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Characterising the B-cell response to Hepatitis C virus infection in patient cohorts: impact on clinical outcomes and implications for vaccine design.

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A thesis submitted in the fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Hepatitis C virus (HCV) infection is one of the major causes of liver morbidity and mortality worldwide. While effective therapies are now available, if eradication of this virus is to be achieved globally, an effective vaccine is still necessary. During hepatitis C virus (HCV) infection, broadly neutralizing antibody (bNAb) responses targeting E1E2 envelope glycoproteins are generated in many individuals. It is unclear if these antibodies play a protective or a pathogenic role during chronic infection or if they could prevent infection or reinfection with the virus.

I investigated the presence and clinical associations of bNAb responses in three cohorts of individuals infected with or exposed to HCV infection. One with chronic HCV infection at differing disease states, one with chronic HCV infection at an early disease state and one group of individuals at high risk of HCV exposure who remained uninfected by conventional testing. I also studied bNAb responses in an individual from a HCV-HIV co-infected cohort who experienced spontaneous clearance of HCV after a post-therapy relapse (‘secondary spontaneous clearance’).

I found a proportion of individuals when exposed to or infected with HCV produce a polyclonal bNAb response which may contribute to viral clearance in some cases. Host genetics and the ability to target multiple neutralising epitopes on the envelope protein are associated with such responses, although resistance mutations to bNAbs do exist *in vivo*. The presence of bNAbs is associated with lower levels of liver fibrosis. Using next generation sequencing technology in the study of B cell receptors in HCV infection revealed subtle changes in the B cell repertoire on HCV infection, this technology may be used in future to gain insight into the generation of bNAb responses.

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Publications:

Arising from work described in this thesis (included as additional material)

Anti-envelope antibody responses in individuals at high risk of hepatitis C virus who resist infection. Swann RE, Mandalou P, Robinson MW, Ow MM, Foung SK, McLauchlan J, Patel AH, Cramp ME. ***J Viral Hepat***. 2016 Nov;23(11):873-880.

Broad Anti-Hepatitis C Virus (HCV) Antibody Responses Are Associated with Improved Clinical Disease Parameters in Chronic HCV Infection. Swann RE, Cowton VM, Robinson MW, Cole SJ, Barclay ST, Mills PR, Thomson EC, McLauchlan J, Patel AH. ***J Virol***. 2016 Apr 14;90(9):4530-43.

Arising from work related to this thesis but not directly described within it

Tracking TCRβ Sequence Clonotype Expansions during Antiviral Therapy Using High-Throughput Sequencing of the Hypervariable Region. Robinson MW, Hughes J, Wilkie GS, Swann R, Barclay ST, Mills PR, Patel AH, Thomson EC, McLauchlan *J.* ***Front Immunol***. 2016 Apr 5;7:131

Viral genotype correlates with distinct liver gene transcription signatures in chronic hepatitis C virus infection. Robinson MW, Aranday-Cortes E, Gatherer D, Swann R, Liefhebber JM, Filipe Ada S, Sigruener A, Barclay ST, Mills PR, Patel AH, McLauchlan *J.* ***Liver Int.*** 2015 Oct;35(10):2256-64.

Elevated interferon-stimulated gene transcription in peripheral blood mononuclear cells occurs in patients infected with genotype 1 but not genotype 3 hepatitis C

virus. Robinson MW, Swann R, Sigruener A, Barclay ST, Mills PR, McLauchlan J, Patel AH. ***J Viral Hepat.*** 2015 Apr;22(4):384-90.

Non cell autonomous upregulation of CDKN2 transcription linked to progression of chronic hepatitis C disease. Robinson MW, McGuinness D, Swann R, Barclay S, Mills PR, Patel AH, McLauchlan J, Shiels PG. ***Aging Cell***. 2013 Dec;12(6):1141-3.

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Author's declaration

This work was completed at the University of Glasgow between October 2011-2015 and has not been submitted for another degree. All work presented in this thesis was obtained by the author's own efforts, unless otherwise stated.

Abbreviations

°C degrees Celsius

% percentage

μ micro (10^{-6})

aa amino acid

A_{450} absorbance at a wavelength of 450 nanometres

ALT alanine aminotransferase

Apo apolipoprotein

ARFP alternative reading frame protein

BCR B cell receptor

bNAbs broadly neutralising antibodies

bp base pair(s)

BSA bovine serum albumin

C- carboxy-terminus

cDNA complementary DNA

CHCV Chronically infected with HCV cohort

CLDN claudin

DAA direct acting antiviral drug

dH2O deionised molecular biology grade water

DMEM Dulbecco's modified eagle's media

DNA deoxyribonucleic acid

DNase deoxyribonuclease

DNTPs deoxynucleotides triphosphates

E.coli Escherichia coli

EC50 half maximal effective concentration

EDTA ethylenediaminetetra-acetic acid

EIA enzyme immunoassay

ELISA enzyme-linked immunosorbant assay

ET patient "ET" from Dr Emma Thomson's HIV HCV coinfected cohort (NB this abbreviation does not relate to any identifiable information for this individual).

FCS foetal calf serum

g gram

GAG glycosaminoglycan

GST glutathione-s-transferase

gt genotype

h hour(s)

HAV hepatitis A virus

HBSS Hanks Balanced Salt Solution

HBV hepatitis B virus

HBc hepatitis B core protein

HC Healthy control

HCC hepatocellular carcinoma

HCl hydrochloric acid

HCV hepatitis C virus

HCVcc HCV cell culture-infectious virus

HCVpp HCV pseudoparticles

HIV human immunodeficiency virus

HLA human leukocyte antigen

HmAb Human monoclonal antibody

HRP horseradish peroxidase

Huh-7 human hepatoma 7 (cells)

HVR hypervariable region

IC50 Inhibitory concentration at the 50% level

IDU injection drug use

IFN interferon

IgG immunoglobulin G

IGHV immunoglobulin heavy chain variable region gene

IgVR intergenotypic variable region

IL28 interleukin 28 also known as IFL3

IRES internal ribosome entry site

IRF interferon regulatory factor

ISG IFN-stimulated gene

JFH Japanese fulminant hepatitis

kbp kilobase pair(s)

kDa kilodalton(s)

LB Luria Broth

LB2 cell lysis buffer

L litre(s)

LDL low-density lipoprotein

LFTs liver function tests

LVP lipoviroparticle

m milli (10^{-3})

M molar

mAb monoclonal antibody

MC Mixed Cryoglobulinaemia

min minute

MR Dr Mark Robinson (MRC University of Glasgow Centre for Virus Research)

mRNA messenger RNA

nano (10-9)

