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Maximum levels of hepatitis C virus lipoviral particles are associated with early and persistent infection.

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Abbreviations: HCV, hepatitis C virus; LVP, lipoviral particle; ATAHc, Australian Trial in Acute Hepatitis ; HITS-p, Hepatitis C Incidence and Transmission Study in prison;

Conflict of Interest: BA is the operations director of HB innovations Ltd that devised the maxi-LVP assay; JG is a consultant/advisor and has received research grants from Abbvie, Bristol Myers Squibb, Gilead Sciences and Merck. GD is a consultant/advisor and has received research grants from Abbvie, Bristol Myers Squibb, Gilead, Merck, Janssen and Roche. The authors have no conflict of interest to disclose

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Author Contributions:

Authors GJD, GVM designed the original ATAHC study.

Authors ARL designed the HITS-p study.

Authors MFB, DN, BA, FF and DS developed the maxi-assay

Author DS conducted the laboratory work related to maxi-LVP in ATAHC and HITS p samples

Authors JGr and DS drafted the primary statistical analysis plan, which was reviewed by GVM, ARL, GD and JG.

JGr, TA, JG, GM coordinated ethical review and access to the ATAHC and HITS p samples

Authors JG and MD provided laboratory and technical support for the analysis.

The primary statistical analysis was conducted by BH and JGr,

All authors reviewed data analysis.

Author DS wrote the first draft of the manuscript.

All authors contributed to and have approved the final manuscript.

Abstract

Background & aims. Hepatitis C virus (HCV) is bound to plasma lipoproteins and circulates as an infectious lipoviral particle (LVP). Experimental evidence indicates that LVPs have decreased susceptibility to antibody mediated neutralisation and higher infectivity. This study tested the hypothesis that LVPs are required to establish persistent infection, and conversely, low levels of LVP in recent HCV infection increase the probability of spontaneous HCV clearance.

Methods. LVP in non-fasting plasma was measured using the concentration of HCV RNA bound to large >100 nM sized lipoproteins after ex vivo addition of a lipid emulsion, that represented the maximum concentration of LVP (maxi-LVP). This method correlated with LVP in fasting plasma measured using iodixanol density gradient ultracentrifugation. Maxi-LVP was measured in a cohort of 180 HCV participants with recent HCV infection and detectable HCV RNA from the Australian Trial in Acute Hepatitis C (ATAHC) and Hepatitis C Incidence and Transmission Study in prison (HITS-p) cohorts.

Results. Spontaneous clearance occurred in 15% (27 of 180) of individuals. In adjusted analyses, low plasma maxi-LVP level was independently associated with spontaneous HCV clearance [≤ 827 IU/mL; adjusted odds ratio 3.98, 95% CI: 1.02, 15.51, $P=0.047$], after adjusting for interferon lambda-3 rs8099917 genotype, estimated duration of HCV infection and total HCV RNA level.

Conclusions. Maxi-LVP is a biomarker for the maximum concentration of LVP in non-fasting samples. Low maxi-LVP level is an independent predictor of spontaneous clearance of acute HCV.

Key words: Hepatitis C virus; lipoviral particles; spontaneous clearance

Key Points

- Spontaneous clearance occurs in ~25% following exposure to hepatitis C virus (HCV). Lipoviral Particles (LVPs) are the infectious fraction of HCV associated with lipoproteins in plasma.
- We investigated whether low levels of LVP are independently associated with spontaneous HCV clearance in 180 participants in the ATACH and HITS-p studies of early HCV infection.
- We measured the maximum concentration of LVP (maxi-LVP) using a size filtration method, demonstrating close correlation with iodixanol density gradient ultracentrifugation, following addition of lipid emulsion to control for post-prandial lipaemia in non-fasting samples.
- Low maxi-LVP level is an independent predictor of spontaneous clearance in early HCV.

Introduction

An essential component of the hepatitis C virus (HCV) lifecycle is the formation of 'lipoviral particles' (LVP) (1). HCV is co-assembled with the hepatocyte's machinery for export of very-low density lipoproteins (VLDL) (2). LVP contain not only HCV viral proteins and RNA, but also have physical properties of lipoproteins, being of low-buoyant density, large size and containing apolipoprotein's B (apoB), apoE and apoA1 (3). The lipoprotein association increases infectivity of LVPs (4, 5) and decreases susceptibility to antibody mediated neutralisation (6). We therefore

hypothesised that formation of LVP is likely to be important in the natural history of early acute HCV infection in determining the probability of spontaneous clearance or persistent infection.

