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Viral Hepatiti

# PCSK9, apolipoprotein E and lipoviral particles in chronic hepatitis C genotype 3: Evidence for genotype-specific regulation of lipoprotein metabolism

Simon H. Bridge[1,2,*], David A. Sheridan[1,3], Daniel J. Felmlee[1,4], Mary M.E. Crossey[5], Fiona I. Fenwick[1], Clare V. Lanyon[2], Geneviève Dubuc[6,9], Nabil G. Seidah[7,9], Jean Davignon[6,9], Howard C. Thomas[5], Simon D. Taylor-Robinson[5], Geoffrey L. Toms[1], R. Dermot G. Neely[1,8], Margaret F. Bassendine[1,*]

[1]*Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom;* [2]*Faculty of Health and Life Sciences, Northumbria University, Newcastle upon Tyne, United Kingdom;* [3]*Institute of Translational & Stratified Medicine, Plymouth University Peninsula School of Medicine & Dentistry, United Kingdom;* [4]*Inserm U1110, University of Strasbourg and Center for Liver and Digestive Diseases, Strasbourg University Hospitals, 3 Rue Koeberlé, F-67000 Strasbourg, France;* [5]*Liver Unit, Department of Medicine, Imperial College London, St Mary's Hospital Campus, Praed Street, London, United Kingdom;* [6]*Hyperlipidemia and Atherosclerosis Research Group, Clinical Research Institute of Montréal (IRCM), Montréal, Canada;* [7]*Laboratory of Biochemical Neuroendocrinology, Clinical Research Institute of Montréal, Montréal, Canada;* [8]*Department of Clinical Biochemistry, Newcastle upon Tyne Hospitals NHS Foundation Trust, Royal Victoria Infirmary, United Kingdom;* [9]*University of Montréal, Montréal, Canada*

**Background & Aims**: Hepatitis C virus (HCV) associates with lipoproteins to form "lipoviral particles" (LVPs) that can facilitate viral entry into hepatocytes. Initial attachment occurs via heparan sulphate proteoglycans and low-density lipoprotein receptor (LDLR); CD81 then mediates a post-attachment event. Proprotein convertase subtilisin kexin type 9 (PCSK9) enhances the degradation of the LDLR and modulates liver CD81 levels. We measured LVP and PCSK9 in patients chronically infected with HCV genotype (G)3. PCSK9 concentrations were also measured in HCV-G1 to indirectly examine the role of LDLR in LVP clearance.

**Methods**: HCV RNA, LVP ($d$ <1.07 g/ml) and non-LVP ($d$ >1.07 g/ml) fractions, were quantified in patients with HCV-G3 (n = 39) by real time RT-PCR and LVP ratios (LVPr; LVP/(LVP + non-LVP)) were calculated. Insulin resistance (IR) was assessed using the homeostasis model assessment of IR (HOMA-IR). Plasma PCSK9 concentrations were measured by ELISA in HCV-G3 and HCV-G1 (n = 51).

**Results**: In HCV-G3 LVP load correlated inversely with HDL-C (r = −0.421; $p$ = 0.008), and apoE (r = −0.428; $p$ = 0.013). The LVPr varied more than 35-fold (median 0.286; range 0.027 to 0.969); PCSK9 was the strongest negative predictor of LVPr ($R^2$ = 16.2%; $p$ = 0.012). HOMA-IR was not associated with LVP load or LVPr. PCSK9 concentrations were significantly lower in HCV-G3 compared to HCV-G1 ($p$ <0.001). PCSK9 did not correlate with LDL-C in HCV-G3 or G1.

**Conclusions**: The inverse correlation of LVP with apoE in HCV-G3, compared to the reverse in HCV-G1 suggests HCV genotype-specific differences in apoE mediated viral entry. Lower PCSK9 and LDL concentrations imply upregulated LDLR activity in HCV-G3.



Keywords: HCV lipoviral particles; HDL-C; Apolipoprotein E; PCSK9; Low-density lipoprotein receptor; Apolipoprotein A1.

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* Corresponding authors. Addresses: NB119, Northumberland Building, Faculty of Health and Life Sciences, Northumbria University, Newcastle upon Tyne NE1 8ST, United Kingdom. Tel.: +44 (0) 191 227 4808 (S.H. Bridge). Institute of Cellular Medicine (Hepatology), 2nd Floor, William Leech Building, The Medical School, Newcastle University, Newcastle upon Tyne NE2 4HH, United Kingdom. Tel.: +44 (0) 191 213 7208; fax: +44 (0) 191 208 0723 (M.F. Bassendine).

*E-mail addresses:* simon.bridge@northumbria.ac.uk (S.H. Bridge), margaret.bassendine@newcastle.ac.uk (M.F. Bassendine).

