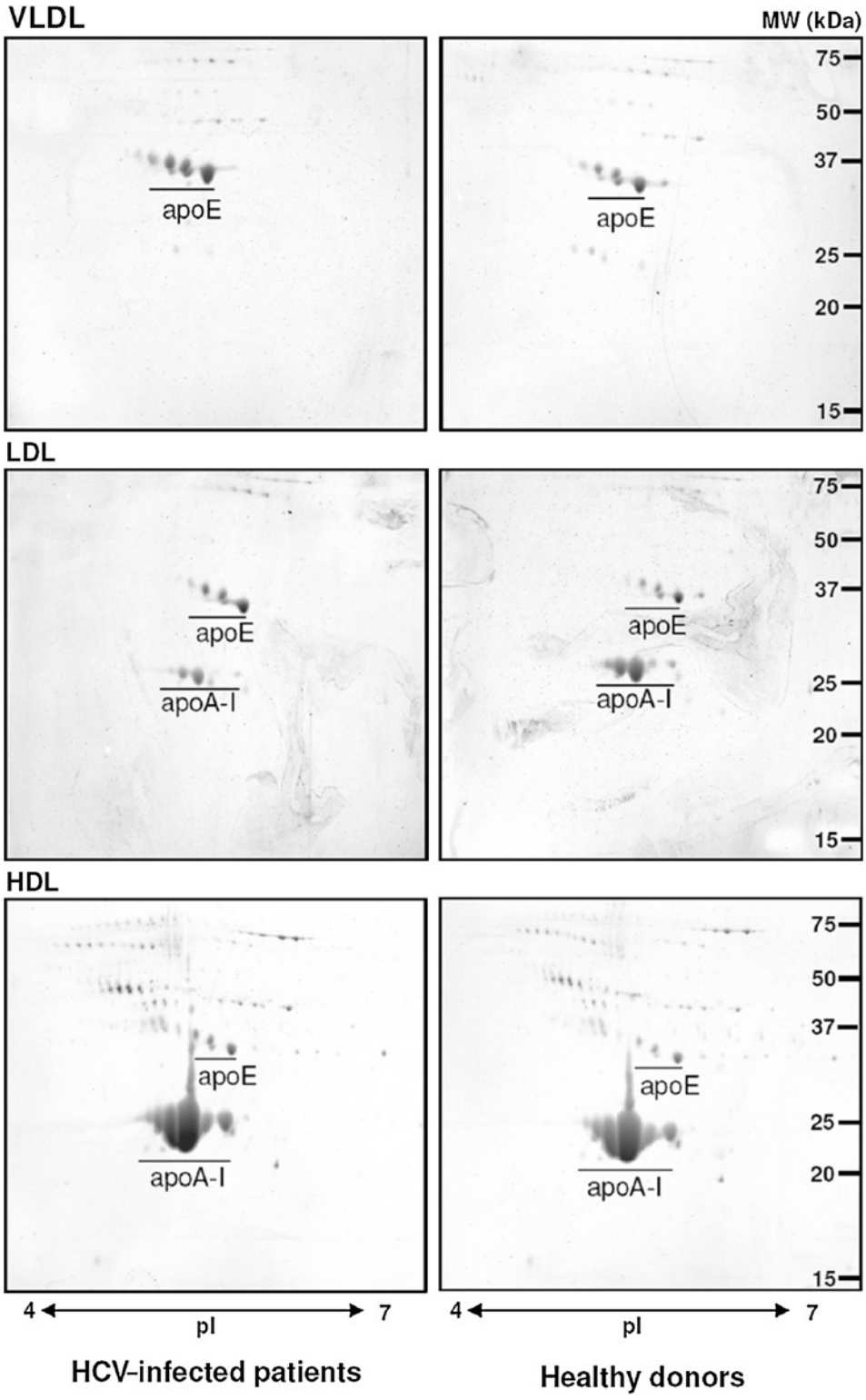

Figure 1 Two-dimensional gel electrophoresis (2-DE) protein pattern of lipoproteins isolated from patients infected with hepatitis C virus (HCV) or healthy donors (HD). Proteins (300 μg/sample) extracted from VLDL, LDL and HDL of the first set of donors (HCV n=10; HD n=9) were separated by 2-DE, detected by Sypro Ruby staining and analysed by MALDI-TOF/TOF mass spectrometry. ApoE and apoA-I protein spots, orientation of the pH gradient and approximate apparent molecular mass ranges are indicated. apo, apolipoprotein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

proteins present in the different lipoprotein fractions were extracted, resolved by 2-DE and stained with Sypro Ruby (figure 1). Densitometric analysis showed a statistically significant decrease in four protein spots, which were identified as isoforms of apolipoprotein (apo)A-I by MALDI-TOF/TOF analysis (data not shown), specifically in the LDL fraction of HCV-infected patients (central panels; normalised volume spot ratios 2.64-fold between HD vs HCV-infected patients). No statistically significant differences in the VLDL and HDL fractions (top and bottom panels, respectively) were found by densitometric analysis.

In order to confirm this finding by means of another biological replicate and to measure the variability in apoA-I levels between individuals, we used a second set of donors, isolating lipoprotein fractions from seven in-treatment-naïve genotype 1 HCV-infected patients and 7 HD (table 1). These samples were analysed both in pools by proteomics and separately by western blotting and 1-DE coupled to densitometric analysis.