The natural history of acute HCV infection has not been fully defined, but spontaneous clearance occurs in ~25% of those exposed to the virus (7, 8). Factors related to innate immunity (9) and induction of interferon stimulated genes may be important for early spontaneous clearance (10, 11). Other factors that have been independently associated with spontaneous clearance include sex, interferon lambda-3 (IFNL3) genotype, interferon-gamma-inducible protein-10, HCV genotype, HIV infection and HBV infection (8, 11, 12).

LVP have previously been measured in patients with chronic HCV infection in fasting plasma using iodixanol density gradient ultracentrifugation (13, 14). This method defined a low-density fraction ($d < 1.07$ g/mL), in which all the apoB containing lipoproteins are captured and HCV RNA detected within this fraction was considered to represent apoB associated LVP (14). HCV RNA in the higher density fraction $d > 1.07$ g/mL that did not contain apoB, were considered 'non-LVPs'. In chronic HCV genotype 1 infection, LVP and LVP ratios were associated with insulin resistance, triglycerides (14), apoE concentrations and sustained virological response to pegylated interferon and ribavirin therapy (15). Different metabolic correlations were identified when comparing LVP in HCV genotype 1 to genotype 3 (16). However, variability of density distribution in the post prandial state limits application of this method to non-fasting samples (17). In order to investigate LVP in non-fasting

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samples from a unique cohort of recent HCV infection, a modified LVP assay measuring sized large HCV particles >100nm after *ex vivo* addition of lipid emulsion (maxi-LVP) was used. It was hypothesised that concentrations of LVP will be higher in early acute HCV infection compared to later duration of infection and that lower LVP levels would independently predict spontaneous HCV clearance. Hence, we evaluated whether i) maxi-LVP is increased in early acute HCV infection (<26 weeks) compared to later (>26 weeks) duration of infection; and ii) the concentrations of maxi-LVP predict spontaneous clearance during recent (acute and early chronic) HCV infection.

Patients and Methods

Study Population and Design

The role of LVPs during recent HCV infection was investigated using stored plasma and data from two studies of recent HCV infection. The methods of both studies have been previously described in detail (18, 19). The Australian Trial in Acute Hepatitis C (ATAHC) was a prospective study of the natural history and treatment of recent HCV (18). The Hepatitis C Incidence and Transmission Study in prison (HITS-p) is an ongoing study of prison inmates at-risk of acute HCV in correctional centres (19). Stored plasma samples were used to measure maxi-LVP in both ATAHC and HITS-p acute HCV cohorts.

For inclusion, participants from these cohorts had to have recent HCV infection defined by an initial positive anti-HCV test and either 1) a negative anti-HCV test within two years prior to the initial positive anti-HCV test or 2) acute clinical hepatitis (either jaundice or ALT >400 IU/mL) within 12 months of the initial positive anti-HCV result.

Among individuals with anti-HCV negative and HCV RNA positive at the time of recent HCV detection, the estimated date of HCV infection was 4 weeks prior to diagnosis date (20, 21). Among individuals with HCV seroconversion and no acute symptomatic infection, the estimated date of infection was calculated as the midpoint between the last negative anti-HCV and first positive anti-HCV or RNA test. Among individuals with acute symptomatic infection, the estimated date of infection was calculated as 6 weeks prior to the onset of acute clinical hepatitis (22). All participants provided written informed consent. The study protocols were approved by St Vincent's Hospital Sydney, Corrective Services New South Wales, and Justice Health Human Research Ethics Committees.

Laboratory testing

Sample selection

All participants with sufficient available EDTA or acid citrate dextrose (ACD) plasma samples that were HCV RNA positive at the time of acute HCV detection were identified. The laboratory analysis was conducted blinded to the outcomes. In addition, 30 participants from the ATAHc study with high volumes of available EDTA

or ACD plasma samples (>1 mL) were randomly selected for duplicate testing to calculate the coefficient of variation (CV) for the maxi-LVP methods described below.