*Abbreviations:* PCSK9, proprotein convertase subtilisin-like kexin type 9; HCV, hepatitis C virus; LVP, lipoviral particle; LDLR, low-density lipoprotein receptor; G, genotype; HCV RNA, hepatitis C virus ribonucleic acid; RT-PCR, reverse transcriptase polymerase chain reaction; IR, insulin resistance; HOMA-IR, homeostatic model of assessment of insulin resistance; ELISA, enzyme-linked immunoabsorbent assay; HCV-G3, hepatitis C virus genotype 3; HCV-G1, hepatitis C virus genotype 1; HDL-C, high-density lipoprotein cholesterol; r, coefficient of correlation; $p$, $p$ value; ApoE, apolipoprotein E; LVPr, lipoviral particle ratio; $R^2$, coefficient of determination; HIV, human immunodeficiency virus; VLDL, very low density lipoprotein; ApoB, apolipoprotein B; ApoC-I, apolipoprotein C-I; ApoA-I, apolipoprotein A-I; HSPG, heparan sulphate proteoglycans; LDL-C, low-density lipoprotein cholesterol; PEG-IFNα, pegylated interferon alpha; RBV, ribavirin; HBV, hepatitis B virus; HDV, hepatitis D virus; BMI, body mass index; SVR, sustained virological response; TG, triglyceride; ApoC-III, apolipoprotein C-III.

## Introduction

Deaths from hepatitis C virus (HCV) have now superseded HIV as a cause of mortality in the United States and these deaths occur disproportionately in middle-aged persons [1]. HCV genotype 3

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(HCV-G3) is the dominant genotype circulating in the UK and Southern Asia, and is most commonly found in European intravenous drug users [2]. Globally, it is estimated to account for 10–15% of the total number of HCV infections [3]. Chronic HCV-G3 patients have been found to have higher rates of late-stage liver disease and death [4], and HCV-G3 is now potentially the most difficult-to-treat genotype [5], most notably in these patients with decompensated cirrhosis.

HCV hijacks host lipid metabolism (reviewed in [6,7]) leading to steatosis and hypocholesterolaemia, which resolves with successful HCV treatment [8]. One striking feature of infectious HCV particles is their buoyant density, which is unusually low for an RNA virus [9], due to interaction with lipoproteins [10]. In patients' sera, HCV particles are found associated with very-low-density lipoprotein (VLDL) components (cholesterol, triglyceride, apolipoprotein (apo) B, apoE and apoCs), forming hybrid particles termed lipoviral particles (LVP). LVP can be immunoprecipitated with antibodies to apoB, apoE, and apoC1 [11,12]. ApoE is enriched on infectious particles and correlates with infectivity [13]; electron microscopy studies have visualised apoE on the HCV envelope [14,15]. Recent cryoelectron tomography studies have provided low-resolution 3D structural information on highly infectious virions and have shown that LVP incorporate apoB and apoA-I in addition to apoE [16].

Evidence has indicated that one function of HCV association with lipoproteins is the co-opting of lipoprotein receptors for attachment and entry into hepatocytes. The initial binding of LVP to cells occurs via interaction with low-density lipoprotein receptor (LDLR) and glycosaminoglycans present on heparan sulphate proteoglycans (HSPGs) [17] (reviewed in [18]). Both LDLR and HPSGs can interact with virion-associated apoE [19,20]. CD81 then mediates a post-attachment event in HCV entry [21].

Proprotein convertase subtilisin kexin type 9 (PCSK9) is a protease made by the liver. PCSK9 normally acts to enhance the degradation of the LDLR [22]. PCSK9 also modulates liver CD81 levels [23]. The circulating concentrations of human PCSK9 are directly correlated with LDL and total cholesterol concentrations in healthy human volunteers [24,25]. Reducing the concentration or activity of PCSK9 enables greater numbers of LDLR on the cell surface, thereby increasing the clearance of LDL particles from the circulation and reducing plasma LDL cholesterol. LDLR binds and internalises LDL via its unique proteins, apoB100 and apoE.

We have previously measured low-density apoB-associated LVP in patients with chronic HCV-G1 and found a positive association with serum triglycerides, insulin resistance (IR) [26], and serum apoE levels [27]. However, there is evidence that virus-host interaction impacts on host lipid metabolism in ways, which may be HCV genotype-specific [28–30]. Therefore, we have, for the first time, examined LVP in the plasma of patients chronically infected with HCV-G3. We have measured PCSK9 concentrations in these patients and compared to patients chronically infected with HCV-G1 [26] to indirectly examine the role of LDLR in LVP clearance.

## Patients and methods

### *Ethics*

The Newcastle upon Tyne Hospitals NHS Foundation Trust acted as sponsor and the study was approved by Northumberland Research Ethics Committee (REC number-07/H0902/45).

### *Patients*

Patients with chronic hepatitis C (CHC), attending the viral hepatitis clinic at the Freeman Hospital, Newcastle upon Tyne and St Mary's Hospital, Imperial College Healthcare Trust, London, were invited to participate and given a patient information leaflet explaining the study. Both treatment naïve and previous non-responders to a combination of pegylated interferon-α and ribavirin (PegIFNα/RBV) antiviral therapy were eligible for inclusion. Patients were excluded if they were alcohol dependent, being treated with concurrent lipid lowering therapy, co-infected with HBV, HDV, or HIV or had poor venous access due to injecting drug use. Non-responders were also invited to participate in a separate lipid-modulating intervention study [31] which, in addition to the above, excluded participants with a body mass index (BMI) $\geqslant$30. BMI was calculated as weight divided by the square of the height (kg/m$^2$). Thirty-nine patients with chronic HCV-G3 aged >18 years, who provided written informed consent, attended after a 12 h fast (fasting samples were essential in view of the post-prandial changes in buoyant density of HCV particles [32] and assessment of IR). Twenty-six of 39 patients were non-responders to PegIFNα/RBV. Eleven patients achieved a sustained virological response (SVR) and two patients were treatment naïve.