Proteins from pooled lipoproteins were extracted, resolved by 2-DE and stained with Sypro Ruby. Image analysis confirmed the results obtained with the first set of samples, showing a decrease

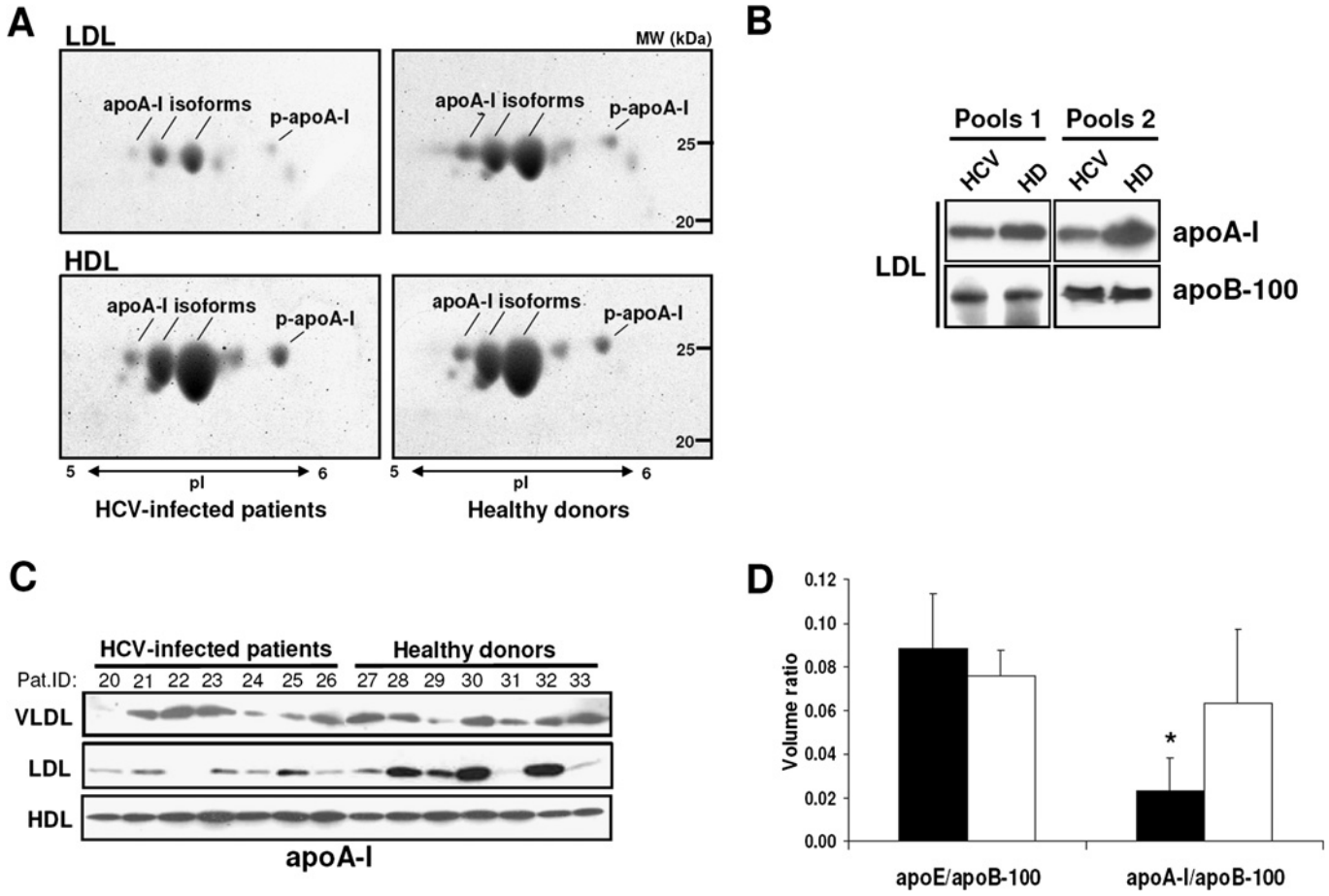

Figure 2 Association of apolipoprotein A-I (apoA-I) with low-density lipoprotein (LDL) is decreased in hepatitis C virus (HCV)-infected patients. (A) Magnification area of apoA-I protein spots in the two-dimensional electrophoresis gels of LDL (300 μg/sample) or HDL (100 μg/sample), purified from a second set of donors (HCV n=7; HD n=7). pH gradient and approximate apparent molecular mass ranges are indicated. (B) Western blotting for apoA-I and apoB-100 in the LDL of the two set of pools analysed by proteomics in figures 1 and 2A. (C) Western blotting for apoA-I in VLDL, LDL and HDL fractions purified individually from donors of the second set of pools analysed in figure 2A. Different amounts of proteins were used: VLDL, 10 μg; LDL, 1 μg; HDL, 0.5 μg. (D) ApoE/apoB-100 and apoA-I/apoB-100 ratio in healthy donors (black columns) and HCV-infected patients (white columns) were determined by densitometric analysis. Proteins (1 μg) of the LDL were separated by SDS-PAGE, stained with Sypro Ruby and the fluorescence intensities of the protein bands corresponding to apoE, apoA-I and apoB-100 were measured (see online material for details). Data are reported as mean±SD. *p<0.01. apo, apolipoprotein; HCV, hepatitis C virus; HD, healthy donors; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