Maxi- LVP assay

The maxi-LVP assay was performed by incubating 50 μ L of EDTA or ACD plasma with an equal volume of diluted lipid emulsion, (27.8 μ L of Intralipid 20% (SigmaAldrich) diluted in 2 mL PBS) at 37 °C for 2 hours. When the incubation was complete, the intralipid-plasma was then further diluted 1 in 20 with addition of 900 μ L of PBS. 200 μ L of the dilute Intralipid-plasma sample was transferred to a Ultrafree [®] MC Durapore [®] PVDF membrane (0.1 μ M) Millipore size filter (Merck-Millipore Ultrafree UFC30VV00) and centrifuged at 10,000 x G for 30 minutes. All of the filtrate (HCV RNA bound to particles size <0.1 μ M) was transferred to an Eppendorf containing Qiagen protease. HCV RNA retained by the filter (particle size >0.1 μ M) was extracted by addition of Qiagen protease (25 μ L protease with 200 μ L PBS) and vortexing for 15 seconds. 200 μ L of RNA extraction lysis buffer was then added to the filters with further vortexing for 15 seconds. The contents (retentate) were then transferred to a new Eppendorf tube and incubated for 15 mins at 56 °C. Viral RNA was extracted from the filtrate and from the retentate using a Qiagen Virus spin Minelute Virus Spin kit according to the manufacturer's instructions.

HCV RNA quantitation

HCV RNA from the retentate (maxi-LVP) and filtrate (non-LVP) were reverse transcribed using AMV Reverse Transcriptase (Promega) and sequence specific primer NCR3 as previously described (23). cDNA was quantitated using a two-step

Taq Man Real time PCR as previously described using primers NCR-3 and NCR-5 and dual labelled probe SN1 to the 5' UTR of the HCV genome (23).

Maxi-LVP was defined as the HCV RNA concentration (IU/mL) in the retentate of the filter after incubation with dilute intralipid. HCV RNA was also measured from the filtrate (non-LVP) and the maxi-LVP ratio calculated as retentate / (retentate + filtrate).

Paired stored EDTA plasma in 53 HCV genotype 1 patients were tested using the two LVP assays; i) iodixanol density gradient ultracentrifugation defined LVP as HCV-RNA detected at density <1.07 g/ml (2), ii) Maxi-LVP assay defined LVP as HCV-RNA retained by the $0.1 \mu\text{M}$ size filter, after incubation with a lipid emulsion.

Pearson's correlation was used to determine the agreement between the two LVP assays (figure 1).

Study outcome

The association of maxi-LVP and non-LVP levels at the first detection of HCV infection with spontaneous HCV clearance was investigated. Spontaneous clearance was defined as two subsequent consecutive undetectable HCV RNA tests (<10 IU/mL) ≥ 4 weeks apart following documented infection. Treated individuals with an estimated duration of infection <26 weeks were excluded from analyses of spontaneous clearance to reduce misclassification bias because there is uncertainty around whether these individuals would have demonstrated spontaneous clearance in the absence of treatment.

Statistical analyses

The co-efficient of variation (CV) of maxi-LVP was determined by calculating the standard deviation/mean of the maxi-LVP assay in the ATAHc cohort of 30 randomly selected non-fasting samples run in duplicate. Nonparametric statistical tests were used for analyses, given that maxi-LVP and non-LVP levels (IU/mL) and log₁₀ transformation of maxi-LVP and non-LVP levels (log IU/mL) were not normally distributed. Median maxi-LVP levels, non-LVP levels and maxi-LVP ratio were compared between spontaneous clearance and persistent infection groups using the Wilcoxon–Mann–Whitney test. The proportion of individuals with detectable and undetectable maxi-LVP was compared between spontaneous clearance and persistent infection groups using Chi Square test. Similarly, distribution of maxi-LVP levels, non-LVP levels and maxi-LVP ratio were compared between individuals with estimated duration of infection <26 weeks at the time of HCV detection (early acute HCV infection) and those with an estimated duration of infection >26 weeks at the time of HCV detection.

The association of low maxi-LVP levels and non-LVP levels with spontaneous clearance was assessed using logistic regression. Low maxi-LVP levels and non-LVP levels were defined as equal or lower than median (i.e. ≤ 827 IU/mL as low maxi-LVP levels and ≤ 1854 IU/mL as low non-LVP levels). In the adjusted models, the association of low maxi-LVP levels and low non-LVP levels with spontaneous clearance was adjusted for potential confounders including interferon-lambda rs8099917 genotype, HCV RNA levels, and estimated duration of infection. Potential confounders in the adjusted models included variables associated with spontaneous

clearance in this study ($P < 0.200$). All analyses were performed using Stata v12.0 (College Station, TX, United States).