### *Clinical and laboratory assessment*

Each subject was assessed for past alcohol intake and medication history and the following data were collected: sex, age, weight, height, waist and hip circumference. A fasting serum sample was collected for lipid analysis. Total cholesterol, triglyceride (TG), HDL-cholesterol (HDL-C), and glucose were measured by standard automated enzymatic methods using an Olympus AU 640 analyser (Olympus, Watford, UK). LDL cholesterol (LDL-C) was calculated using the Friedewald equation [33]. Insulin was measured by immunoassay (ELISA – Dako UK Ltd, Ely, UK) and assessment of insulin resistance was performed by calculation of HOMA-IR using the formula: HOMA-IR = (fasting glucose [mmol/L] × insulin [mU/L])/22.5). Apolipoproteins A-I, E and B were measured on each sample by automated rate nephelometric methods (BNII, Dade Behring Marburg GmbH, Marburg, Germany). ApoC-III was measured using a quantitative sandwich ELISA (AssayMax – AssayPro, St. Charles, USA). Fasting plasma PCSK9 concentrations were measured by ELISA [25], which was developed and validated in a group of 254 healthy individuals (the method is described in the Supplementary material). In addition, we measured PCSK9 in a well-characterised HCV-G1 infected cohort (n = 51) [26].

### *Iodixanol gradient ultracentrifugation*

The LVP-containing fraction was harvested using a method described in the Supplementary material and previously described [26].

### *Quantification of HCV RNA*

The HCV RNA was quantitated using a method described in the Supplementary material.

### *PCSK9 ELISA*

Plasma PCSK9 concentrations were determined using a validated ELISA, using a method described in the Supplementary material.

### *Statistical methods*

All statistical methods used in this study are described in the Supplementary material.

## Results

### *Clinical characteristics of the patients with chronic HCV-G3*

The physical and metabolic characteristics of the 39 patients with CHC-G3 infection recruited in this study are summarised in Table 1 and fully detailed in Supplementary Table 1. Ethnicities

**Table 1. Characteristics of the patients with chronic hepatitis C virus genotype 3.**

| Characteristic | HCV-G3 (n = 39) | High LVP ratio (n = 20) | *p* value* |
|---|---|---|---|
| Sex, n (M/F) | 30/9 | 17/3 | - |
| Age (yr), mean ± SD | 48.2 ± 10.5 | 47.9 ± 6.8 | 0.86§ |
| Liver stiffness ≥13 kPa, n (%)† | 12 (30.8) | 8.8 (9.1) | 0.92¶ |
| Waist (cm), mean ± SD | 89 ± 10.1 | 89.8 ± 9.5 | 0.63§ |
| Waist/hip ratio, median (IQR) | 0.96 (0.08) | 0.97 (0.06) | 0.61¶ |
| BMI (kg/m²), mean ± SD | 25.3 ± 3.0 | 25.2 ± 2.6 | 0.92§ |
| Cholesterol (mmol/L), mean ± SD | 3.74 ± 0.90 | 3.73 ± 0.8 | 0.92§ |
| LDL-C (mmol/L), mean ± SD | 2.04 ± 0.67 | 2.2 ± 0.7 | 0.24§ |
| TG (mmol/L), median (IQR) | 0.85 (0.50) | 0.86 (0.63) | 0.88¶ |
| ApoB (g/L), median (IQR) | 0.60 (0.30) | 0.7 (0.20) | 0.07¶ |
| HDL-C (mmol/L), median (IQR) | 1.20 (0.68) | 1.1 (0.60) | 0.20¶ |
| ApoA-I (g/L), mean ± SD | 1.41 ± 0.35 | 1.35 ± 0.31 | 0.25§ |
| TG/HDL-C ratio, median (IQR) | 0.60 (0.66) | 0.72 (1.19) | 0.39¶ |
| ApoC-III (μg/ml), median (IQR) | 62.4 (55.22) | 62.3 (57.9) | 0.99¶ |
| ApoE (mg/L), median (IQR) | 30.0 (19.5) | 28.5 (14.0) | 0.39¶ |
| PCSK9 (ng/ml), median (IQR) | 60.7 (39.0) | 50.4 (45.1) | 0.04¶ |
| Glucose (mmol/L), median (IQR) | 5.20 (1.30) | 5.2 (1.30) | 0.92¶ |
| Insulin (mU/L), median (IQR) | 6.95 (5.62) | 8.1 (5.90) | 0.61¶ |
| HOMA-IR‡, median (IQR) | 1.53 (1.62) | 1.62 (1.79) | 0.64¶ |
| HCV genotype, n (3a/3b/unknown) | 27/1/11 | 14/0/5 | - |
| Total viral load $\log_{10}$(IU/ml), median (IQR) | 6.06 (0.78) | 5.87 (1.03) | 0.4¶ |
| LVP load $\log_{10}$(IU/ml), mean ± SD | 5.22 ± 0.90 | 5.53 ± 0.94 | 0.02§ |
| Non-LVP load $\log_{10}$(IU/ml), mean ± SD | 5.62 ± 0.86 | 5.42 ± 1.03 | 0.13§ |
| LVP ratio, median (IQR) | 0.286 (0.432) | 0.50 (0.36) | <0.001¶ |

ALT, alanine transaminase; BMI, body mass index; apoB, apolipoprotein B; apoA; apolipoprotein A-I; apoE, apolipoprotein E; apoC-III, apolipoprotein C-III; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, Homeostasis Model Assessments of Insulin Resistance; LDL-C, low-density lipoprotein cholesterol; LVP, lipoviral particle; PCSK9, proprotein convertase subtilisin/kexin type 9; TG, triglyceride.

