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Spontaneous loss of Hepatitis C virus RNA from serum is associated with genotype 1 and younger age at exposure.

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	HCV - viraemic	HCV-non-viraemic
Genotype	Number (%)	
1	98 (45.0)	40 (64.5)
2	16 (7.3)	4 (6.5)
3	102 (46.8)	16 (25.8)
4	2 (0.9)	0
5	0	1 (1.6)
6	0	1 (1.6)
	N = 218	N = 62

Table I: Results of HCV genotyping and serotyping (excluding mixed genotypes and serotyping failures).

		N=321	Univariable	Multivariable	N=280	Univariable	Multivariable
Factor	Level	Non-Viraemic/Total (%)	RR (95% CI), p-value	RR* (95% CI), p-value	Non-Viraemic/Total (%)	RR (95% CI), p-value	RR* (95% CI), p-value
Gender	Male	65/202 (32.2)	1.03 (0.74-1.45), 0.84	1.08 (0.80-1.45), 0.61	44/181 (24.3)	1.34 (0.82-2.18), 0.25	1.31 (0.84-2.03), 0.24
	Female	37/119 (31.1)	Baseline		18/99 (18.2)	Baseline	Baseline
Country of Birth	UK	66/147 (44.9)	Baseline	Baseline	33/114 (28.9)	Baseline	Baseline
	Non-UK	4/46 (8.7)	0.19 (0.07-0.50), 0.001	0.24 (0.09-0.64), 0.004	3/44 (6.8)	0.24 (0.76-0.73), 0.01	0.24 (0.08-0.74), 0.01
	Unknown	32/128 (25.0)	0.56 (0.39-0.79), 0.001	0.62 (0.44-0.86), 0.004	26/122 (21.3)	0.74 (0.47-1.15), 0.18	0.73 (0.48-1.09), 0.12
Ethnicity	White	64/239 (26.8)	Baseline		47/221 (21.3)	Baseline	
	Non-White	5/23 (21.7)	0.81 (0.36-1.81), 0.61		2/20 (10.0)	0.47 (0.12-1.79), 0.27	
	Not-known	33/59 (55.9)	2.09 (1.53-2.84), <0.001		13/39 (33.3)	1.57 (0.94-2.61), 0.085	
Mode of acquisition	IDU	77/211 (36.5)	Baseline		49/183 (26.8)	Baseline	
	Transfusion	18/43 (41.9)	1.15 (0.77-1.70), 0.50		9/33 (27.3)	1.02 (0.56-1.87), 0.95	
	Not-known	7/67 (10.4)	0.29 (0.14-0.59), 0.001		4/64 (6.3)	0.23 (0.88-0.62), 0.004	
Alcohol consumption	Safe alcohol	71/195 (36.4)	Baseline	Baseline	38/161 (23.6)	Baseline	Baseline
	Excess alcohol	29/117 (24.8)	0.68 (0.47-0.98), 0.04	0.61 (0.43-0.85), 0.004	22/110 (20)	0.85 (0.53-1.35), 0.49	0.72 (0.50-1.05), 0.09
	Not-known	2/9 (22.2)	0.61 (0.18-2.1), 0.43	0.98 (0.28-3.48), 0.98	2/9 (22.2)	0.94 (0.27-3.30), 0.93	0.92 (0.24-3.56), 0.90
Genotype	Type-3				16/118 (13.6)	Baseline	Baseline
	Type-1				40/138 (29.0)	2.14 (1.26-3.61), 0.005	2.07 (1.25-3.43), 0.005**
	Type-2,4,5,6				6/24 (25.0)	1.84 (0.08-4.23), 0.15	2.94 (1.74-4.97), <0.001**
Age at exposure	<20 years	46/94 (48.9)	Baseline	Baseline	32/80 (40.0)	Baseline	Baseline
	>20 years	36/117 (30.8)	0.63 (0.45-0.88), 0.008	0.67 (0.49-0.93), 0.015	14/95 (14.7)	0.37 (0.21-0.64), <0.001	0.42 (0.25-0.72), 0.002
	Not-known	20/110 (18.2)	0.37 (0.24-0.58), <0.001	0.45 (0.28-0.70), <0.001	16/105 (15.2)	0.38 (0.23-0.64), <0.001	0.43 (0.25-0.73), 0.002
Time since exposure	<20 years	27/78 (34.6)	Baseline		17/68 (25.0)	Baseline	
	>20 years	55/133 (41.4)	1.19 (0.83-1.72), 0.34		29/107 (27.1)	1.08 (0.65-1.82), 0.76	
	Not-known	20/110 (18.2)	0.53 (0.32-0.87), 0.01		16/105 (15.2)	0.61 (0.33-1.12), 0.11	