Results

Participant characteristics

The baseline characteristics of the study cohorts are shown in Table 1. Among 206 participants in ATAHc and HITS-p, 180 individuals had detectable HCV RNA at initial HCV diagnosis and are included in this analysis (69% male, 18% HIV infected, median total HCV RNA: $4.87 \log_{10}$ IU/mL).

Assay development and validation

The validity of the maxi-LVP assay in comparison to iodixanol density gradient ultracentrifugation method was assessed by paired analysis using the two assays from the same stored samples. There was strong correlation between LVP (HCV RNA \log_{10} IU/ml) as measured by iodixanol density gradient vs maxi-LVP methods (R^2 57.2%, $r = 0.756$, $P < 0.001$). The iodixanol ratio also correlated significantly with the maxi-LVP ratio (R^2 36.9%, $r = 0.421$, $P = 0.006$). Likewise there was significant correlation between non LVP (HCV RNA \log_{10} IU/ml) as measured by iodixanol density gradient ($d > 1.07$ g/ml) and HCV RNA in the size filtrate (R^2 36.9%, $r = 0.608$, $P < 0.001$) (figure 1). Bland-Altman plots describe the test agreement between the two assays (Figure 1 B and D). Maxi LVP and non-LVP consistently under-measured HCV RNA compared to iodixanol density gradients by $-0.97 \log_{10}$ IU/ml for LVP and $-1.15 \log_{10}$ IU/mL for non-LVP, which was not clinically significant for analysis in this study.

Maxi-LVP sensitivity and co-efficient of variation

Maxi-LVP assay was performed in duplicate on 30 non-fasting samples from ATAHC. The CV varied according to baseline HCV RNA level. For samples with HCV RNA level >1,500 IU/ml, filtrate (non-LVP) CV = 0.09, retentate (Maxi-LVP) CV = 0.19, maxi-LVP ratio CV = 0.16.

LVP-max in early (<26 weeks) vs late (>26 weeks) HCV infection

At first acute HCV detection, the median maxi-LVP level was 827 IU/ml [inter-quartile range (IQR): 0, 6436], non-LVP level was 1854 IU/ml (IQR: 0, 19999) and maxi-LVP ratio was 0.30 (IQR: 0.03, 0.48). In early HCV infection (<26 weeks) there was a trend towards increased median maxi-LVP concentrations compared to later HCV infection (>26 weeks) [1140 IU/mL (IQR: 199, 8090) vs. 686 IU/mL (IQR: 0, 4335); $P=0.071$; Figure 2).

Maxi-LVP and spontaneous HCV clearance

Spontaneous HCV clearance occurred in 15% (27 of 180) overall. Lower median maxi-LVP levels were observed among people with subsequent spontaneous clearance than in those subsequently developing persistent infection [253 IU/mL (IQR: 0, 2012) vs. 986 IU/mL (IQR: 116, 5761); $P=0.074$; Figure 3].

The proportion of individuals with spontaneous clearance was 9% (8 of 87) among those with plasma maxi-LVP levels >827 IU/mL (median) vs 20% with maxi-LVP levels \leq 827 IU/mL (19 of 93; $P=0.035$). Of those with spontaneous clearance, maxi-LVP were undetectable in 41%, compared to 24% in those developing persistent infection ($P=0.060$).

In adjusted analyses, low plasma maxi-LVP level (≤ 827 IU/mL) was independently associated with spontaneous HCV clearance [adjusted odds ratio (AOR) 3.98, 95% CI: 1.02, 15.51, $P=0.047$], after adjusting for IFNL3 rs8099917 genotype, estimated duration of HCV infection and HCV RNA level (Table 2). In contrast, non-LVP was not independently associated with spontaneous HCV clearance after adjusting for IFNL3 rs8099917 genotype, estimated duration of infection and HCV RNA level (Table 3)

Discussion:

This is the first study to report LVP in the natural history of recent HCV and spontaneous clearance. The key findings are that low maxi-LVP at the detection of recent HCV infection independently predicted subsequent spontaneous clearance.

This was consistent with results demonstrating that people with spontaneous clearance had lower median maxi-LVP levels compared to those who developed persistent HCV infection.