Parametric data is shown with plus-minus values, which are the means ± standard deviation. Non-parametric data is shown as the median value plus the interquartile range.

*$p$ values are for the comparison between high/low LVP ratios (LVPr). $p$ <0.05 were considered significant. High LVP ratio (n = 20) is defined as greater than or equal to the median value of 0.286.

†Liver stiffness measured by transient elastography using a Fibroscan™ machine with a standard probe [52]. A score ⩾13.0 kPa represents cirrhosis [51].

‡Assessment of insulin resistance was performed by calculation of Homeostasis Model Assessments of Insulin Resistance (HOMA-IR) from a fasting blood sample using the formula: (fasting glucose (mmol/L) × fasting insulin (mU/L))/22.5.

§$p$ value calculated using the parametric $t$ test.

¶$p$ value calculated using the non-parametric Kruskal–Wallis test.

were self-reported: Caucasian (n = 31), South Asian (n = 3), Middle Eastern (n = 2), East Asian (n = 1) and Mixed-other (n = 2; East/South Asian). BMI was normal (<25 kg/m$^2$) in 17 patients, overweight (25–30 kg/m$^2$) in 21 patients, and obese (>30 kg/m$^2$) in 1 patient. According to the criteria of the International Diabetes Federation [34], 7 patients had metabolic syndrome. Of these, 2 patients were receiving treatment for type 2 diabetes mellitus. The virological characteristics of the patient cohort are also summarised in Table 1. The mean plasma HCV RNA viral load was 5.91 $\log_{10}$ (IU/ml) but ranged from 3.66 to 7.62 $\log_{10}$ (IU/ml). The mean HCV RNA LVP load was 5.22 $\log_{10}$ (IU/ml). The mean LVPr was 0.333, but varied widely between patients ranging from 0.027 to 0.969 and the median LVPr was 0.286.

*Correlations between plasma viral load and metabolic factors*

The relationship between host metabolic factors with specific viral parameters (total viral load, LVP, and LVPr) was examined using either Pearson's or Spearman's (r) rank correlation analysis. Using Spearman's rank correlation analysis, a number of metabolic factors correlated with total viral load (Fig. 1). There was a significant negative correlation between total viral load and TC (Fig. 1A; r = −0.450; $p$ = 0.004), LDL-C (Fig. 1B; r = −0.444; $p$ = 0.005), apoB (Fig. 1C; r = −0.352; $p$ = 0.028) and apoA-I (Fig. 1D; r = −0.409; $p$ = 0.010). In multivariable stepwise regression analysis, many of the significant correlates identified interacted, but after 3 rounds of backward elimination of the weakest predictors, LDL-C was the significant independent determinant of total viral load ($R^2$ = 10.7%; $p$ = 0.042). Thus, in HCV-G3, the higher viral load, the lower the concentration of LDL-C.

*Correlations between LVP and metabolic factors*

The association of LVP load with metabolic factors was evaluated with univariate correlation analysis (Fig. 2). LVP load correlated negatively with HDL-C (Fig. 2A; r = −0.421; $p$ = 0.008), apoA-I (Fig. 2C; r = −0.394; $p$ = 0.013), and apoE (Fig. 2E; r = −0.428; $p$ = 0.013). The comparison of these factors with LVP was further evaluated by separating LVP load into high LVP (defined as above the median value of 5.375 $\log_{10}$ HCV RNA (IU/ml)) and low LVP (defined as below the median value of 5.375 $\log_{10}$ HCV RNA (IU/ml)). High LVP load was associated with significantly lower

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HDL-C (Fig. 2B; 1.14 ± 0.40 mmol/L *vs.* 1.40 ± 0.46 mmol/L, $p = 0.047$), apoA-I (Fig. 2D; 1.31 ± 0.32 g/L *vs.* 1.53 ± 0.34 g/L, $p = 0.044$), and apoE (Fig. 2F; 29.8 ± 13.2 mg/L *vs.* 41.13 ± 19 mg/L, $p = 0.020$). Multivariable stepwise regression analysis of the relationship between LVP load and these correlates (with backward elimination of the weakest predictive factors) showed that after two rounds HDL-C was the most significant determinant of LVP load ($R^2 = 17.8\%$; $p = 0.008$). Thus, in HCV-G3 the higher the HDL-C the lower the LVP load.