Table II: Univariable and multivariable analyses of those individuals for whom genotyping and serotyping data was available (N=280) and for all those included in the study with and without typing data (N=321). * Relative risks obtained using binomial regression with a log link. ** Note that using logistic regression rather than binomial regression with a log-link gives OR=3.03 (1.53-6.00), P=0.002 for type-1 and OR = 2.75 (0.84-9.02), P=0.10 for types 2, 4, 5 and 6.

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3 **Spontaneous loss of Hepatitis C virus RNA from serum is associated with**
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5 **genotype 1 and younger age at exposure.**
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10 **Running title: HCV genotype 1 and spontaneous loss of HCV-RNA**
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Abstract

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A variety of factors have been associated with spontaneous loss of hepatitis C virus (HCV-)RNA from serum, including infecting HCV type, although results are conflicting. This study aimed to investigate further whether infecting HCV type was linked to spontaneous loss of HCV-RNA. Serum samples from 321 untreated HCV antibody positive patients presenting at the Hepatology clinic at Addenbrooke's Hospital, Cambridge between 2004 and 2007 were tested. These individuals were classified either as HCV antibody and HCV-RNA positive (viraemic, n=219) or HCV antibody positive and repeatedly HCV-RNA negative (non-viraemic, n=102). Infecting HCV type was identified by genotyping (viraemic) or serotyping (non-viraemic). Binomial regression analysis investigated the independent effect of HCV type on spontaneous loss of HCV-RNA from serum by comparing the two groups. Ninety one per cent of patients were found to be either genotype 1 or genotype 3. The prevalence of type 1 infection was greater among non-viraemic (64.5%) than viraemic individuals (45%). After controlling for the effects of potential confounding factors, multivariable analyses showed that individuals with type 1 infections were more likely to be non-viraemic than genotype 3 infections (RR=2.07; 95%CI: 1.25, 3.43; P=0.005). Individuals infected at an older age were also less likely to become HCV-RNA negative spontaneously (RR=0.42 comparing those infected at ≥ 20 years of age against those infected at < 20 years of age, 95%CI: 0.25, 0.72; P=0.002). In conclusion, the results suggest that HCV genotype 1 infections are more likely than genotype 3 infections to become spontaneously non-viraemic, as are infections acquired at younger age.

Introduction

Spontaneous resolution of HCV infection, defined by normalisation of liver function tests (LFTs) and loss of HCV-RNA from serum, occurs in 26 - 36% of HCV-infected individuals (Santantoni *et al*, 2003, 2006, Micallef *et al*, 2006). This group is of particular interest since an understanding of the mechanisms associated with spontaneous loss of HCV-RNA from serum may aid vaccine development. A number of factors are linked to spontaneous loss of HCV-RNA from serum including clinical factors such as symptomatic infection (Gerlach *et al*, 2003), HBV co-infection (Piasecki *et al*, 2004) and host factors including female gender (Alric *et al*, 2000; Micallef *et al*, 2006;) and white ethnicity (Piasecki *et al*, 2004; Thomas *et al*, 2000).

Innate and host immune responses to HCV are likely to play an important role in spontaneous loss of HCV-RNA from serum, which has been linked in some, but not all studies with: genetic polymorphisms that modify IL-10, TNF- α or IFN- γ production (Kusumoto *et al*, 2006; Lio *et al*, 2003; Mangia *et al*, 2004; Minton *et al*, 2005); polymorphisms in interferon-induced genes (Knapp *et al*, 2003) and HLA class II alleles (Minton *et al*, 1998). Recent studies have demonstrated a significant association between a single polymorphism upstream of the IL28B gene (which encodes the type III interferon IFN- λ 3) and both spontaneous (Thomas *et al*, 2009) and treatment-induced (Ge *et al*, 2009) HCV resolution. In a large cohort of women infected with HCV from a single source, HLA-B27 allele was present more frequently in non-viraemic individuals compared to those with viraemia (McKiernan *et al*, 2004).