LVPs are considered to represent the infectious fraction of plasma by direct virus-lipoprotein interactions (1). Analysis of this unique cohort of people with recent HCV infection permitted exploration of the hypothesis that formation of LVP in early infection influences the outcome of spontaneous clearance or persistent infection, independently of other host genetic and immune factors. Previous analysis of the ATAHc and HITS-p cohorts has indicated that factors associated with spontaneous clearance included IFNL3 genotype, female sex, and estimated duration of infection <26 weeks (8). These factors were taken into account in the multivariate analysis, and concentrations of maxi-LVP below the median value remained independently associated with spontaneous clearance.

High concentrations of LVP indicate that HCV has established efficient viral assembly within the liver by co-opting the secretory machinery of VLDL export from hepatocytes (1). The association of HCV with lipoproteins *in vivo* is dynamic and variable. Quantities of very low density LVP have previously been observed to increase after a high fat meal, and are rapidly cleared (17). Post prandially, HCV in plasma associates with both liver derived VLDL containing apoB100, and intestinally derived chylomicrons containing apoB48 (17, 24). In the fasting state, only a minority of HCV RNA (27-37%) were detectable in a very low density fraction ($d < 1.025$ g/L), but following *ex vivo* addition of post prandial plasma from a healthy volunteer, the amount of HCV RNA in the very low density fraction increased (53-78%) (17). The significance of this observation is that measurement of LVP by density or size in randomly selected non-fasting samples will be subject to variation dependent on the nature and timing of sampling in relation to ingestion of the last meal. Previous work has reported that addition of a dilute lipid emulsion (Intralipid) increases concentrations of very low-density HCV RNA to the same extent as with addition of post-prandial plasma (17). These previous observations indicated therefore that the maximum concentration of LVP apparent post-prandially can be recapitulated by *ex vivo* addition of a lipid emulsion. In the present study, the property of re distribution of HCV RNA to large triglyceride rich lipoproteins has been utilised to devise a novel method to measure the maximum concentration of LVP (maxi-LVP), which fractionates non-fasting plasma using a size filter after *ex vivo* addition of a lipid emulsion. Thus *ex vivo* addition of lipid emulsion generates the maximum concentration of LVP and overcomes the limitation of not having fasting samples available in this unique cohort of early HCV. Maxi-LVP not only measures HCV RNA bound to large VLDL, but also measures HCV RNA that has redistributed onto large

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sized lipid emulsion particles by *ex vivo* transfer during the incubation. Maxi-LVP may therefore measure both large sized, hepatocyte assembled LVPs and *de novo* LVPs formed by coalescence of smaller / high density sub-viral 'empty' LVPs (25) and HCV-RNA containing nucleocapsids or HCV RNA containing exosomes (26). The described phenomenon of HCV RNA redistributing onto large triglyceride rich lipoproteins including intestinally derived chylomicrons in the vascular compartment following ingestion of a high fat meal (17) leads to the hypothesis that this dynamic component of the HCV lifecycle may be an important factor in establishing persistent infection.

The conceptual model of an LVP is one in which the virus is wholly or partially encapsulated within the native lipoprotein (2). Thus the lipoprotein masks viral epitopes exposed to antibody mediated neutralisation (3). Our data supports this model. There is considerable variation in the patterns of viraemia observed in acute HCV (27) particularly in the early stages, and divergence of viraemia does not become apparent until months 3-5 following exposure (28). Some of this variation could be explained by the interplay between LVPs and neutralising antibody responses to HCV. LVP have decreased susceptibility to antibody mediated neutralisation (6). Evaluation of broadly neutralising antibody responses in acute HCV has indicated that individuals that spontaneously clear HCV have cross-reactive broadly neutralising antibody responses to heterologous viral pseudo-particles that are rarely detected in chronic infection (29, 30). Our model implies that these neutralising antibody responses are more likely to occur in those with low or undetectable LVP concentrations. When broadly neutralising antibody responses have been studied longitudinally in chronic HCV infection, the literature supports the

model that immunological selection pressures drive viral escape mutations in defined neutralising epitopes, leading to loss of recognition by monoclonal antibodies against these epitopes and decreased neutralisation of corresponding pseudo-particles (31).