in apoA-I levels in the LDL fraction of HCV-infected patients compared with those of HD. Figure 2A shows the magnification area of the four apoA-I protein spots in the LDL (top panels) or HDL (lower panels) preparative gels, as identified by MALDI-TOF/TOF analysis (data not shown). The more basic isoform (indicated with p-apoA-I) contains the N-terminal six amino acid propeptide RHFWQQDEPPQSPWDR, as revealed by the presence of a strong signal at 2108.98 mass/charge in the mass spectrum (data not shown); the electrophoretic mobility of the other three isoforms suggests that they differ in the phosphorylation status.[30] Image analysis of these gels showed that the normalised volume spot ratios of p-apoA-I and apoA-I isoforms were decreased in the HCV-LDL-like fraction compared with those of HD by 2.4-fold and 3.0-fold, respectively; again, no differences were found in the HDL fraction. Western blot analysis for apoA-I confirmed the proteomics results of both sets of LDL pools (figure 2B).

Since at least some viral particles in the plasma of HCV-infected patients are associated with apoB-100, increasing their density in the range of LDL although having a TG/apoB-100 ratio normally defining VLDL, we measured this ratio in the two sets of pools analysed by proteomics. A slight increase in the TG/apoB ratio was found in the lipoproteins of HCV-infected patients compared with HD (0.534 vs 0.480 and 0.833 vs 0.782 for HCV vs HD in pools 1 and 2, respectively), suggesting that part of our samples could contain viral particles. However, in our analysis we did not find viral proteins, at least in the amount of total protein used for the 2-DE preparative gel.

The variability in individual apoA-I levels was measured in VLDL, LDL and HDL particles by western blot analysis. As shown in figure 2C, variability among donors was found in VLDL and LDL fractions, while little variation was observed in HDL. The major differences in apoA-I levels between the two groups of samples were seen in the LDL fraction which had a generally decreased level in the HCV-infected patient group, thus confirming the results obtained with pools.

The results described so far were obtained by comparing samples normalised by total protein content. The limited number of proteins present in lipoprotein particles may render questionable this normalisation and it could be argued that the decrease in apoA-I levels observed in HCV-infected patients are due to a general decrease in the levels of the circulating LDL-like density fraction. In order to circumvent this problem, taking into account that each LDL particle contains only one apoB-100 molecule,[31] we calculated apoA-I/apoB-100 and apoE/apoB-100

Table 1 Characteristics of donors

	HCV genotype 3*	HCV genotype 1*	HD*
Age (years)	50.57±5.10	53.43±7.89	35.43±9.54
Sex (% male)	71.4	71.4	71.4
HCV RNA (IU/mL)	4.316.911±4.233.514	3.745.501±2.962.205	ND
ApoA-I (mg/dL)	140.57±43.17	145.66±22.05	143.60±18.79
ApoB-100 (mg/dL)	75.43±26.81	75.44±22.05	77.50±24.55
Chol-Total (mg/dL)	156.57±35.93	147.00±29.46	158.86±26.42
Chol-HDL (mg/dL)	47.43±23.44	48.86±16.65	46.86±8.32
Chol-LDL (mg/dL)	100.17±33.33	79.57±20.60	93.43±26.99
Triglycerides (mg/dL)	91.26±37.70	92.14±34.50	91.00±52.01
ALT (mU/mL)	89.67±41.24†	92.50±86.29†	23.43±15.30
γGT (mU/mL)	38.57±20.02	97.67±57.51‡	21.71±5.38
Glucose (g/L)	0.97±0.10	0.97±0.13	0.99±0.07

*n=7.
†p<0.005 vs HD.
‡p<0.001 vs HD and p<0.05 vs genotype 3.
ALT, alanine transaminase; apoA, apoB, apolipoprotein A and B; Chol, cholesterol; γGT, γ-glutamyl transferase; HCV, hepatitis C virus; HD, healthy donors; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

ratios by one-dimensional electrophoresis, Sypro Ruby staining and densitometric analysis. A decrease in the apoA-I/apoB-100 ratio was found in HCV-infected patients, while no significant differences were found in the apoE/apoB-100 ratio (figure 2D). Moreover, since no significant differences in the apoB-100 levels were found by densitometric analysis (not shown) or by western blotting (figure 2B), we conclude that, in HCV-infected patients, the reduction in apoA-I is due to a specific impairment of the association of apoA-I with LDL-like particles rather than a general decrease in the level of these lipoproteins.

We then investigated whether this observation could be extended to other HCV genotypes. The LDL levels of in-treatment-naïve patients chronically infected with HCV genotype 3 (ie, the most steatogenic viral genotype) were analysed. Lipoproteins were isolated from the plasma of seven patients, apoA-I and apoB-100 levels measured by ELISA assays, the apoA-I/apoB-100 ratios were calculated and then compared with those of patients infected with HCV genotype 1 or HD. As shown in figure 3, the average apoA-I/apoB-100 ratio of patients infected with HCV genotype 3 was significantly lower than those of patients with HCV genotype 1 and HD. Again this decrease was due to a lower apoA-I level (figure 3; central panel).

Taken together, these results show that, during chronic HCV infection, the association of apoA-I with circulating LDL particles is specifically impaired.

HCV replication affects the intracellular apoA-I association with LDL

LDL is mainly derived from plasma VLDL catabolism. However, cell experiments and studies in animal models support the concept that the liver is also capable of producing apoB-100-containing lipoproteins of IDL/LDL densities.[32–35] Using the HCV cellular models, we determined whether the presence of the virus directly affects the association of apoA-I with LDL.