N- amino terminus

NAbs neutralising antibodies

NGS next generation sequencing

NK natural killer

NP non-progressor group in the Historical cohort

OCLN occluding

OD optical density

ORF open reading frame

P progressor group in the Historical cohort

PBMC Peripheral Blood Mononuclear Cell

PBS phosphate-buffered saline

PBST Tween added to phosphate buffered saline

PBSTM 2% Milk powder added to PBST

PCR polymerase chain reaction

pH potential of hydrogen

PI protease inhibitor

PWID people who inject drugs

RBR Dr Rachael Bashford-Rogers

RhF Rheumatoid Factor

RLU relative light unit

RNA ribonucleic acid

RNase ribonuclease

RT room temperature

RT-PCR reverse transcription PCR

RT-qPCR reverse transcription-quantitative PCR

sec second(s)

sE2 soluble E2

SD standard deviation

SNP single nucleotide polymorphism

SR-BI scavenger receptor class B member I

SVR sustained virological response

TMD transmembrane domain

TP Time point

TRIS 2-Amino-2-(hydroxymethyl)-1,3-propanediol

TV trypsin/versene

U unit

UTR untranslated region

uv ultraviolet

V volts

VLDL very-low density lipoprotein

wt wild-type

w/v weight/volume ratio

One and three letter amino acid abbreviations

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F

Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1 Introduction

1.1 Overview of the impact of Hepatitis C virus infection

Hepatitis C virus (HCV) infection is one of the major causes of liver morbidity and mortality worldwide. It affects 2-3% of the global population(1). In up to 75% of those infected, the host is unable to clear the virus and develops chronic infection (2, 3). Once chronic infection is established, it can cause liver inflammation and fibrosis leading to cirrhosis, end stage liver failure and hepatocellular carcinoma, being one of the leading indications for liver transplant in the UK and the United States of America(4, 5). In addition to liver related harm, infection is also strongly associated with pathological B cell disorders such as cryoglobulinaemia(6) and may contribute to neurocognitive disturbances(7, 8). While effective directly acting antiviral (DAA) therapies are now available, these are unlikely to result in global eradication of the virus in the short to medium term without further preventative strategies. One such strategy is the development of a universal HCV vaccine. As vaccination candidates to date have not met the standards required for widespread use(9), further study of the interaction of HCV and the adaptive immune system is helpful to inform future development.

1.2 The structure, function and pathogenesis of HCV

HCV, first identified in 1989(10), is a single-stranded positive-sense RNA virus belonging to the hepacivirus genus of the *flaviviridae* family (Fig. 1.1) which includes yellow fever, dengue and Japanese encephalitis in addition to the more recently identified pegiviruses.

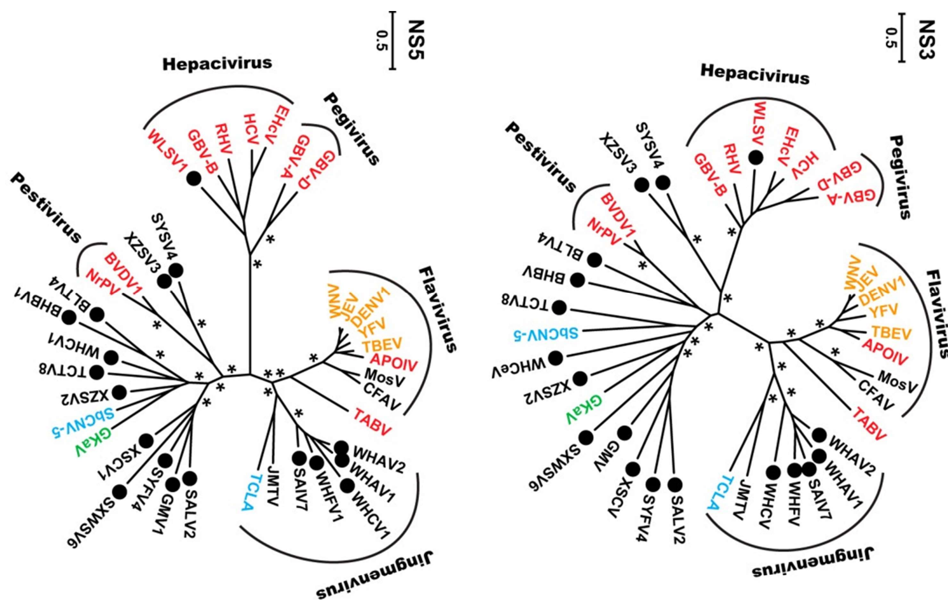


Figure 1.1 Phylogenetic tree of the Hepatitis C virus alongside related viruses in the Flaviviridae family.

Phylogenetic tree (unrooted) of the family *Flaviviridae* and jingmenviruses based on conserved domains in the NS3 (right panel) and NS5 (left panel). Based on their host types, the virus names are shaded red (vertebrate only), yellow (vertebrate and arthropod), green (plant), blue (non-arthropod invertebrates), or black (arthropod only). [Figure and Legend reproduced from Shi et al J. Virol 2016(11) Copyright American Society of Microbiology, permission granted for reuse in academic thesis]

HCV has a narrow host specificity being a predominantly human pathogen although other primates can sustain chronic infections(12). It primarily targets hepatocytes with recent reports suggesting that it may infect and replicate in other tissues including lymphocytes and neurons (13).

There are 7 accepted genotypes of HCV with at least 67 confirmed subtypes(14). Considerable genetic diversity exists both between and within the differing genotypes. The nucleotide sequence of different subtypes within a genotype differ by >15% whereas there can be as much as a 30-40% variation between different genotypes(15). This genetic diversity in part explains why HCV has been so successful in quickly evolving to overcome host defences.