We hypothesise that LVP may be increased in the early stages of acute infection prior to the occurrence of high titre neutralising antibody responses. In later chronic HCV infection, neutralising antibody responses may generate increased levels of immune complexed HCV, thereby decreasing the fraction of viral particles circulating as LVP. Taken together, these data support the model that HCV interacts with lipoproteins early in acute HCV infection and that this interaction influences the balance of probabilities of persistent infection or spontaneous clearance. Efficient formation of LVPs is likely to mask viral epitopes from antibody mediated neutralisation and support immune escape and establishment of persistent infection, whereas failure to form LVP exposes HCV to antibody mediated neutralisation, and favours spontaneous clearance.

In conclusion, this study reports the application of a novel candidate viral biomarker (maxi-LVP) to a unique cohort of people with recent HCV. The data support the concept that LVPs are likely to be important in establishing persistent HCV infection. Future work is required to define the specific mechanisms and sequence variants that mediate HCV-lipoprotein association. Disruption of HCV-lipoprotein interactions may be relevant for development of an efficacious anti-HCV vaccine to 'unmask' viral epitopes from the LVP.

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Table 1. Baseline characteristics of study cohorts

	Overall n (%) <i>n=180</i>	ATAHC n (%) <i>n=105</i>	HITS n (%) <i>n=75</i>
Age; years; median (IQR)	29 (25, 37)	33 (26, 41)	27 (23, 30)
Sex			
Female	56 (31)	32 (30)	24 (32)
Male	124 (69)	73 (70)	51 (68)
<i>IFNL3</i> genotype (rs12979860)			
TT/CT	90 (50)	53 (50)	37 (49)
CC	80 (44)	49 (47)	31 (41)
Unknown	10 (6)	3 (3)	7 (9)
<i>IFNL3</i> genotype (rs8099917)			
GG/GT	71 (39)	41 (39)	30 (40)
TT	103 (57)	60 (57)	43 (57)
Unknown	6 (3)	4 (4)	2 (3)
HCV genotype			
Genotype non-1	90 (50)	54 (51)	36 (48)
Genotype 1	71 (39)	49 (47)	22 (29)
Unknown	19 (11)	2 (2)	17 (23)
HCV RNA levels; log IU/mL; median (IQR)	4.8 (3.6, 5.7)	4.5 (3.3, 5.7)	4.9 (4.2, 5.7)
Estimated time since infection			
≤ 26 weeks	89 (49)	48 (46)	41 (55)
> 26 weeks	91 (51)	57 (54)	34 (45)
HIV co-infection			
No	148 (82)	73 (71)	75 (100)
Yes	32 (18)	32 (30)	0 (0)

Table 2: Unadjusted and adjusted model evaluating the association of high plasma maxi-LVP (HCV RNA detected at size ≥ 100 nM) with spontaneous clearance

	Unadjusted model		Adjusted model 1*:		Adjusted model 2**	
	OR (95% CI)	P	Adjusted OR (95% CI)	P	Adjusted OR (95% CI)	P
Maxi-LVP						
> 827 IU/mL	1.00		1.00		1.00	
≤ 827 IU/mL	2.53 (1.05, 6.14)	0.039	3.46 (1.33, 9.00)	0.011	3.98 (1.02, 15.51)	0.047
Age	0.98 (0.93, 1.03)	0.338				
Sex						
Female	1.00					
Male	0.92 (0.38, 2.25)	0.857				
IFNL3 (rs12979860)						
TT/CT	1.00					
CC	0.56 (0.29, 1.10)	0.096				
IFNL3 (rs8099917)						
GG/GT	1.00		1.00		1.00	
TT	2.74 (1.10, 6.80)	0.030	3.20 (1.22, 8.36)	0.018	3.23 (1.23, 8.48)	0.017
HCV genotype						
Genotype non-1	1.00					
Genotype 1	0.89 (0.37, 2.14)	0.795				
HCV RNA levels (log IU/mL)	0.77 (0.55, 1.07)	0.116			1.07 (0.66, 1.73)	0.778
Estimated time since infection						
≤ 26 weeks	1.00		1.00		1.00	
> 26 weeks	0.29 (0.11, 0.72)	0.008	0.25 (0.09, 0.65)	0.005	0.24 (0.09, 0.64)	0.004

* Adjusted for the variables with a $P < 0.05$ in unadjusted analysis, including *interferon lambda* genotype (rs8099917) and estimated time since infection

** Adjusted for the variables with a $P < 0.20$ in unadjusted analysis, including *interferon lambda* genotype (rs8099917), estimated time since infection and HCV RNA levels

Table 3: Unadjusted and adjusted models evaluating the association of high plasma non-LVP levels (HCV RNA detected at size <100 nM) with spontaneous clearance