First, we used the JFH1 HCVcc (genotype 2a) which generates infectious HCV particles in the Huh7-5.1 hepatoma cell line, thus recapitulating the complete viral life cycle.[7] After 4 days of cultivation the LDL released in the culture medium by either the parental or JFH1-infected cells were isolated using the standard KBr ultracentrifugation method. As shown in figure 4A, the presence of apoA-I was specifically reduced in the LDL fraction of JFH1-infected cells (top panel) while its level was unchanged in the culture medium and intracellularly. These results indicate that, in hepatocytes, HCV infection induces an impairment in the association of apoA-I with nascent LDL rather than a reduction in apoA-I synthesis or release.

In order to determine which stage of the HCV life cycle affects the apoA-I/LDL association, we used cells carrying the subgenomic HCV replicons which recapitulate exclusively the intracellular steps of RNA replication.[36] To minimise clone-specific and drug-specific effects, two different Huh7-derived cell lines were used: rep60 cells, which are resistant to G418 and derived from single cell cloning,[27] and repBlast cells, which are resistant to blasticidine and derived from a mixed population (unpublished); the two cell lines share the same HCV subgenomic replicon (genotype 1b). ApoA-I levels were measured in LDL-like density lipoproteins, in culture medium and whole cell extracts by western blotting. Figure 4B shows that compared with the parental cell line Huh7, both rep60 and repBlast cells had decreased apoA-I content specifically in the LDL-like density lipoproteins. These results were also confirmed by ELISA assays, which quantified an average of 50% reduction in the apoA-I/apoB-100 ratio in

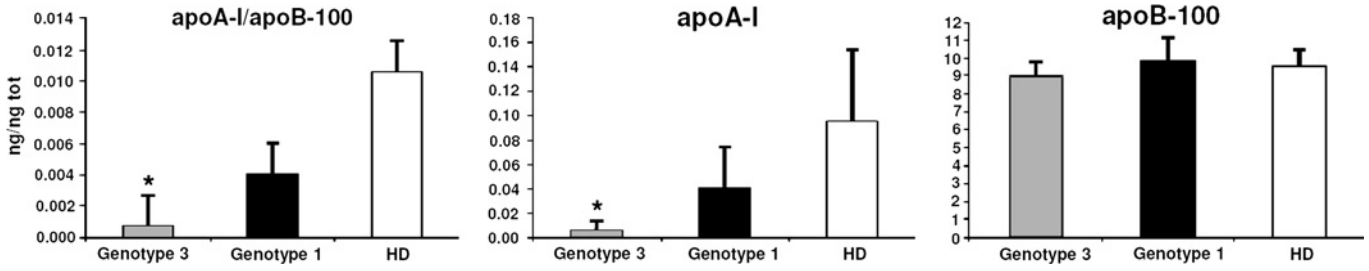

Figure 3 Association of apolipoprotein AI (apoA-I) with low-density lipoprotein (LDL) is decreased in patients infected with hepatitis C virus (HCV) genotypes 1 and 3. LDL fractions were isolated individually from either in-treatment-naïve HCV-infected patients (genotype 1, n=7; genotype 3, n=7) or healthy donors (HD, n=7). ApoA-I and apoB-100 amounts were determined by specific ELISA assay and reported as mean±SD. *p<0.02 vs healthy donors.