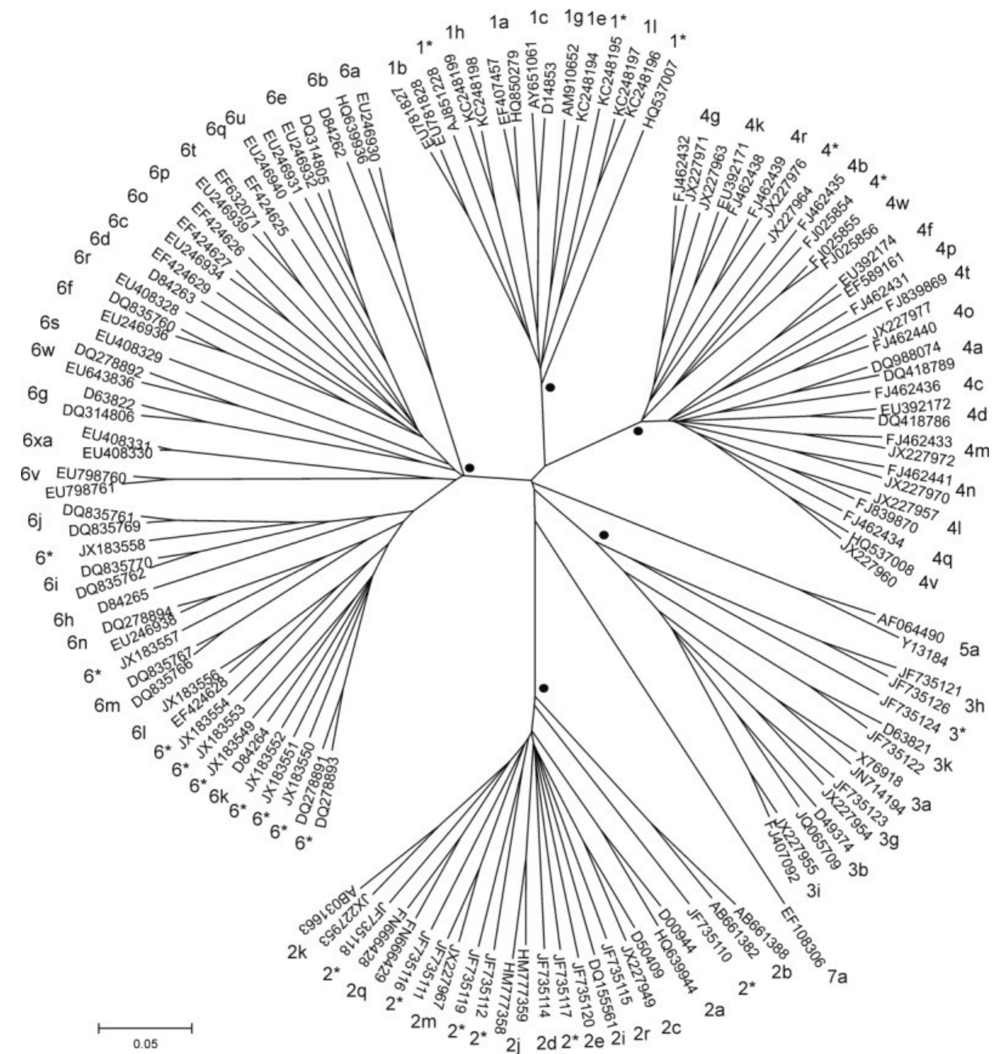


Figure 1.2 Phylogenetic tree of representative sequences of genotypes and subgenotypes of Hepatitis C

Phylogenetic tree of 129 representative complete coding region sequences. Up to two representatives of each confirmed genotype/subtype were aligned (together with a third extreme variant of subtypes 4g and 6e) and a neighbor joining tree constructed using maximum composite likelihood nucleotide distances between coding regions using MEGA (Molecular Evolutionary Genetics)software(16). Sequences were chosen to illustrate the maximum diversity within a subtype. Tips are labeled by accession number and subtype (*unassigned subtype). For genotypes 1, 2, 3, 4, and 6, the lowest common branch shared by all subtypes and supported by 100% of bootstrap replicates (n = 1,000) is indicated by ·. [Figure and Legend reproduced from Smith *et al* 2014, Hepatology(14) , permission granted through Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0/.]

The geographical distribution of the different genotypes shows marked regional variations with genotype (gt) 1 being most common in north America, gt 3 predominant in Asia and gt 4 most prevalent in north east Africa (Fig. 1.3). Within the UK, due to immigration patterns, gt 1 and gt 3 are almost equally distributed in the population(17).

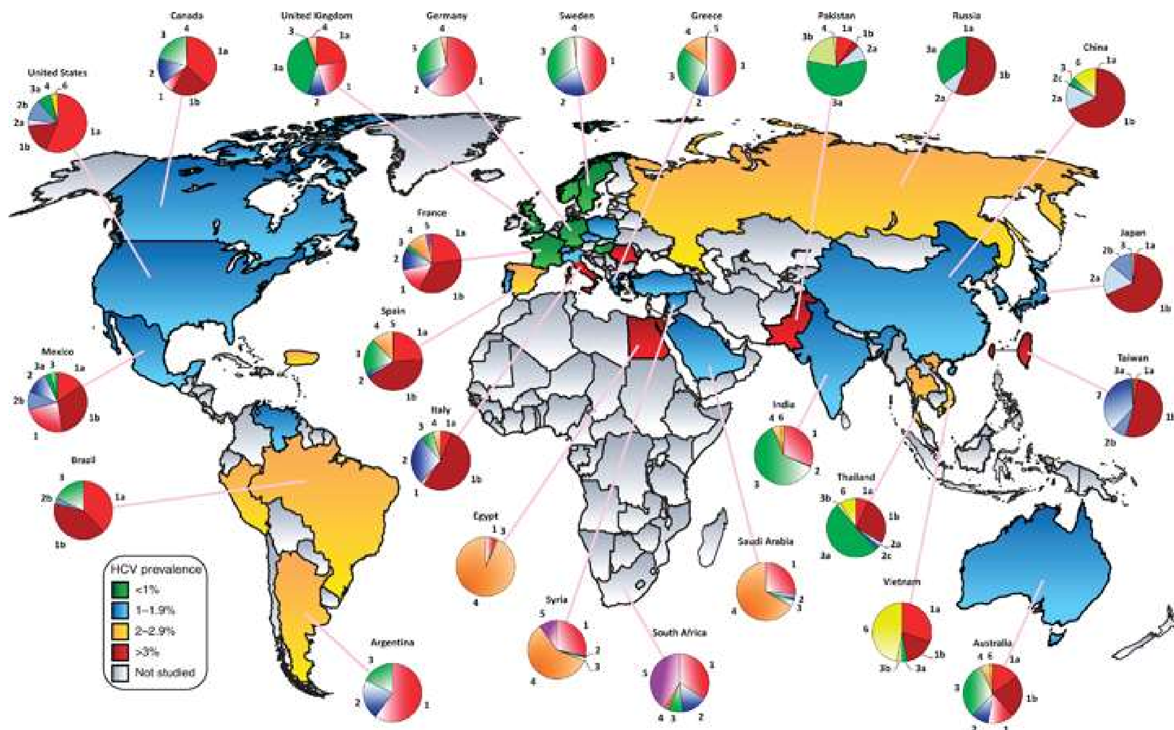


Figure 1.3 Global prevalence of HCV and distribution of genotypes.

Map displaying overall prevalence of HCV infection (indicated by shading of country) and genotype distribution within those countries where information is available.