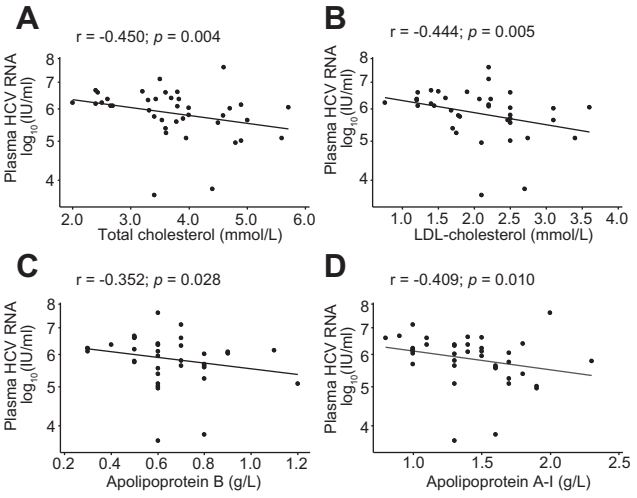


**Fig. 1. Relationship between total viral loads and lipoproteins/apolipoproteins in HCV-G3.** Total viral loads correlated negatively with (A) total cholesterol ($r = -0.450$; $p = 0.004$), (B) LDL-C ($r = -0.444$; $p = 0.005$), (C) apolipoprotein B ($r = -0.352$; $p = 0.028$), and (D) apolipoprotein A-I ($r = -0.409$; $p = 0.010$).

*Correlation between LVP ratio and metabolic factors*

In patients with HCV-G3, neither LVP load (Supplementary Fig. 1A, $r = -0.029$; $p = 0.863$) or LVP ratio (Supplementary Fig. 1B, $r = 0.010$; $p = 0.952$) showed evidence of an association with HOMA-IR. We found a negative correlation between LVPr with PCSK9 in HCV-G3 (Fig. 3A; $r = -0.478$, $p = 0.002$) and the correlation remained significant after excluding the two outliers ($r = -0.403$; $p = 0.015$), whereas in HCV-G1, LVPr [26] trended towards a positive correlation with PCSK9 (Fig. 3C, $r = 0.267$; $p = 0.058$). In HCV-G3, regression analysis (this model excludes unusual observations (outliers) with very high or very low PCSK9 concentrations) showed that PCSK9 concentration was a significant independent predictor of LVPr ($R^2 = 16.2\%$; $p = 0.012$).

*Stratification of LVP ratio into high and low LVP ratio*

The comparison of clinical and metabolic variables in patients with chronic HCV-G3 with a low LVPr (defined as below the median value of 0.286; n = 19) and those with a high LVPr (defined as above the median value of 0.286; n = 20) is shown in Table 1. The only significant difference between patients with a high or low LVPr was found in PCSK9 concentrations (50.4 ng/ml *vs.* 76.3 ng/ml; $p = 0.044$) which is shown in Fig. 3B. Therefore, in patients with chronic HCV-G3, we have shown that higher LVP ratio is associated with lower PCSK9 concentration. We compared the PCSK9 concentrations between high and low LVP ratios in the HCV-G1 cohort and found that higher LVP ratios are associated