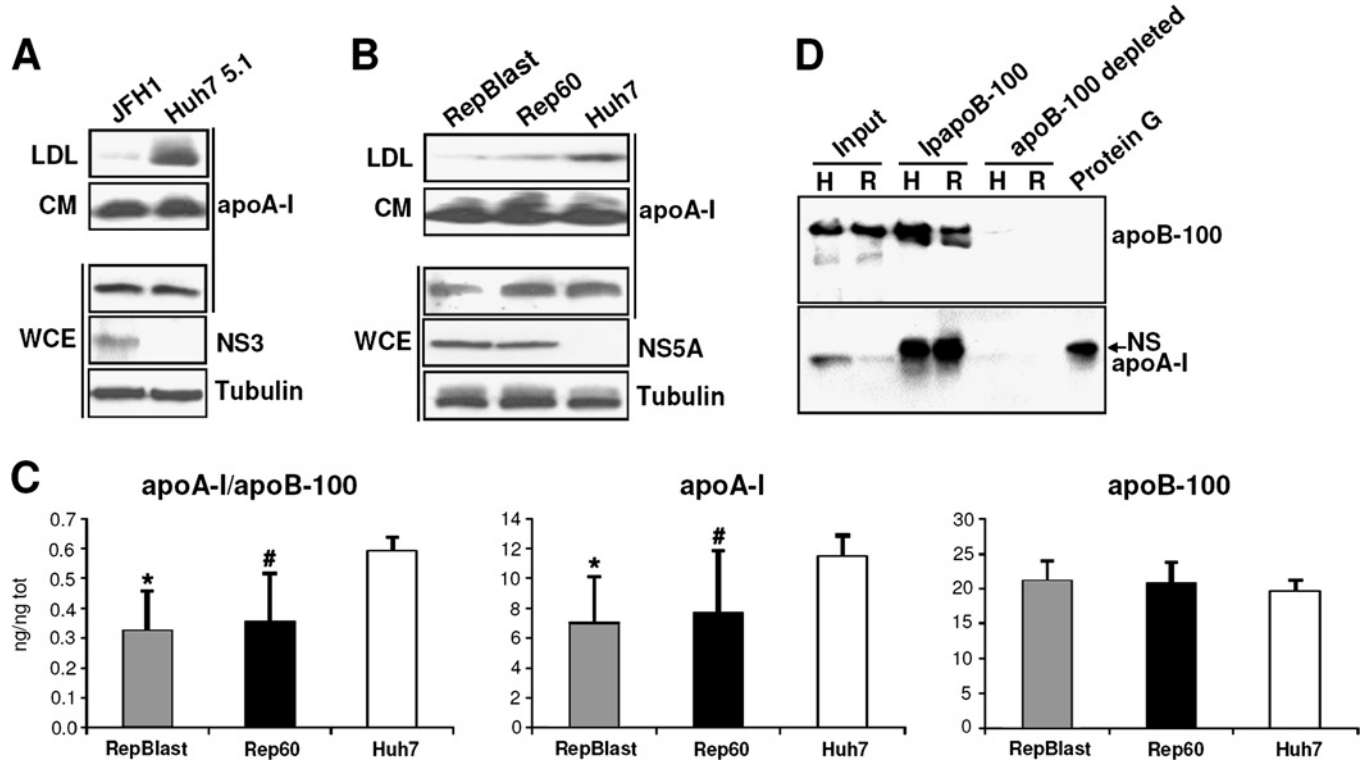

Figure 4 Expression of hepatitis C virus (HCV) non-structural (NS) proteins is sufficient to impair the association of apolipoprotein A-I (apoA-I) with nascent low-density lipoprotein (LDL). Western blot analysis of apoA-I in the low-density fraction (LDL), conditional medium (CM) and whole cell extract (WCE) of (A) parental (Huh7.5.1) and JFH1-infected cells or (B) parental (Huh7) and sub-genomic replicon-carrying cells rep60 and repBlast. Different amounts of samples were analysed in the LDL-like density lipoproteins (20 μg), CM (2 μl) and WCE (10 μg). NS3 and NS5A proteins were used as controls for the presence of viral proteins; tubulin was used as loading control. One representative experiment out of three is shown. (C) Levels of apoA-I and apoB-100 in the LDL fraction produced by rep60, repBlast and Huh7 cells were measured by ELISA assay. Four experiments were performed and reported as mean±SD. *$p<0.05$; #$p<0.02$ vs Huh7 cells. (D) LDL-like density fraction isolated from Huh7 (H) and repBlast (R) cells were immunodepleted of apoB-100 by two consecutive rounds of immunoprecipitation. LDL-like density lipoproteins (input), apoB-100-immunoprecipitated (IpapoB-100) and apoB-immunodepleted samples were analysed for the presence of apoA-I and apoB-100 by western blotting. IpapoB-100 shows a NS band close to the apoA-I signal due to a component of protein G (protein G lane). One representative experiment out of three is shown.

both cell lines (figure 4C, left panel). This was mainly caused by a decrease in the level of apoA-I (figure 4C, central panel) rather than an increase in the apoB-100 content (figure 4C, right panel).

We then investigated whether the decreased apoA-I level was associated with apoB-100-containing lipoproteins rather than with non-apoB-100 lipid-rich particles. We immunoprecipitated apoB-100 and performed western blotting against apoA-I. Unfortunately, all the anti-apoA-I antibodies tested (n=6) cross-reacted with some component of the protein G or A released in the immunoprecipitated samples (data not shown and NS band in figure 4D), so we immunodepleted apoB-100 in the LDL-like density lipoproteins generated by Huh7 and repBlast cells and tested the unbound material for the presence of apoA-I. As shown in figure 4D, after two rounds of apoB-100 immunoprecipitation, apoA-I was not detected in the flow-through (ie, apoB-100-depleted material), which suggests that all the apoA-I present in the LDL-like density particles produced by the two cell lines are associated with apoB-100. This result therefore strengthens the hypothesis that HCV affects the association of intracellular apoA-I with nascent apoB-100-containing lipoproteins rather than apoA-I detachment upon secretion.

Taken together, these results show that HCV affects the association of intracellular apoA-I with nascent LDL-like density lipoproteins, a specific phenomenon that could not be ascribed to a general reduction in apoA-I. Notably, the sole viral replication apparatus (ie, non-structural (NS) proteins) was sufficient to induce this impairment.

ApoA-I downregulation affects HCV replication and virion production

The results indicating that HCV NS proteins affect the association of apoA-I with LDL recall previous reports showing a direct interaction between apoA-I with NS5A in vivo and in vitro.[37 38] The pivotal role of NS5A in viral replication and virus production led us to speculate an involvement of apoA-I in these processes.

In order to test this hypothesis, apoA-I expression was silenced by specific small interfering RNA (siRNA). Three siRNAs targeting different regions of apoA-I were tested and the most efficient apoA-I downregulator was selected (data not shown) and used for the analyses.

To analyse a possible role for apoA-I in HCV replication, rep60 and repBlast cells were transfected with either the siRNA targeting apoA-I or a scrambled control siRNA containing a similar GC content (CTR-L). Only apoA-I siRNA significantly reduced the protein and mRNA levels of apoA-I, assessed 3 and 6 days after transfection (figure 5A and 5B), while it did not change the level of apoE (figure 5A) and did not cause a general cellular toxicity as assessed by total RNA and protein synthesis (data not shown).