[Figure extracted from Negro and Alberti Liver International 2011(18) reuse authorised by John Wiley and Sons]

The HCV genome has an open reading frame (ORF) of approximately 9600 base pairs in length which encodes for a polyprotein of around 3000 amino acids (aa), although this varies between genotypes and individual isolates. Before the N-terminus of the ORF sits an uncapped 5'UTR. This combines with a portion of the

core coding region to form a characteristic RNA secondary structure, the internal ribosomal entry site (IRES), responsible for binding ribosomal subunits to enable subsequent translation of the ORF.

The ORF encodes a polyprotein that is co-translationally processed by both host and viral encoded proteases into structural and non-structural proteins. The structural proteins are core and the two envelope glycoproteins named E1 and E2. The virus shares basic structural characteristics with other members of the Flaviviridae family, encompassing a nucleocapsid composed of core protein containing the RNA genome. This is covered in a phospholipid bilayer which incorporates the envelope glycoproteins E1and E2 involved in cell attachment (Figure 1.4). The non-structural proteins are p7, NS2, NS3, NS4 and NS5. With further understanding of the function of these proteins, different subunits have been identified which have distinct functions and act as specific targets for drug therapy. The functions and characteristics of each of these proteins are addressed below.

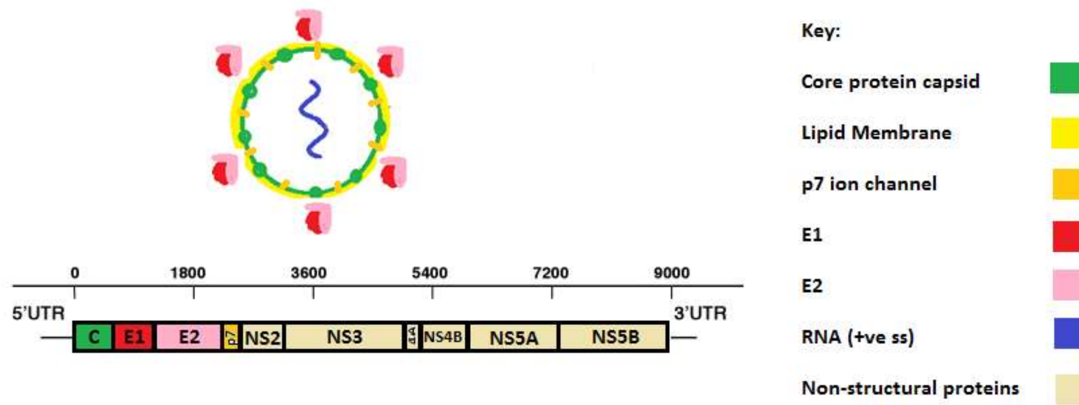


Figure 1.4 Structure of the HCV genome and viral particle

The diagram shows the accepted structure of the HCV virion with positive sense single stranded RNA encapsulated in a capsid constructed of core protein and also likely containing p7 ion channels. The outer surface of the virion incorporates a lipid membrane layer with E1E2 heterodimers protruding from the surface. Below is shown the relative position of the genes for these and the non-structural proteins in the HCV genome.

1.2.1 Core and Alternative Frameshift (REF) proteins

The core protein is usually 191 aa in length and highly basic in nature. It is one of the most highly conserved proteins in the HCV polyprotein(19). It consists of 3 domains, a hydrophilic domain, a hydrophobic domain and a final region which is important for correct initiation of E1 translation. Core protein is first generated in an immature form then cleaved from E1 by the host signal peptidase and finally cleaved again at aa 177 by the host signal peptide peptidase to form the mature core protein (20). It is this mature core protein which forms the nucleocapsid.

In addition to its structural role as part of the virus, the core protein has been proposed to have several pathogenic properties. It has been shown to interact with a

wide range of intracellular proteins including the Jak/STAT pathway and the TGF-β pathway (21) which may impair host immunity to the virus.

Although the core region is one of the most conserved regions of the viral genome, specific mutations may produce a survival advantage. A particular strain of core proteins are thought to interact with the cellular protein DDX3 in replication and affect sensitivity to interferon (IFN) (22). Similarly, several mutations in core have been associated with resistance to exogenous IFN therapy (23) .

Core protein may also be play role in the adverse liver outcomes in HCV infection. Transgenic mice studies have shown that those expressing core protein have a greater risk of insulin resistance, steatosis and possibly hepatocellular carcinoma (24).

1.2.1.1 The alternative frameshift Protein (REF)

One other incompletely understood function of the core encoding region is its capacity to undergo a translational frame-shift leading to the synthesis of an alternative frame protein (REF). This REF protein is produced when core is translated in the -2/+1 frame rather than from the ORF. The functions of this protein are less well described. It has been shown to interact with iron metabolism and may play a role in cellular proliferation and influence viral replication (25, 26) .