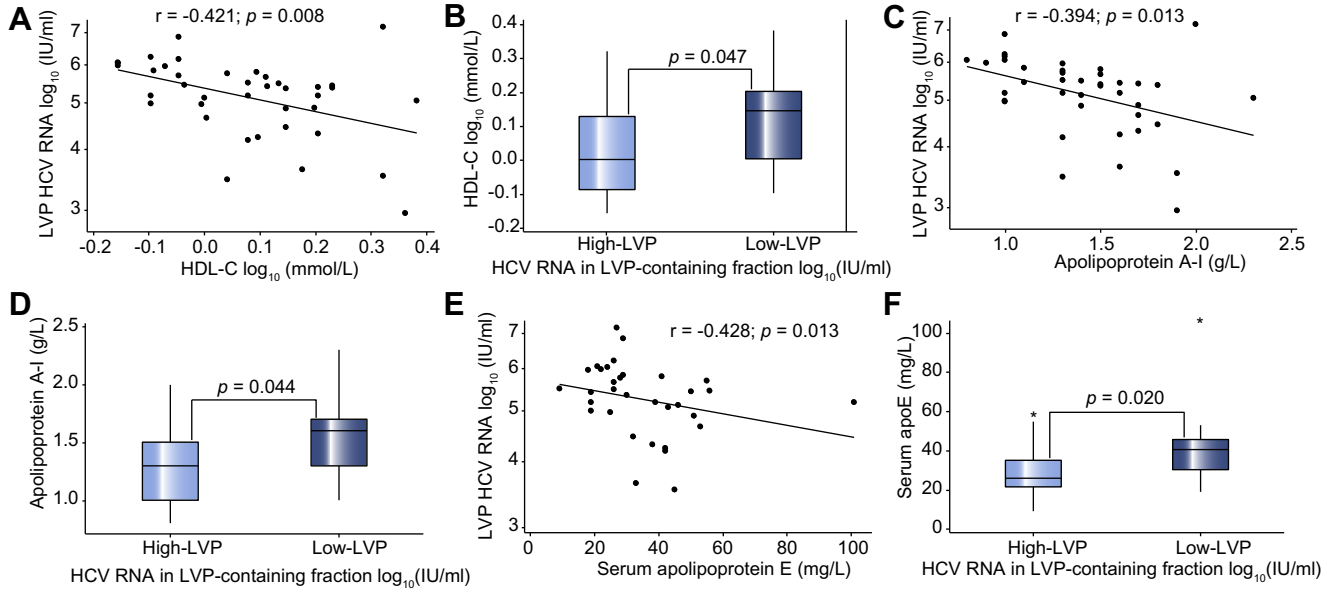


**Fig. 2. Relationship between lipoviral particle (LVP) load and high-density lipoprotein cholesterol (HDL-C), apoA-I, and apoE.** (A) Negative correlation between HDL-C and LVP load ($r = -0.421$; $p = 0.008$). (B) Boxplot showing high LVP load is associated with significantly lower HDL-C (1.14 ± 0.40 mmol/L *vs.* 1.40 ± 0.46 mmol/L, $p = 0.047$). (C) Negative correlation between apoA-I and LVP load ($r = -0.394$; $p = 0.013$). (D) Boxplot showing high LVP is associated with significantly lower apoA-I (1.31 ± 0.32 g/L *vs.* 1.53 ± 0.34 g/L, $p = 0.044$). (E) Negative correlation between apoE and LVP load ($r = -0.428$; $p = 0.013$) and (F) boxplot showing high LVP load is associated with significantly lower apoE (29.8 ± 13.2 mg/L *vs.* 41.13 ± 19 mg/L, $p = 0.020$). The box height represents the interquartile range (Q1–Q3), the line within the box is the median value, the lower whisker represents Q1 − 1.5 (Q3–Q1), and the upper whisker represents Q3 + 1.5 (Q3–Q1).

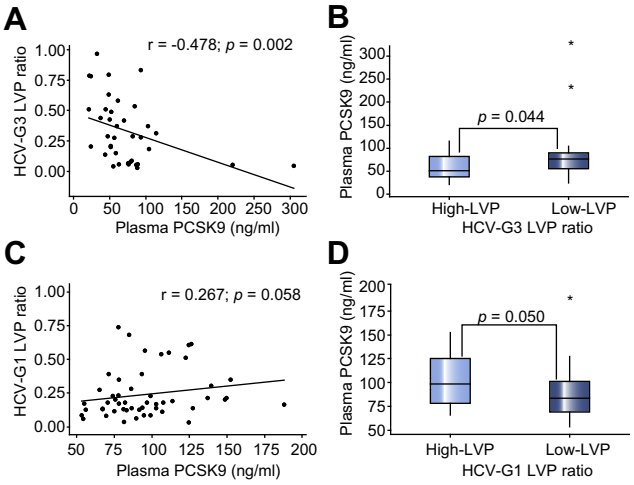


**Fig. 3. Relationship between plasma proprotein convertase subtilisin/kexin type 9 (PCSK9) concentration, as measured by ELISA, and LVP ratio in HCV-G3 and HCV-G1 infection.** (A) Negative correlation in HCV-G3 patients between PCSK9 concentration and LVP ratio ($r = -0.478$; $p = 0.002$). (B) Boxplots showing the relationship between plasma PCSK9 concentration with high and low LVP ratio (50.4 ng/ml *vs.* 76.3 ng/ml; $p = 0.044$) in HCV-G3 patients. (C) Trend towards a significant positive correlation patients between plasma PCSK9 concentration and LVP ratio ($r = 0.276$; $p = 0.058$) in HCV-G1 and (D) boxplots showing the relationship between plasma PCSK9 and high and low LVP ratio (98 ng/ml *vs.* 83.2 ng/ml; $p = 0.05$) in HCV-G1 patients.

with higher PCSK9 concentrations (98.0 ng/ml *vs.* 83.2 ng/ml; $p = 0.05$) (Fig. 3D).

*PCSK9 concentrations are lower in HCV-G3 compared to HCV-G1*

PCSK9 was measured in both the HCV-G3 and HCV-G1 infected patients (n = 51; [26]) and compared to the PCSK9 concentrations in HCV negative individuals (n = 254; [25]), Supplementary Fig 2A. PCSK9 concentrations were significantly lower in HCV-G3 patients compared to HCV-G1 (Fig. 2A; 73.8 ± 52.2 ng/ml *vs.* 96.1 ± 28.7 ng/ml; $p < 0.001$). PCSK9 concentrations were also significantly lower in HCV-G3 patients compared to HCV negative individuals (Fig. 2A; 73.8 ± 52.2 ng/ml *vs.* 89.4 ± 31.9 ng/ml; $p = <0.001$). The concentration of PCSK9 in HCV-G1 patients was found to be significantly higher than in HCV negative subjects (Fig. 2A; 96.1 ± 28.7 ng/ml *vs.* 89.4 ± 31.9 ng/ml; $p = 0.049$). Although the PCSK9 concentrations were relatively low in HCV-G3, given the low concentrations of LDL-C, the plasma PCSK9 and PCSK9/LDL-C ratio were somewhat higher than expected. In HCV-G3, the PCSK9/LDL-C ratio was 43.9 ± 46.3 and HCV-G1 39.9 ± 19.9, compared to 33.2 ± 12.9 in 254 HCV negative individuals. There were no statistically significant differences between the groups as shown in Supplementary Fig. 2B. The differences remained insignificant after exclusion of the two HCV-G3 outliers with very high PCSK9 concentrations and PCSK9/LDL-C ratios (36.4 ± 18.7 *vs.* 39.9 ± 19.9; $p = 0.264$).