Interestingly, in both rep60 and repBlast cells, apoA-I downregulation resulted in a significant decrease in the levels of NS5A protein (figure 5A) and HCV RNA (figure 5B) 6 days after transfection with only a slight effect 3 days after transfection, as measured by western blotting and quantitative real-time PCR,

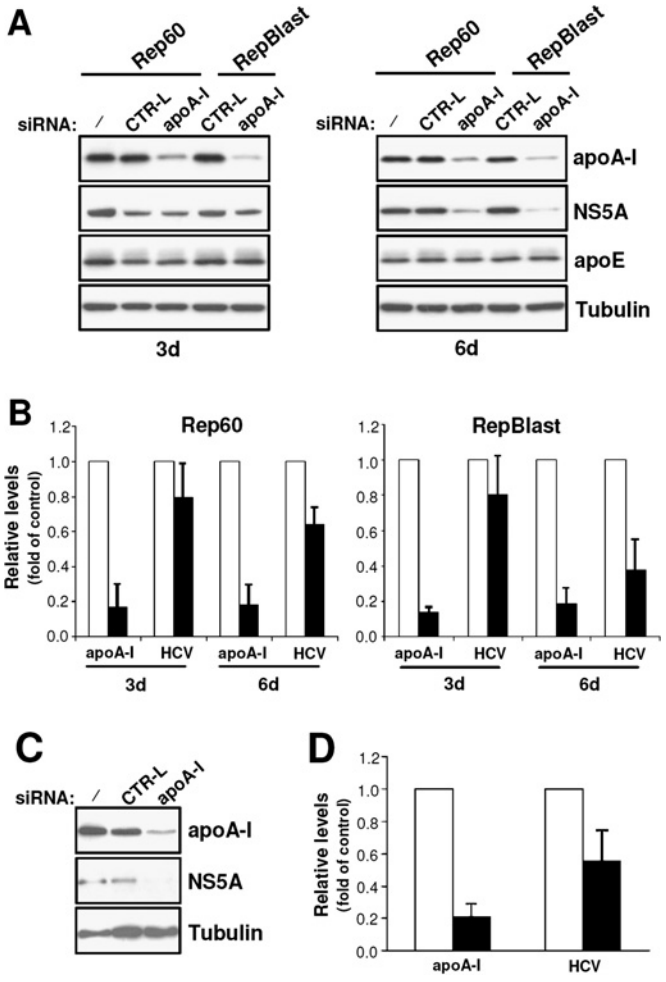

Figure 5 Apolipoprotein A-I (apoA-I) is required for replication of hepatitis C virus (HCV) in the replicon systems. (A,B) Downregulation of apoA-I expression in rep60 and repBlast cells was obtained by specific siRNA. (A) Western blotting analysis for apoA-I, NS5A and apoE was performed at 3 and 6 days after transfection in cells untransfected (/) or transfected with either scrambled control siRNA (CTR-L) or siRNA targeting apoA-I. (B) Levels of apoA-I mRNA and HCV RNA were measured by quantitative real-time PCR (qPCR) in cells transfected with CTR-L (white bars) or apoA-I (black bars) siRNA. (C,D) Downregulation of apoA-I expression in full-length genomic replicon harbouring cells by siRNA. (C) Western blotting analysis for apoA-I and NS5A was performed 6 days after transfection as described in (A). (D) Levels of apoA-I mRNA and HCV RNA were measured as described in (B). Western blotting analysis of one representative experiment out of at least four is shown; qPCR values were normalised to L34 mRNA levels and reported as fold changes relative to control samples (mean±SD of at least four experiments).

respectively. The same results were obtained in the full-length genomic replicon-carrying cells transfected with the specific apoA-I siRNA 6 days after transfection (figure 5C and 5D).

Overall, these data suggest a functional role for apoA-I in HCV replication.

Next, we tested whether apoA-I is involved in the production of infectious HCV particles by hepatocytes. To this aim, Huh7 cells were transfected with either apoA-I-specific siRNA or CTR-L siRNA and, after 24 h, infected with HCV at MOI=0.1 for 2 h. The effect of apoA-I siRNA on HCV production was assessed by quantifying the viral RNA released in the medium at different time points after infection. In control cells transfected with the CTR-L siRNA, the HCV RNA copy number increased by ≥100-fold during the 4 days of culture (figure 6A). In cells transfected with apoA-I siRNA, HCV production was significantly reduced (ranged 70–90%), starting from the second day after infection (figure 6A). These reduced levels of HCV RNA copy number were similar to those obtained by cells transfected with apoB-100 siRNA (reduction of 85–95%) that we used as a control for the inhibition of HCV viral production (data not shown).[13 17 19 21] Importantly, the decreased amount of HCV RNA present in the medium of apoA-I siRNA-treated cells is not caused by the inhibition of viral RNA synthesis; in these cells the intracellular HCV RNA levels remained unaffected from day 1 to day 3 after infection (figure 6B). However, it is worth noting that, at day 4 after infection, the intracellular HCV RNA level was decreased by ≈50%, thus confirming the results obtained with the replicon-carrying cells. The intracellular apoA-I mRNA levels were significantly decreased over the duration of the experiments in the apoA-I siRNA-treated cells (figure 6C).