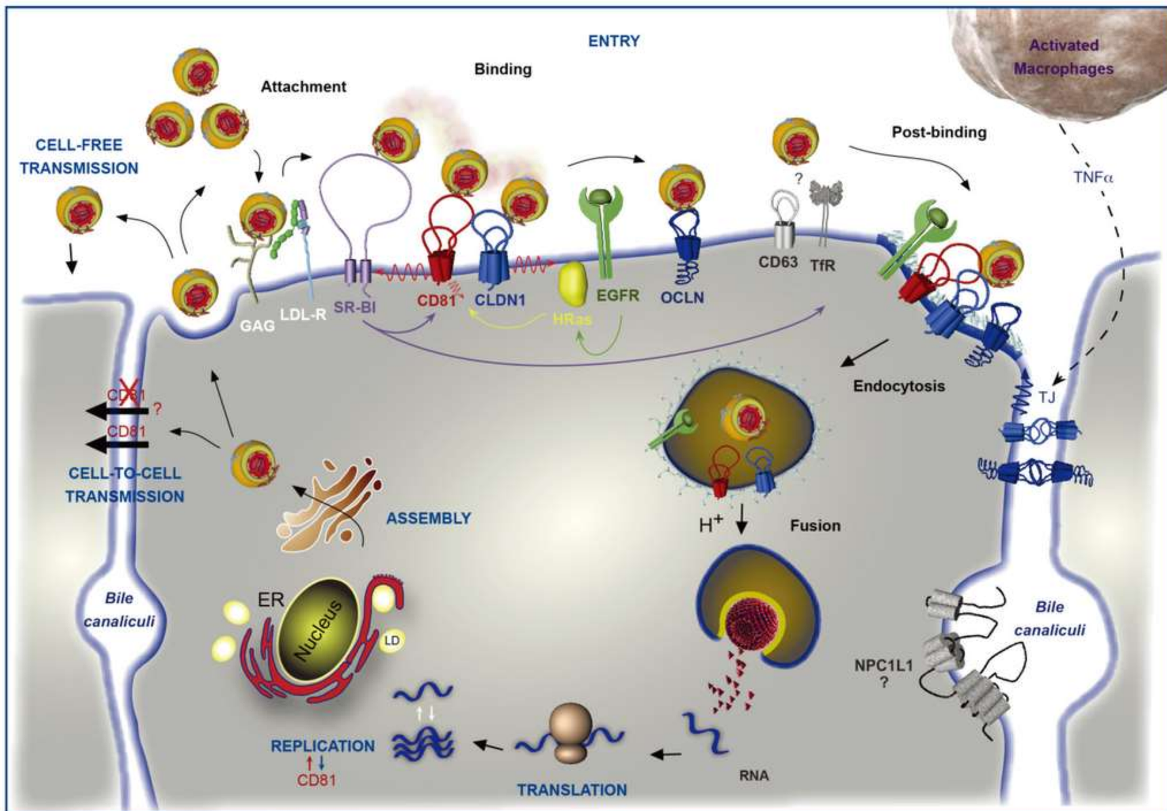
1.2.2 Envelope proteins E1 and E2

E1 and E2 are type I transmembrane glycoproteins. These envelope proteins form a highly structured noncovalent heterodimer on the surface of the virion in association with lipids and apolipoprotein E (Apo E). Correct folding of the protein is dependent

on the interaction between E1 and E2 with evidence that E1 forms trimers in the presence of E2 which may then organise into pentamers (27). There is evidence that rather than relying on conserved domains in both proteins, this interaction may be isolate-specific with Carlsen *et al* showing that generating chimeric envelope proteins using E1 and E2 from different isolates can disrupt this interaction(28).

The envelope protein interacts with lipids and Apo E. Replication of the HCV virion is associated with lipid droplets so that in vivo the virion is coated in VLDL. This lipid coating is important for infectivity and may mediate resistance to neutralisation by antibodies. For example, an amino acid mutation at position 451 alters the density of lipoproteins associated with viral particles and enhances sensitivity to neutralisation *in vitro* (29).

This lipoprotein coated envelope is responsible for attachment to hepatocytes and in facilitating entry into them by interacting with various cellular proteins. There is evidence that interaction with the LDL lipid receptor may be an initial step in entry(30). In addition, binding to sulphated glycosaminoglycans (GAGs) on the cell surface has also been shown to play a role in cell attachment (31). The best characterised interaction in HCV cell entry is the binding of envelope to the cell surface proteoglycans, CD81 and SR-BI to attach to the cell and initiate cell entry (32). Subsequently this appears to activate a pH dependent cellular endocytic pathway. This is facilitated by interaction of cellular proteins Claudin (33) and Occludin (34) with the envelope protein / CD81 complex (Figure 1.5). The viral envelope is also able to attach to the adhesion molecules DC-SIGN and LC-SIGN(35). While these are not found on the hepatocyte cell surface, they are found on other hepatic cells, thus this attachment may direct circulating virus towards hepatocytes.



Legend for Figure 1.5 on following page

Figure 1.5 Diagram showing steps in HCV cell entry and interaction with cell surface molecules.

HCV initiates its infection into hepatocytes by an attachment step at the cell surface in which virions interact with non-specific factors such as glycosaminoglycans (GAG). Due to the association of viral particles with lipoproteins, the Low Density Lipoprotein-Receptor (LDL-R) likely plays a role in this initial step of entry. HCV first interacts with the scavenger receptor class B type I (SR-BI), which in turn probably facilitates the association of viral envelope proteins with CD81. CD81 and the tight junction protein claudin-1 (CLDN1) naturally form a complex that is essential to HCV entry and which is likely regulated by the Epidermal Growth Factor-Receptor (EGFR) and the GTPase HRas. After interaction with the CD81/CLDN1 complex, HCV interacts with occludin (OCLN), another tight junction protein. Other molecules, such as the transferrin receptor (TfR), the tetraspanin CD63, and the Niemann-Pick C1-like1 (NPC1L1) cholesterol transporter, which is mainly localized in bile canaliculi (BC), have been shown to also be involved in HCV entry but for which mechanisms need to be elucidated. The membrane diffusion of CD81 (depicted by the red ↯) is another important element regulating HCV entry. The virus is next internalized by clathrin-mediated endocytosis, possibly in association with CD81/CLDN1 complex and EGFR. Internalization is likely favoured by the lipidic transfer properties of SR-BI. After fusion at low pH with the membrane of an early endosome, the viral genome is released into the cytosol. Next, translation and polyprotein processing take place and the viral RNA is replicated. In the late stages of the cycle, new virions are assembled in an ER-related compartment in close connection with the Very Low Density Lipoproteins (VLDL) biogenesis pathway. This process seems to occur in the proximity of lipid droplets (LD). Virions that are released can infect new cells by cell-free transmission. Particles can also be transferred directly to the neighbouring cells by cell-to-cell transmission for which CD81-independent and CD81-dependent routes have been described but are still controversial.

[Figure and Legend reproduced from from Feneant et al (2015) (36) under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/)].

The structure of a truncated but correctly folded E2 has recently been resolved using X ray crystallography (37, 38). This shows previously characterised CD81 binding regions identified by alanine scanning as being adjacent to one another along one face of the molecule (Figure 1.6). The E2 protein consists of several relatively conserved regions associated with CD81 binding or structural stability and 3 variable regions termed HVR-1, HVR-2 and IgVR (Figure 1.7). Even within these variable regions some amino acids are highly conserved suggesting a role in cell entry or other envelope functions (39). Towards the C –terminus of the protein a hydrophobic 'heptad repeat' region is located (aa 675-699). This is structural motif found in several viral fusion proteins and consists of a highly conserved repeating sequence of 7 aa. It has been shown to be important in the heterodimerisation of the envelope proteins and also in cell entry (40).

The structure of the majority of E1 protein has not yet been resolved, and in general it has been less well characterised than E2. However a 'fusogenic' region (aa 274-291) crucial for cell entry has been identified. Specific mutations may also modulate binding to the cell surface receptor SRB1 (41). Interactions between the two envelope proteins are still incompletely understood. A recent in silico study has proposed a structure of the whole E1/E2 protein and has suggested that E1 residues 192-215, 258-268, 290-306 and the transmembrane domain 352-378 interact with the E2 protein for correct heterodimerisation (27) .