Viral factors have also been reported to influence spontaneous loss of HCV-RNA from serum. A prospective study of 92 anti-HCV positive males with a history of

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3 injecting drug use demonstrated a greater prevalence of genotype 3 in individuals
4 who cleared HCV-RNA from serum spontaneously (86% of type 3 cleared compared
5 with 7% of type 1) (Lehmann *et al*, 2004). Infection with genotype 1, specifically
6 subtype 1b, has been linked to chronic infection (Amoroso *et al*, 1998; Hwang *et al*,
7 2001). Conversely, in a large study, Harris and colleagues (2007) suggested that
8 HCV genotype 1 infection was more likely to be associated with spontaneous loss of
9 HCV-RNA from serum than non-1 genotype infection. A recent meta-analysis of 31
10 longitudinal studies with 675 study subjects concluded that the only factors
11 significantly associated with loss of HCV-RNA from serum were female gender and
12 acute symptomatic HCV infection (Micallef *et al*, 2006).
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29 The aim of this study was to describe the prevalent HCV genotypes in a cohort of
30 patients presenting to the hepatology service at Addenbrooke's Hospital, Cambridge
31 and to investigate whether spontaneous loss of HCV-RNA from serum was linked to
32 infecting HCV genotype.
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41 **Materials and Methods**

42 *Study sample*

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45 Consecutive patients presenting as new referrals to the Hepatology clinic at
46 Addenbrooke's Hospital, Cambridge between April 2004 and April 2007 who were
47 HCV antibody positive by routine testing were identified. Inclusion and exclusion
48 criteria were: included if confirmed to be HCV antibody positive using the ADVIA
49 Centaur[®] HCV assay (Siemens Healthcare Diagnostics, Surrey, UK); excluded if an
50 HCV antibody test was indeterminate; included if tests for HIV were negative;
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3 excluded if patients had ever received immune suppression; excluded if patients had
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5 ever been treated with antiviral therapy.
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10 Serum was tested, as part of routine clinical management, for the presence of HCV-
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12 RNA using an in-house reverse transcription PCR. This is a real-time Taqman PCR
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14 assay which targets a 90 bp section of the conserved 5' non-coding region on a
15
16 Rotor-Gene™ 3000 instrument (Corbett Lifescience, Cambridge, UK). HCV
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18 quantitation is carried out using a serial dilution of recombinant plasmid standards,
19
20 incorporating the HCV target site as an external calibration system. These standards,
21
22 which have been pinned to the WHO international HCV-RNA standard (96/798), are
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24 included in each run alongside the samples to be tested. A standard curve is
25
26 generated by the Rotor-Gene by plotting the threshold cycle (Ct) versus the
27
28 concentration of the standards. The HCV-RNA quantity of each unknown sample is
29
30 determined by locating its Ct on the standard curve. Probit analysis (Stats Direct,
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32 www.statsdirect.com) revealed a lower limit of detection of 25 IU/mL (6.3-38.6, 95%
33
34 CI) (unpublished data). The HCV-RNA PCR assay results were used to classify the
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36 cohort into viraemic (HCV-RNA positive) and non-viraemic (HCV-RNA negative);
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38 patients were only included if at least one follow up sample was available and where
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40 results were consistent.
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50 After exclusions as above, 336 patients were identified. Clinical details were collated
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52 including: sex, age, ethnicity (white or non-white), country of birth (UK or non-UK),
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54 mode of HCV acquisition (injecting drug use or known transfusion), alcohol
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56 consumption (safe alcohol < 21 units per week for males and <14 units for females;
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58 excess alcohol >21 units per week for males and >14 units for females), age at
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3 exposure (derived from time of first injecting drug use or from date of first transfusion)
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5 and time since exposure (calculated as the date of sample minus the reported date of
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7 exposure). Sufficient residual sera were available for further testing in 321 of these
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9 individuals (219 viraemic and 102 non-viraemic patients) which were subjected to
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11 investigation as below.
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15 16 17 *HCV typing*