Finally, we analysed the infectivity of the HCV virions generated by HCV-infected and siRNA-transfected Huh7 cells (ie, 4 days after infection) by infecting naïve Huh7.5.1 cells and measuring, after 48 h, the intracellular HCV RNA copy number by RT-qPCR. Huh7.5.1 cells infected with HCV generated by apoA-I-silenced cells showed a reduction of about 70% in the amount of intracellular HCV RNA compared with those infected with HCV produced by CTR-L siRNA-transfected cells (figure 6D), consistently with decreased HCV copy numbers measured in the culture medium (figure 6A).

Taken together, these data show that apoA-I function influences several steps of the HCV life cycle such as replication and virion production.

DISCUSSION

HCV gene expression has been associated with lipid dysmetabolism in both humans and animals.[39–41] Moreover, there is increasing evidence to indicate that the virus uses the host lipoprotein machinery for its life cycle and dissemination.[42] Analysis of the quality and quantity of lipoprotein components may reveal important information about the mechanisms responsible for virus-mediated lipid dysmetabolism. The recent development of ionisation methods coupled to mass spectrometry has allowed an in-depth biochemical analysis of the proteins associated with lipoproteins. In this study, by means of a 2-DE MALDI-TOF/TOF proteomic approach, we compared the protein composition of the main subclasses of circulating lipoproteins isolated from HCV-infected patients with those from healthy subjects.

The main finding of our studies was the identification of an LDL-specific reduction of apoA-I in HCV-infected patients, showing for the first time a lipoprotein structural alteration induced by this virus. This variation has not been observed previously, possibly because the parameters usually monitored in clinical studies are total plasma lipids and apolipoprotein concentrations and not the molecular composition of the lipoprotein particles. Indeed, in our analysis, no significant differences in the total lipid concentration or in total circulating apoA-I levels were measured between HCV-infected patients and HD (table 1).

ApoA-I, the major structural protein of the HDL, mediates most of the anti-atherogenic properties of these lipoproteins. It has been described as protecting against the development of premature atherosclerosis and acting as an anti-inflammatory agent.[43 44] Conversely, to the best of our knowledge, its role in the physiology of VLDL/LDL is as yet unknown, probably because it has only recently been associated with VLDL and LDL by proteomic analyses.[45 46] Since we recently found that lipoproteins derived from HCV-infected patients induce altered cellular lipid metabolism,[25]