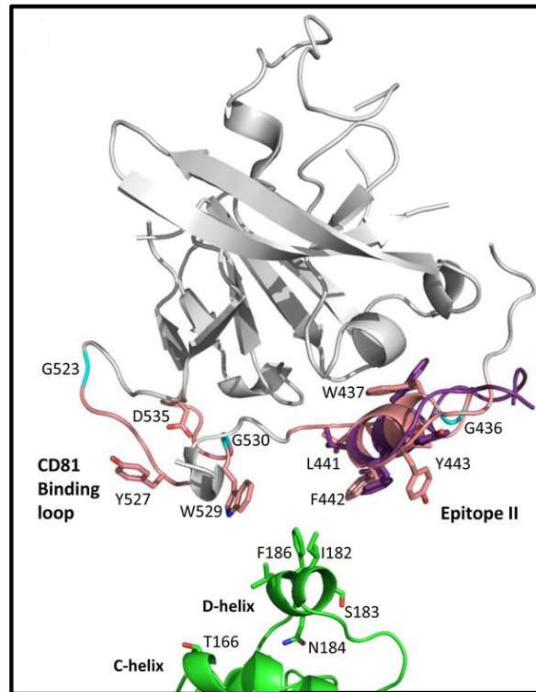


Figure 1.6 Diagram of the proposed structure of the E2 core structure with additional detail from CD81 binding regions superimposed (PDB ID 4MWF).

This shows the E2 core in grey, with regions involved in CD81 binding highlighted pale pink and specific side chains associated with CD81 binding displayed as follows: carbons in pale pink, oxygens in red, and nitrogens in blue, with the exception of glycines, which are shown in cyan. The docked epitope II peptide conformer superimposed on the same sequence in E2 is shown with side chains displayed in stick representation, with carbons shown in purple, oxygens in red, and nitrogens in blue. The CD81-LEL dimer is shown the side chains of the D-helix shown in stick representation and positioned as a reference to how this region may interact with the E2 helix motif, $W^{437}LAGLFY^{443}$. [Figure and legend reproduced from Harman *et al* 2015 (42)Copyright American Society of Microbiology, permission granted for reuse in academic thesis]

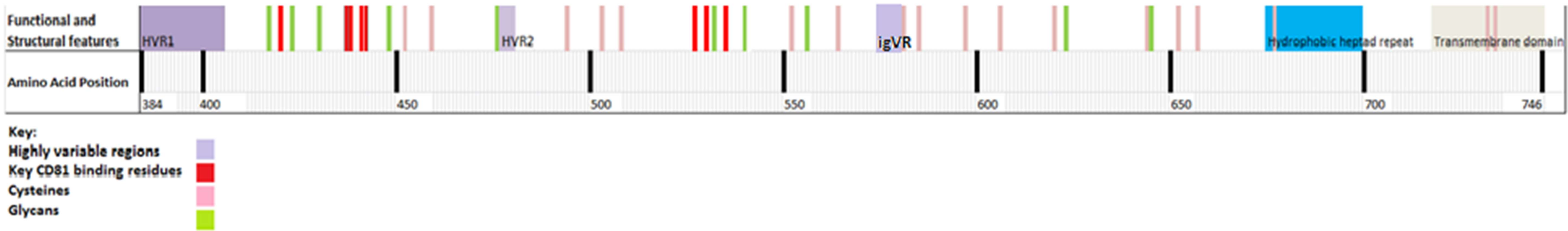


Figure 1.7 Linear diagram of E2 structure showing position of key CD81 binding residues and variable regions.

There are several glycosylation sites throughout the envelope proteins, 4 in E1 and 11 in E2. Of these, 5 located in E2 play an important role in infectivity forming part of a 'glycan shield'. These reduce sensitivity to neutralisation and are largely conserved. Loss of glycosylation at these sites causes considerable deterioration in viral fitness(43). At the other glycosylation sites, variable glycosylation can alter viral infectivity and susceptibility to antibody binding. The envelope proteins also have conserved Cysteines (44) which are important for correct folding, and Histidines (45) which contribute to CD81 binding.

While there is relatively little data on potential pathogenic effects of the envelope proteins on host beyond facilitating virus entry, one group has proposed that envelope binding to the CD81 ligand in turn activates the MAPK/ERK pathway (46) which may impact on cell proliferation and differentiation. This may have a pathological effect on hepatocytes. In addition, envelope binding to CD81 is one proposed mechanism of B cell dysregulation in the pathogenesis of HCV-associated cryoglobulinaemia (47).

1.2.3 p7

The p7 protein spans amino acids 751-814 of the HCV polyprotein. The function of p7 is incompletely understood. Recent structural analysis has shown that p7 has a structure consisting of two transmembrane alpha helices and a positively charged cytosolic loop. This is similar to transmembrane ion channels present in other viruses. It is mainly present on the endoplasmic reticulum, mitochondria and plasma membranes within infected cells. In vitro culture studies have also shown that, although it is not essential for replication, it is vital for particle assembly and release, likely having a role in cleaving an E2/p7/NS2 intermediate protein (48). Some work

has also proposed a role for p7 in cell entry but it is not essential for this process (49-51). A recent study has also identified a region of p7 which is key for reducing the sensitivity of HCV to IFN (52). Whether the p7 protein has additional pathological effects on host cells has not been determined.

1.2.4 Non Structural proteins

The non-structural proteins NS2, NS3, NS4 and NS5A/B are generally concerned with viral reproduction and assembly. Their sequences also code for the proteases required to cleave each functional part of the protein from its neighbour. These proteases are vital for viral replication and have been successfully targeted by direct acting anti-viral therapies in the clinic(53).

1.2.4.1 NS2

NS2 forms part of the NS2/3 protease, important for cleaving NS2 from p7 and NS3. NS2 is also thought to play a complex role in virus assembly separate from its protease role, however this function is likely to involve interaction with many other viral proteins and has not been fully described (54).

1.2.4.2 NS3/NS4

In addition to forming part of the NS2/3 protease, the N-terminal third of NS3 acts as a serine protease in conjunction with NS4a to cleave the HCV polyprotein at 4 sites: NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5a/NS5B. This protease contains a catalytic triad essential for protein cleavage. The C-terminal 2 thirds of the NS3 protein act as a ‘RNA helicase’. As there is no requirement for a RNA helicase in the HCV lifecycle it has been proposed that this may interact with HCV RNA during

replication to facilitate this process, or help protect viral RNA from host defences which use RNA binding proteins(55).

The NS4B protein, produced by cleavage with the NS3B/4A protease, is 261 amino acids long. It localises to the endoplasmic reticulum. It is also thought to bind intracellular ‘lipid rafts’ creating an intracellular membrane which provides an anchor for replication complexes(56).