Using univariate correlation analysis, we found that PCSK9 concentrations did not correlate with either total cholesterol or LDL-C in either HCV-G1 or HCV-G3 infected patients, which is in contrast to the HCV negative subjects, where a correlation was found between the PCSK9 concentrations with either total or LDL cholesterols (Fig. 4). The relationship between PCSK9 concentrations and biomarkers of liver inflammation, e.g., ALT, AST and GGT were also evaluated (Supplementary Fig. 3). It is noteworthy that the two PCSK9 outliers with very high PCSK9 concentrations were found to have low LDL-C and much higher liver stiffness measurements (>13 kPa), as determined by transient elastography.

## Discussion

In chronic HCV infection, viral load is not a useful prognostic indicator of the severity of liver disease [35] and is influenced by a large number of demographic, viral, and human genetic factors [36]. In this study of HCV-G3 patients, we found that LDL-C was a significant determinant of total viral load; i.e., higher viral load, lower LDL-C. It has previously been shown that liver steatosis is independently associated with HCV-G3 [37] and that steatosis correlates with lower LDL-C and hypobetalipoproteinaemia [38]. In addition, steatosis grade correlates with higher viral load [39], and steatosis is a predictor of virological relapse to antiviral therapy in HCV-G3 [40]. Our finding emphasises the link between HCV-G3 replication, steatosis, viral load, and LDL-C. This suggests that LDL-C should be included in the parameters examined as predictors of virological relapse in the era of direct acting antivirals [41].

The poor correlation between viral load and disease severity in chronic HCV could be partially explained by the increasing evidence that not all of the HCV RNA is equally infectious. Infectious HCV particles have a low buoyant density, due to interaction with lipoproteins to form hybrid lipoviral particles [10] that can facilitate virus entry into hepatocytes (reviewed in [18]). This is the first study to quantitate LVP in the plasma of patients with HCV-G3, and a striking finding is that LVP load is negatively correlated with apoE; i.e., higher LVP load, lower serum apoE. This might seem surprising, as LVP can be immunoprecipitated with anti-apoE [11,42] and apoE is enriched on the surface of HCVcc [15]. There is also evidence that apoE mediates attachment of clinical HCV to hepatocytes by binding to cell surface heparan sulphate proteoglycan receptors [20]. Our finding in HCV-G3 is the opposite of HCV-G1, where we showed that LVP load positively correlates with apoE [27]. One interpretation of our opposing findings in HCV-G3 *vs.* HCV-G1 is that apoE is depleted by LDLR-mediated uptake of LVP, thereby mediating viral entry into hepatocytes in HCV-G3 *in vivo*.

In this study, we found that the most significant determinant of fasting LVP load in G3 is HDL-C; i.e., higher HDL-C, lower LVP load. We have previously shown that HCV particles in the serum exhibit post-prandial shifts in buoyant density [32], moving on to very-low-density apoB-associated triglyceride-rich lipoproteins (TRLs) after a fatty meal. The reciprocal relationship between HDL-C and LVP found in HCV-G3 infection suggests slower clearance of HDL-bound HCV RNA containing particles retained in the higher density fraction.

LVP load in HCV-G3 did not correlate with insulin resistance as measured by HOMA-IR, unlike HCV-G1 [26]. Insulin plays a central role in coordinating lipoprotein metabolism and promotes the uptake of TRL remnant particles [43,44], however, insulin resistance is associated with overproduction of TRLs. Our finding in HCV-G3 *vs.* HCV-G1 again implies genotype-specific differences in the regulation of the pathways of TRL production and TRL remnant clearance, the latter being more important in HCV-G3 compared with HCV-G1.

This is also the first report of PCSK9 concentrations in HCV patients. PCSK9 regulates recycling of the LDLR and plasma PCSK9 concentrations normally correlate inversely with LDLR

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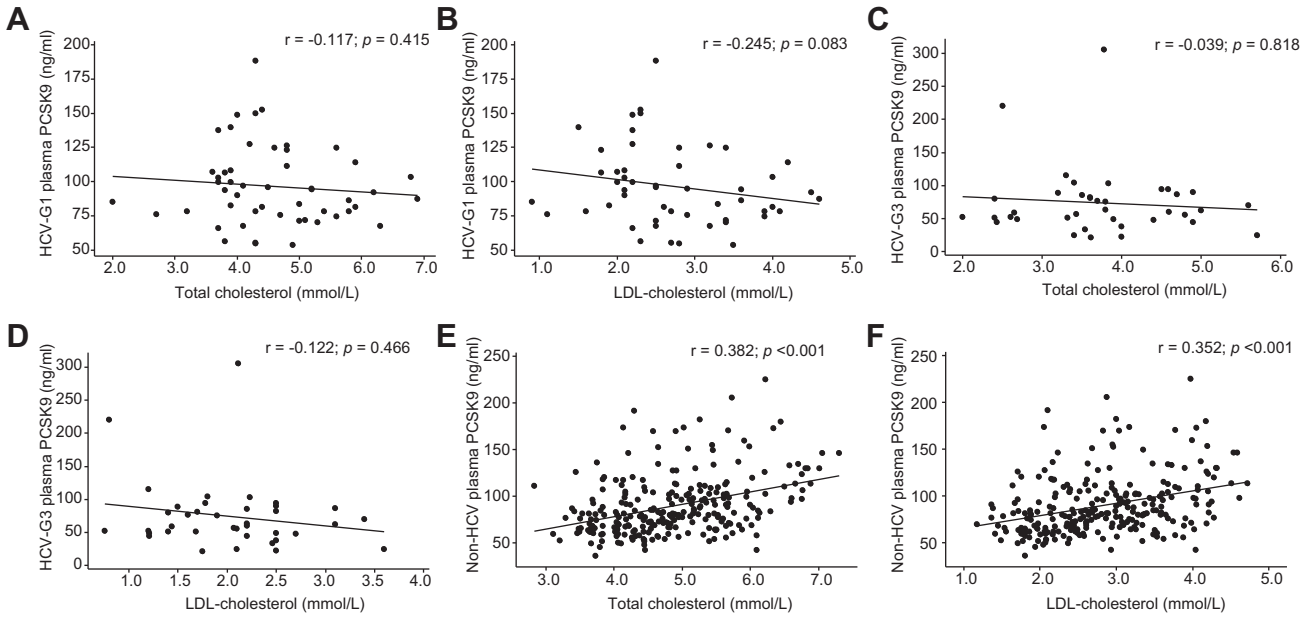