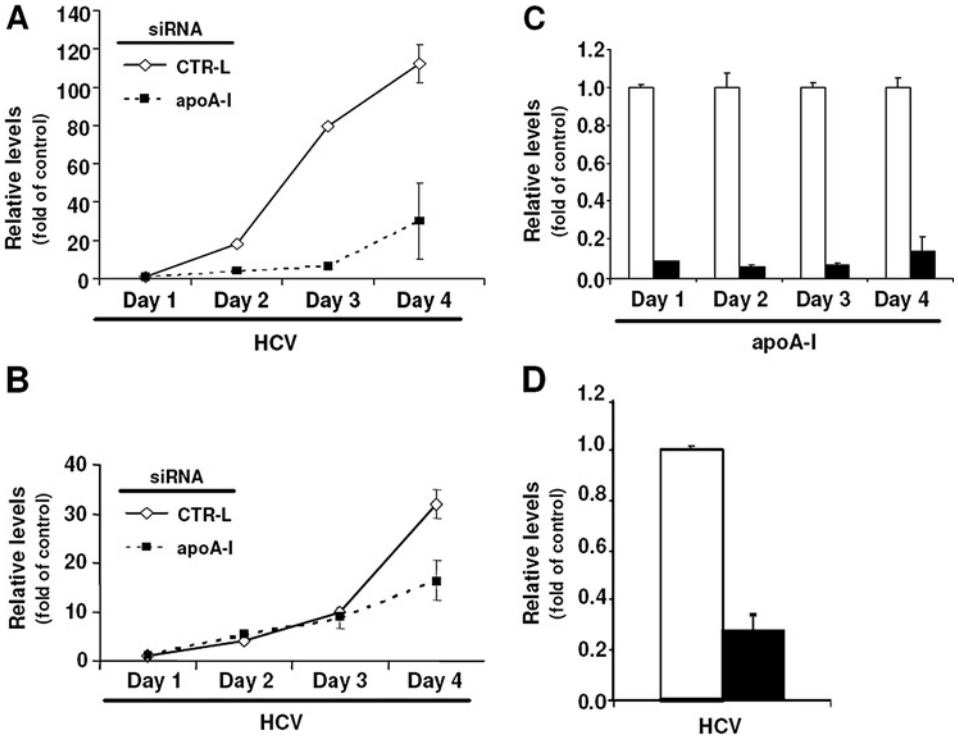

Figure 6 Apolipoprotein A-I (apoA-I) is required for production of hepatitis C virus (HCV) in culture. Huh7 cells were transfected with control siRNA (CTR-L) or apoA-I siRNA and, after 24 h, infected with HCV at an MOI of 0.1 (as described in the online supplement). The RNA present in the culture medium or intracellularly was collected at different time points. Results from three independent experiments are shown. (A) HCV RNA copy number present in the culture medium of CTR-L or apoA-I siRNA-treated cells, as indicated, was determined by RT-qPCR using the vector coding for the HCV JFH-1 genome as standard curve (see online supplement) and reported as mean±SD fold changes relative to CTR-L siRNA-treated cells on day 1 after infection. (B) Intracellular levels of HCV RNA of CTR-L or apoA-I siRNA-treated cells, as indicated, were measured by RT-qPCR, normalised for L34 mRNA levels and reported as mean±SD fold changes relative to CTR-L siRNA-treated cells on day 1 after infection. (C) Levels of apoA-I mRNA were measured by RT-qPCR in cells transfected with CTR-L (white bars) or apoA-I (black bars) siRNA. qPCR values were normalised to L34 mRNA levels and reported as mean±SD fold changes relative to control samples at each day of culture. (D) Infectivity of HCV viral particles generated, after 4 days of culture, by HCV-infected Huh7 cells transfected with either CTR-L (white bars) or apoA-I (black bars) siRNA. Naïve Huh7.5.1 cells were infected by HCV particles and their intracellular viral RNA levels were measured after 48 h by RT-qPCR. qPCR values were normalised to L34 mRNA levels and reported as fold changes relative to control samples (mean±SD of three independent experiments).

it is tempting to speculate that the decreased apoA-I level could have a role in this dysmetabolism. Experiments on the biogenesis and on the metabolic properties of apoB-100-containing lipoproteins in the absence of apoA-I, either in presence or absence of HCV proteins, are necessary to test this hypothesis.

LDL are derived mainly from VLDL catabolism in the circulation, but they can also be generated by the liver.[32–35] Although it is known that the exchange of apolipoproteins among lipoprotein particles and interconversion of particles occurs in the plasma compartment, our observations indicate that the HCV-induced decreased association of apoA-I with LDL probably has a cellular origin. In fact, among the apoA-I isoforms found to be decreased in the LDL of HCV-infected patients, mass spectrometry identified the intracellular p-apoA-I isoform (figure 2A), which carries the six amino acid propeptide known to be removed soon after the release of apoA-I in the circulation.[47]

Notably, we also observed the HCV-associated LDL-specific reduction in apoA-I in cellular models. This has a number of implications: (1) it supports the hypothesis that the virus-induced decrease in the apoA-I level in apoB-100-containing lipoproteins derives from their biogenesis rather than lipoprotein catabolism occurring in the circulation; (2) it provides a robust correlation between the virus and the LDL altered composition, well beyond the conclusions drawn by the limited numbers of HCV-infected patients analysed in this study; (3) it indicates that, as the secreted or intracellular apoA-I level was unaffected, the impaired apoA-I/LDL association is not due to a general downregulation of apoA-I; (4) the results obtained with subgenomic replicon-carrying cells, which recapitulate only the viral RNA replication model, indicate that the sole NS viral proteins are sufficient to impair the apoA-I/LDL association.

In the light of this latter point, it is tempting to speculate that LDL biogenesis and viral replication share elements which could be limiting factors for both processes. Common site and bridging molecules between viral replication and LDL biogenesis are indeed conceivable. In particular, the HCV replication complex induces the formation of a membranous web that derives from endoplasmic reticulum membranes[48] where the lipoprotein assembly starts to take place.[49] Most importantly, a direct interaction between NS5A and apoA-I has been reported to occur both in vitro and in vivo.[37 38]

The description of the possible interactions occurring between the platforms driving apoB-100-containing lipoprotein biogenesis and viral replication is hampered by the paucity of information regarding these processes. We therefore attempted directly to gather functional evidence on apoA-I involvement in viral replication. We found that the specific downregulation of apoA-I by siRNA in the two subgenomic replicon-carrying cells (rep60 and repBlast) induced a significant reduction in both HCV RNA and NS5A protein levels.

The membranous web, a membrane alteration of the endoplasmic reticulum induced by HCV, is the site of viral RNA synthesis. However, it has been reported that, in the presence of HCV core protein, viral replication is also present on lipid droplets.[50] Our data, obtained with both a genomic replicon non-infectious system (figure 5C) and with HCVcc (figure 6), suggest that apoA-I is also necessary in the presence of viral structural proteins, thus indicating that apoA-I function is required for HCV replication in vitro. Interestingly, it has been reported that, in liver biopsies of HCV-infected patients, viral RNA could be found associated with apoA-I,[51] indicating that this apolipoprotein could also play a role in viral replication in vivo.

Since in the absence of apoA-I expression viral replication is significantly hampered starting 5–6 days after siRNA transfection while a considerable reduction in apoA-I protein levels is observed as early as 2 days after transfection, it is likely that apoA-I acts

indirectly in the replication complex through other molecules involved in this process. However, we can recall as a possible mechanism the apoA-I function in intracellular cholesterol efflux,[52] which is known to be important in the HCV life cycle.[53] Further evidence is needed to confirm this hypothesis and possibly to elucidate the specific molecular mechanism.

In addition to this role in HCV replication, we found that apoA-I also has a role in the formation and/or release of viral particles. ApoA-I joins the other members of the lipoprotein family (apoB-100, apoE, MTP or ACLS3) known to be involved in HCV production,[13 17–22] which suggests that lipoproteins, in addition to viral assembly, release and infectivity, could also have an active role in viral replication, strengthening the link between HCV and the lipoprotein synthesis and secretion pathway.

In conclusion, our study provides an insight into the mechanisms that regulate the relationship between HCV and the metabolism of the host lipoproteins, and suggests that apoA-I may be a possible target for antiviral therapy.

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Competing interests None.

Patient consent Obtained.

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Hepatitis C virus production requires apolipoprotein A-I and affects its association with nascent low-density lipoproteins

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