Additionally, NS4B appears to be immunogenic, being commonly recognised by humoral and cellular immune responses in infected hosts (57). It has also been shown to activate the intracellular NF-kB pathway(58) which may contribute to intrahepatic inflammation and development of fibrosis. Of note, a particular mutation (Q1737H) was associated with relative IFN resistance in one study(59). NS4B has also been shown to have an oncogenic effect in the presence of the HRas gene (60).

1.2.4.3 NS5A and NS5B

Until recently, the function of the 447 amino acid NS5A protein was incompletely understood. It is now known to consist of 3 domains. Domains 1 and 2 are involved in replication and may be responsible for recruiting NS5B to the replication complex, whereas domain 3 associates with the core protein and is involved in viral assembly and intracellular transport (61). It is also thought to play a role in preventing cell apoptosis which in turn may have an impact on tumorigenesis within the infected liver(62, 63).

NS5B consists of a 591 aa protein which acts as a RNA-dependant RNA polymerase, essential for viral replication (64). Because of the essential role of both NS5 proteins, they have been attractive target for small molecule direct acting

antivirals and agents targeting these regions are now the mainstay of recommended IFN-free treatment regimens (65).

1.3 Virus transmission, the immune response to HCV and its ability to escape from host defences

HCV is a blood borne virus and therefore is most commonly transmitted by inoculation of blood contaminated bodily fluids into another individual. While HCV has managed to infect up to 3% of the global population, it is relatively less infective per exposure than the other hepatotropic blood borne viruses, HBV and HIV. Its successful spread is partly a factor of the chronic nature of most infections, thus providing a long period over which to transmit the virus. It has also benefited greatly from human behaviours which have assisted its spread, some of which are detailed below.

1.3.1 Routes of transmission

While many bodily fluid contacts have theoretical potential for transmitting HCV, the most common route of transmission is subcutaneous insertion of a needle contaminated with blood from an infected individual.

In developed countries, the major route of transmission remains the use of injection drugs and sharing of injection equipment by injection drug users (IDUs) (66). Symptomatic acute HCV infection is rare and HCV has the potential to spread undetected within these populations. Even though effective antiviral medication is now available(53), treatment to date has remained costly and ineffectively

implemented with the prevalence of HCV in IDUs rising in England in recent years(67).

Other common sources of HCV transmission include tattoos or piercings using multiple use equipment and iatrogenic transmission. As HCV screening of blood products was only introduced in 1991, many individuals were infected through blood or blood product transfusions prior to this date. The details of these transmissions have recently been analysed in the Penrose enquiry (68). Groups particularly at risk include the haemophiliac population who received pooled clotting factors derived from multiple donations of blood and plasma. While screening has almost completely eliminated the risk to current blood product recipients, there are still occasional cases of transmission in the medical setting e.g. through haemodialysis equipment (69).

More recently, sexual transmission has been recognised as a key component of acute HCV infection, particularly in men who have sex with men participating in high risk sexual practices. This is well documented in those co-infected with HIV(70). However, among heterosexual couples the risk of transmission from unprotected sex is low(71). Vertical transmission is recognised but infants of infected mothers are at relatively low risk (approximately 5% infection rate) if relevant precautions are taken (72).

In the developing world, causes of transmission vary. In the Asian subcontinent and parts of Africa, use of inadequately sterilised needles for mass vaccination programmes has undoubtedly contributed to the high prevalence of HCV in these countries. In Egypt, which has the highest world prevalence of HCV at 14.7%, the

main risks for transmission have been identified as medical procedures ranging from injection of medications to surgery (73).

1.3.2 Viral and host factors associated with spontaneous resolution of infection

While most infected individuals will progress to chronic infection without therapy, some clear the virus spontaneously. This usually occurs in the first 6 months following infection. Studies of high risk individuals have identified exposed groups at lower risk of contracting HCV and with a higher probability of viral clearance if infected.

Due to the rapid multiplication of virus and error rate of HCV RNA polymerase, even infection with a single strain of virus soon evolves into infection with multiple quasispecies. Viral factors associated with progression to chronic infection include a diverse range of quasispecies detected shortly after transmission(74). This either suggests a virus which could rapidly diversify in that particular host environment, or that multiple quasispecies have been transmitted initially.

HCV proteins themselves are thought to play an important part in attenuating the immune response by interaction with intracellular signalling pathways essential for viral defence. For example, the HCV protein complex NS3/4A blocks the action of IFN regulatory factor-3(75) . While specific mutations in these regions associated with chronic infection have not been identified, it is likely some viral quasispecies are better able to supress the immune response than others. Indeed, small studies have also suggested that genotype 3 may be easier for individuals to clear during acute infection(76).

While the host immune response is key in determining whether the virus is cleared during the acute phase, other factors such as female sex and young age at infection in adults are also associated with a higher likelihood of clearance and resolution of reinfection(77, 78). The mechanism by which these confer an advantage is unknown although these effects may also be mediated through an influence on immune activity.

1.3.3 Innate immunity to HCV infection

The first immune response to viral infection is in the form of innate immunity. The majority of innate responses are mediated through the production of IFNs and downstream upregulation of IFN (IFN) stimulated genes. This occurs predominantly in both infected cells and the key antigen sensing cells of the immune system, dendritic cells and macrophages. In general the innate immune response is triggered by binding of viral proteins, often RNA, to host receptors. Two main pathways for this have been described. The cytosolic pathway is activated through RNA binding to the 'RNA helicases retinoic acid inducible gene-I' (RIG-I) or Protein Kinase R receptors in the cytoplasm. This in turn activates the Mitochondrial Anti-Viral Signalling protein (MAVS). The endosomal pathway is triggered by recognition of viral proteins by one of the 3 antiviral Toll-Like Receptors (TLR). Binding to TLR-3 activates the 'Toll-IL-1 receptor domain-containing adaptor inducing IFN-β' (TRIF) and the kinase TBK1 (Fig. 1.8). Receptor binding in both these pathways induces a chain of interactions triggering the release of IFN I and IFN III. Type II IFN (IFN – γ) is produced by NK cells and some adaptive immune cells and potentiates the action of IFN I (79, 80). IFNs then bind to their specific receptors activating the Jak-STAT pathway which in turn upregulate an array of 'IFN Stimulated Genes' with anti-viral activity.