	Unadjusted model		Adjusted model 1*		Adjusted model 2**	
	OR (95% CI)	P	AOR (95% CI)	P	AOR (95% CI)	P
Non-LVP						
> 1854 IU/mL	1.00		1.00		1.00	
≤ 1854 IU/mL	2.47 (1.02, 5.98)	0.045	2.63 (1.03, 6.65)	0.042	2.48 (0.57, 10.72)	0.225
Age	0.98 (0.93, 1.03)	0.338				
Sex						
Female	1.00					
Male	0.92 (0.38, 2.25)	0.857				
IFNL3 genotype (rs12979860)						
TT/CT	1.00					
CC	0.56 (0.29, 1.10)	0.096				
IFNL3 genotype (rs8099917)						
GG/GT	1.00		1.00		1.00	
TT	2.74 (1.10, 6.80)	0.030	2.83 (1.12, 7.16)	0.028	2.83 (1.12, 7.18)	0.028
HCV genotype						
Genotype non-1	1.00					
Genotype 1	0.89 (0.37, 2.14)	0.795				
HCV RNA levels (log IU/mL)	0.77 (0.55, 1.07)	0.116			0.97 (0.58, 1.64)	0.919
Estimated time since infection						
≤ 26 weeks	1.00		1.00		1.00	
> 26 weeks	0.29 (0.11, 0.72)	0.008	0.28 (0.11, 0.72)	0.008	0.28 (0.11, 0.72)	0.008

* Adjusted for the variables with a $P < 0.05$ in unadjusted analysis, including *interferon lambda* genotype (rs8099917) and estimated time since infection

** Adjusted for the variables with a $P < 0.20$ in unadjusted analysis, including *interferon lambda* genotype (rs8099917), estimated time since infection and HCV RNA levels

Figure Legends:

Figure 1: Maxi-LVP assay development and validation.

Hepatitis C virus lipoviral particles (LVP) were measured by two methods using the same fasting EDTA plasma samples. The reference assay defined LVP as low density HCV RNA ($d < 1.07 \text{ g / ml}$), measured from 500 μL of plasma using iodixanol density gradient ultracentrifugation as described previously (14, 15). Maxi-LVP were measured in 50 μL of EDTA plasma, and defined as HCV RNA retained by a size filter (100, 200 and 450 nm), after incubation with diluted lipid emulsion (to mimic post prandial lipaemia), Intralipid 20% (SigmaAldrich) or PBS as a control, at 37 °C for 2 hours. The greatest correlation coefficient between iodixanol gradient low density LVP and size filter retained HCV RNA was obtained using addition of dilute intralipid-plasma and fractionation using a 100 nm Durapore size filter (panel A). A Bland-Altman plot describes the test agreement between maxi-LVP and iodixanol LVP $\log_{10} \text{ IU/mL}$ (panel B). The mean difference was $-0.97 \log_{10} \text{ IU/mL}$, standard deviation 0.47, (95% CI $-1.11, -0.83$). The upper limit of agreement (ULA) was -0.83 , and lower limit of agreement (LLA) $-1.89 \log_{10} \text{ IU/mL}$.

Non-LVP were defined from a reference iodixanol density gradient assay as HCV RNA detected at high density ($d > 1.07 \text{ g/mL}$). There was significant correlation between the filtrate non-LVP (size $< 100\text{nm}$), after addition of dilute Intralipid (panel C). A Bland-Altman plot describes the test agreement between maxi-LVP filtrate and iodixanol non-LVP $\log_{10} \text{ IU/mL}$ (panel D). The mean difference was $-1.15 \log_{10} \text{ IU/mL}$, standard deviation 0.57, (95% CI $-1.32, -0.98$). The upper limit of agreement (ULA) was -0.03 and lower limit of agreement (LLA) $-2.27 \log_{10} \text{ IU/mL}$.

Figure 2

Total HCV RNA levels (A), maxi-LVP levels (B), non-LVP levels (C) and maxi-LVP ratios (D) in early acute (<26 weeks) vs late acute >26 weeks duration of HCV infection. The line represents the median value.

Figure 3: Total HCV RNA levels (A), maxi-LVP levels (B), non-LVP levels (C) and maxi-LVP ratios (D) in acute HCV comparison between spontaneous clearance and persistent HCV infection. The line represents the median value.