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19 For viraemic patients, HCV genotype was identified using an improved Taqman HCV
20
21 genotyping assay (Rolfe *et al*, 2005, 2009). For non-viraemic patients, the infecting
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23 viral type was ascertained by serotyping using the Murex HCV Serotyping kit (Abbott
24
25 Diagnostics, Berkshire), following manufacturers' guidelines. Further tests were
26
27 carried out on all non-viraemic samples to exclude the possibility that these might be
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29 HCV-RNA positive but at levels below the sensitivity of the HCV quantitation assay.
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31 One ml of serum was ultracentrifuged (25,000 xg for 60 min) prior to extraction to
32
33 concentrate any virus particles. The pellet was then re-suspended in 350 µl of ultra-
34
35 PURE™ water. Nucleic acid was extracted on the BioRobot MDx workstation
36
37 (Qiagen, Crawley UK) and amplified using the HCV quantitation assay. HCV-RNA
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39 remained undetectable in all sera ultracentrifuged prior to extraction; the internal
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41 control (which detects amplification inhibition) was amplified in these samples within
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43 the acceptable range.
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50 51 52 *Statistical analysis*

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54 For statistical analysis, age at exposure was examined as a categorical variable (<20
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56 years vs. ≥ 20 years). As 91% of infections were found to be either genotype 1 or
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58 3, genotype was categorised as type 3, type 1 and 'other' genotypes. Missing data
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60 were modelled as a 'not-known' category. Analysis was carried out with STATA®

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3 statistical software (StataCorp LP, Texas, USA), using univariable and multivariable
4 binomial regression (regression with binomial errors and a logarithmic link function)
5
6 to obtain relative risks (RR). Age, gender, genotype and other significant variables
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8 (5% level) were retained in the multivariable analysis. Logistic regression was also
9
10 carried out to verify p-values from binomial regression which are sometimes
11
12 unstable. Analyses were performed initially using all patients for whom typing data
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14 were available and then including the additional 41 that were non-typable.
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22 *Ethical Approval*

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24 HCV genotyping of viraemic patients was carried out as part of routine patient
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26 management. Ethical approval for HCV serotyping of stored serum specimens from
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28 non-viraemic patients was received from the Cambridge Local Research Ethics
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30 Committee (LREC) (06/Q0108/339).
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36 **Results**

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38 Of the 321 specimens, 37 were untypable, 4 were mixed genotypes, 138 were type
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40 1, 118 were type 3, and 24 were other types. For the primary analysis those that
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42 were untypable or of mixed type were not included, leaving 280 typed specimens of
43
44 which 62 were from non-viraemic patients (Table I). The univariable analyses
45
46 demonstrated that spontaneous loss was more likely in those who were UK born,
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48 who were younger at acquisition and who were infected with type 1 virus (Table II). In
49
50 the multivariable analysis the effect of type 1 remained similar with a RR compared to
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52 type 3 of 2.07 (95% CI; 1.25, 3.43). Genotypes 2, 4, 5, and 6 showed some evidence
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54 of having a higher spontaneous loss than type 3 (RR 2.94), although this was based
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56 on small numbers (n=24) and was not significant when using logistic regression
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3 (p=0.10) (Table II). In the multivariable analysis, likelihood of spontaneous loss
4 remained significantly associated with those born in the UK and those who were <20
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6 compared to ≥ 20 years old at acquisition (RR 0.42; 95% CI 0.25, 0.72, for ≥ 20
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8 versus <20) (Table II).
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13 Further analyses on all individuals initially included in the study (n=321), which took
14 into account the 41 that were non-typable or of mixed type, were undertaken (Table
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16 II). Age at exposure and country of birth remained significantly associated with
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18 spontaneous loss of HCV-RNA from serum with broadly similar RR estimates.
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20 Additionally, excess alcohol was associated with a reduced likelihood of spontaneous
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22 loss of HCV RNA in this group (RR 0.61; 95% CI 0.43, 0.85). Genotype could not be
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24 assessed in this analysis since those that were non-typable (with one exception)
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26 were non-viraemic.
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34 35 **Discussion**