**Fig. 4. Relationship between plasma proprotein convertase subtilisin/kexin type 9 (PCSK9) levels and serum total and LDL-cholesterol in HCV-G1 (n = 51; [26]), HCV-G3 (n = 39) and non-HCV infected subjects (n = 254; [25]).** (A) PCSK9 does not correlate with total cholesterol in HCV-G1 (r = −0.117; $p = 0.415$), (B) PCSK9 does not correlate with LDL-C in HCV-G1 (r = −0.245; $p = 0.083$), (C) PCSK9 does not correlate with serum cholesterol in HCV-G3 (r = −0.039; $p = 0.818$), (D) PCSK9 does not correlate with serum LDL-C in HCV-G3 (r = −0.122; $p = 0.466$), (E) PCSK9 correlates with total cholesterol in 254 non-HCV infected study participants (r = 0.382; $p = <0.001$) and (F) PCSK9 correlates with LDL cholesterol in non-HCV infected individuals (r = 0.352; $p = <0.001$).

expression, hence positively with plasma LDL-C concentrations in healthy individuals [45] (reviewed in [22,46]). We found that plasma PCSK9 did not correlate with total cholesterol or LDL-C in HCV-G3 and HCV-G1 patients, indicating disruption of lipid homeostatic mechanisms by HCV. PCSK9 concentrations were significantly lower in HCV-G3 *vs.* HCV-G1. In apoB kinetic studies, fasting PCSK9 concentrations correlate inversely with apoB fractional catabolic rate; i.e., lower PCSK9 is associated with high LDL clearance via LDLR [47]. Thus, a lower PCSK9 concentration in HCV-G3 supports the concept that chronic HCV-G3 is characterised by increased clearance of both LDL-C and LVP mediated by LDLR. It also implies that increased clearance of apobetalipoproteins contributes to the hypobetalipoproteinaemia found in HCV-G3. Our findings suggest that HCV-G3 acts in an analogous manner to statins in that higher viral replication correlates with lower LDL-C and apparent upregulation of LDLR. This would normally be counteracted by upregulation of PCSK9 mediated homeostatic mechanism(s), and increased PCSK9 concentrations, as seen in statin treatment. Studies of the relationship between apoB kinetics and markers of sterol synthesis and absorption are needed in patients with chronic HCV, to elucidate the mechanism underlying these findings and to confirm that the low apoB concentrations, particularly in chronic HCV-G3, are not primarily due to decreased apoB production, as has been assumed as a consequence of MTP inhibition [48]. However, it must be emphasised that the undertaking of such demanding studies is fraught with difficulty in this patients' group. This study also highlights that the use of PCSK9 inhibitors may be hazardous in this patients' group as they could further increase LDLR mediated LVP uptake and enhance the severity of infection in HCV-G3.

In summary, the difference in LVP correlations in HCV-G3 compared to HCV-G1 [26,27] suggests important differences in HCV entry for these 2 genotypes. ApoE-mediated entry via LDLR may be more dominant in HCV-G3 whereas apoE-mediated clearance via the SR-B1 pathway could be more important *in vivo* for HCV-G1 infection. This would mean that statins may be more beneficial as adjunct therapy in HCV-G1 rather than HCV-G3, as has been reported in a recent study [49]. The genotype–specific differences in lipoprotein interactions found *in vivo* in this study are likely to be of relevance, not only for blocking viral entry, but also for designing approaches to targeting host lipid pathways as adjunctive therapy [50].


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## Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

## Authors' contributions

MFB, RDGN, HCT, SDT-R, and GLT conceived the study. SHB, DAS, DJF, MMEC, SDT-R, HCT, GLT, RDGN, and MFB were study investigators. DAS, MMEC, SDT-R, and MFB participated in the

recruitment of patients and reporting of data for the enrolled patients. SHB, DAS, and DJF designed and performed experiments. SHB performed the statistical analysis. GD, NS, and JD contributed important reagents and collaborated with the study investigators. SHB, DAS, DJF, CVL, GLT, RDGN, and MFB contributed to the analysis and interpretation of data. SHB, RDGN, and MFB drafted and wrote the manuscript. All authors reviewed and revised the manuscript for intellectual content, and approved the final version for submission.

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### Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.11.016.

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