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37 Early treatment of HCV can prevent chronic infection. However, antiviral therapy for
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39 HCV infection has substantial side effects and is costly. Spontaneous loss of HCV-
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41 RNA from serum occurs in around twenty to thirty per cent of people with acute HCV
42
43 infection (Santantonio *et al*, 2003; Micallef *et al*, 2006). It would be advantageous to
44
45 identify these people prior to therapy. Spontaneous loss of HCV-RNA from serum
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47 occurs within 3 months of infection (Gerlach *et al*, 2003; Santantonio *et al*, 2005), so
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49 delaying treatment of individuals to target only those who remain HCV-RNA positive
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51 is feasible (Santantonio *et al*, 2006). For the strategy of delayed treatment to be
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53 possible, it would be helpful to identify factors, host or viral, that predict spontaneous
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55 loss of HCV-RNA from serum.
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3 In this study, those without viraemia were defined as negative for HCV-RNA on a
4 number of occasions. It is presently unclear whether spontaneous loss of HCV-RNA
5 from serum is associated with complete eradication of the virus including the liver, or
6 whether low-level viral replication persists but is controlled by cellular and humoral
7 immune responses. Several small studies suggest that HCV-RNA can be
8 consistently undetectable in serum or plasma by laboratory assays, but be
9 identifiable in serum and peripheral blood mononuclear cells (PBMCs) by molecular
10 tests with enhanced sensitivity, in patients thought to have loss of HCV-RNA from
11 serum (Pham *et al*, 2004; Radkowski *et al*, 2005). Increasing the sensitivity of HCV-
12 RNA detection is possible by ultracentrifugation of the serum prior to nucleic acid
13 extraction (Bartolomé *et al*, 2007; Forman *et al*, 2004; McHutchison *et al*, 1999). The
14 HCV quantitation assay utilised in this study, demonstrated good sensitivity (25
15 IU/mL), comparable to commercial assays. HCV-RNA was not detected in any of
16 those HCV-RNA negative in serum despite ultracentrifugation prior to extraction.
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39 Thirty seven isolates (36.3%) were negative by HCV serotyping, which was probably
40 due to degradation of HCV-specific antibodies during storage. Serotyping failure
41 could also be a result of loss of antibody response with time in resolved infection,
42 especially in individuals with a long time period between exposure and subsequent
43 testing. The assay should not exhibit different genotype sensitivity which might have
44 resulted in bias. The possibility of bias was investigated by examining the optical
45 density (OD) readings of all serotyped isolates. No significant difference in OD was
46 observed between genotype 1 and type non-1 isolates. However, the possibility of
47 over-representation of a particular serotype amongst those which failed cannot be
48 completely ruled out.
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3 Despite the steps taken to ensure that samples were HCV-RNA negative, the
4 possibility of occult HCV infection in individuals presumed to have cleared the virus
5 cannot be ruled out. Occult HCV infection has been recently reported and is defined
6 as the presence of HCV-RNA in the liver without serum HCV-RNA (Castillo *et al*,
7 2004). It has been reported in two clinical situations: in anti-HCV negative, serum
8 HCV-RNA negative patients with abnormal LFTs and in anti-HCV positive subjects
9 with normal LFTs without serum HCV-RNA (Carreno *et al*, 2006). In a small study on
10 twelve patients who were anti-HCV positive, serum HCV-RNA negative with normal
11 LFTs, 83% of these individuals had both genomic and anti-genomic HCV-RNA
12 strands detected in the liver, demonstrating HCV replication in the majority of these
13 patients (Carreno *et al*, 2006a). Interestingly, all the infections were found to be with
14 subtype 1b. Recently a study examining liver biopsies from HCV non-viraemic
15 individuals found evidence of an ongoing immune response in the liver, which also
16 often demonstrated fibrosis, supporting the view that HCV persists in the liver of most
17 serum HCV-RNA negative individuals (Hoare *et al*, 2008). These findings suggest
18 that the term 'spontaneous clearance' must be used with care, as these individuals
19 may still have genomic HCV-RNA in liver tissue.
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46 The findings of the present study support those of Harris and colleagues (2007). In
47 their study, the infecting type was ascertained in a large cohort of individuals who
48 were viraemic (n = 508) or had spontaneous loss of HCV-RNA from serum (n = 96).
49 That study showed that the prevalence of genotype 1 in those who were HCV-RNA
50 negative in serum was 69% and in those that had remained viraemic was 51%,
51 suggesting that individuals with HCV genotype 1 infection were more likely to
52 become HCV-RNA negative (OR 0.47, 95% CI 0.29–0.78, P = 0.003). The study
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3 cohort consisted of patients enrolled in the UK HCV National Register and most of
4 these patients (90%) had been traced during the National HCV Lookback
5 Programme and had acquired their infection on a known date via transfusion of
6 infected blood (prior to routine HCV screening) (Harris *et al*, 2000). The population
7 group in the present study is very different. Unlike the aforementioned study, many
8 of the patients in this study would have been identified by virtue of their clinical
9 symptoms and referred to the Hepatology clinic for further investigation post-testing.
10 As such, cohorts of patients from tertiary referral centres are more likely to be biased
11 towards symptomatic disease (Sweeting *et al*, 2006). Also, in contrast to the study
12 by Harris and colleagues, 'date of infection' was often a crude estimate, as high risk
13 activities, like intravenous drug use, gave an uncertain date for HCV acquisition.
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32 The results from the present study strengthen the evidence that patients with
33 genotype 1 infections are more likely to become spontaneously HCV-RNA negative
34 in serum. Infecting genotype was significantly associated with non-viraemia in both
35 univariable and multivariable statistical analyses. Individuals who had type 1
36 infections were more than twice (2.07x) as likely to become HCV-RNA negative.
37
38 Over 91% of HCV infections in this study were genotype 1 or 3, which reflects UK
39 epidemiology (Health Protection Agency, 2009) This study also demonstrated that
40 genotypes 2, 4, 5 and 6 were associated with non-viraemia (RR 2.94) although due
41 to the low numbers of these genotypes, the finding was not significant when using
42 logistic regression (p=0.10). Additionally those who acquired their infections at an
43 older age were less likely to become HCV-RNA negative, with a RR of 0.42 for those
44 infected at ≥ 20 years of age compared to < 20 years of age.
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3 The suggestion that genotype 1 infections are more likely to spontaneously lose
4 HCV-RNA from serum than type 3 infections is contrary to certain other studies
5 (Amoroso *et al*, 1998; Hwang *et al*, 2001; Lehmann *et al*, 2004). Although
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The spontaneous loss of HCV-RNA from serum may be more likely in genotype 1
infection, it is thought that, in those individuals with a chronic genotype 1
disease may be more severe (Harris *et al*, 2007; Lopez-Labrador *et al*, 1997;
Nousbaum *et al*, 1995; Pozzato *et al*, 1994; Tanaka *et al*, 1998). It is plausible that
genotype 1 infections might be more aggressive and therefore more likely to provoke
an immune response leading to resolution, but if the immune response fails then the
disease may be more severe. The association between younger age at exposure
and spontaneous loss of HCV-RNA from serum demonstrated in this study has been
reported in haemophiliacs (Messick *et al*, 2001; Zhang *et al*, 2006) and in childhood
HCV infection (Yeung *et al*, 2007). Older age at exposure is thought to be linked to
increased risk of progression to cirrhosis (Minola *et al*, 2002). It is pertinent to
mention that in the study described, the age of exposure was, for the majority, a
crude estimate which relied on each individual's recollection of risk incident. For
those, such as intravenous drug users, who are involved continually in risk
behaviour, the date of infection may be inaccurate. The association between
younger age at exposure and increased likelihood of spontaneous non-viraemia is
not surprising as the immune system is more responsive and able to fight infection at
a young age and degenerates during the ageing process.

This study has demonstrated that age at exposure and HCV genotype are both
associated with loss of HCV-RNA from serum. Thus, younger patients with acute
genotype 1 HCV infection may be less likely to need antiviral treatment.

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