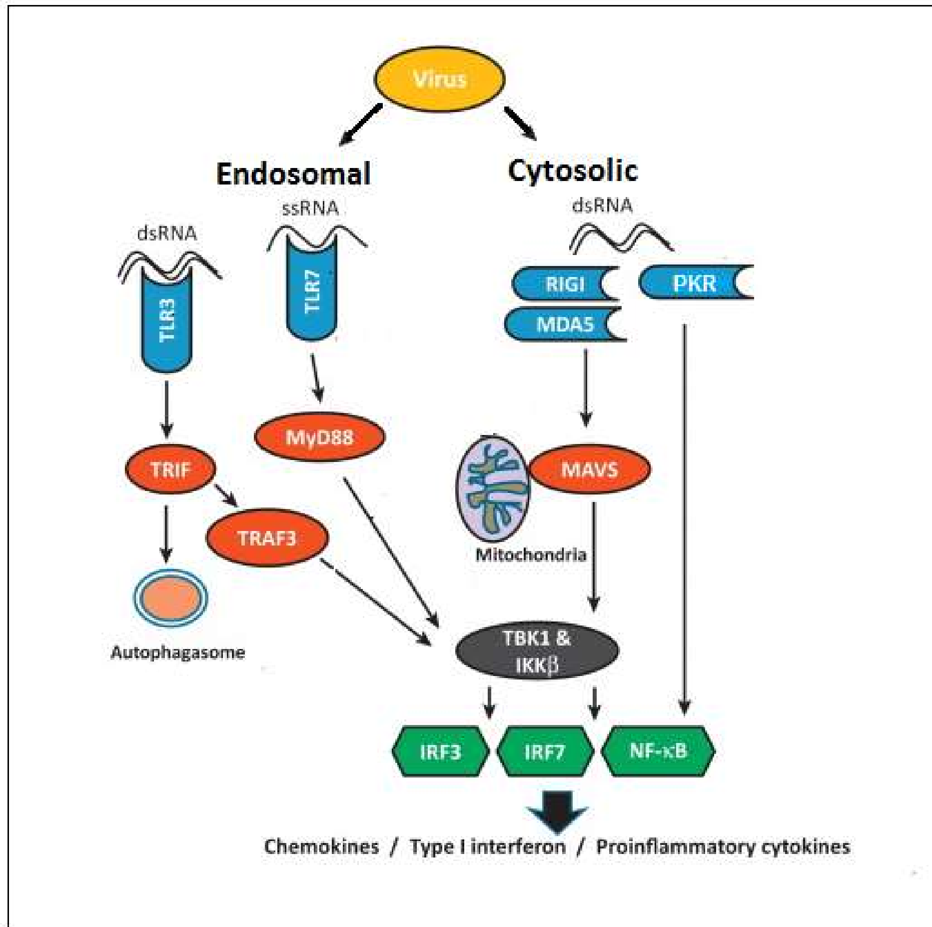


Figure 1.8 Outline of the endosomal and cytosolic innate antiviral pathways

The two main pathways for cellular detection of viruses are shown. In the endosomal pathway, Toll-like receptors results in activation of myeloid differentiation primary response 88 (MyD88) and Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) pathways. In the cytosolic pathway retinoic acid-inducible gene (RIG)-like receptors (RIGI) are triggered, Protein Kinase R (PKR) is also phosphorylated which results in the activation on anti-transcription factors and activation of nuclear factor (NF)-κB. The TRIF signaling pathway can also trigger autophagy. RIGI activation triggers mitochondrial antiviral-signaling protein (MAVS)-dependent pathways. These pathways have common mediators in TANK-binding kinase-1 (TBK1) and inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-β). These in turn activate the transcription factors IFN regulatory factor (IRF) 3, IRF7, and nuclear factor (NF)-κB to promote production of IFN in addition to other inflammatory molecules. [Figure adapted from Carty (2014) Trends in immunology (81)]

HCV proteins such NS3/4 are able to block IFN production to some extent in infected cells. Despite this, surrounding cells are stimulated to produce IFN (predominantly types α, β and γ) generally leading to small clusters of infected cells surrounded by rings of uninfected cells (82). It is interesting to note that while IFN is undoubtedly part of acute immune responses which promote clearance, in chronic infection, pre-treatment high levels of IFN stimulated genes are negative predictors of response to dual therapy with peg-IFN-α and ribavirin (83).

Extracellular innate antiviral defences have also recently been identified as being important in HCV. For example, small molecules such as complement, ficolins and collectins have been proposed as having importance in HCV resistance (84, 85). The role of lipoproteins in facilitating and inhibiting HCV infection has also been explored with the discovery that while ficolins can bind to E1E2 to prevent cell entry, they are inhibited in the presence of ApoE (86).

Genetic factors influencing the innate immune system are associated with differential outcomes in acute infection and exposure. A single nucleotide polymorphism (SNP) at the IFN lambda 3 locus (also known as IL28B) genotype CC is associated with increased likelihood of clearing acute infection, in addition to its association with higher viral clearance rates in chronically infected individuals treated with IFN therapy(87-90).

In cohorts which are at high risk of repeated exposure (i.e. injection drug users who share needles and injecting paraphernalia), a group of individuals have been identified who show no serological evidence of ever having been infected (i.e. standard antibody tests to non-structural proteins are negative). These uninfected

individuals (termed exposed uninfected or EU) have been shown to have innate immune system characteristics which are distinct from both healthy controls and spontaneous clearers (Table 1.1) (91-93). EU cohorts display raised levels of proinflammatory mediators IL6/IL8 and TNF-α in addition to natural killer cell activity compared to IDUs infected with HCV (94, 95). EU cohorts are also genetically distinct from spontaneous resolvers and those with chronic HCV(96) with the alleles for the Killer Cell Immunoglobulin Like Receptor KIR2DL3 and HLA type C1 over represented in both the EU and spontaneous resolver groups whilst prevalence of the IL28B (IFNL3) polymorphism is similar in EU to chronically infected cohorts(93, 95, 97, 98).

Table 1.1: Immunological characteristics of EU and spontaneous HCV resolvers.

Immunological Compartment	Exposed Uninfected	Spontaneous Resolvers	Chronically Infected	Control group	Ref
Natural Killer Cell cytotoxicity	↑/→	↓ (NB spontaneous resolvers and chronically infected not separated)		Healthy controls	(94)
IL28B CC genotype	→	↑	→ (control)	Chronically infected	(96)
KIR2DL3	↑	↑	→ (control)	Chronically Infected	(97, 98)
IL12 genotype (C allele)	↑	↑	N/A	Compared to healthy controls	(93)
IL6/IL8 levels	↑	N/A	N/A (control)	Healthy controls and chronically infected	(95)
TNF-α	↑	→	→	Healthy controls	(95)
HCV specific T cell proliferation	↑	Present in some	→ (control)	Chronically infected	(92, 99)

Summary of studies comparing immune characteristics of individuals chronically infected with HCV, healthy controls, individuals acutely infected with the virus who clear or spontaneously (spontaneous resolvers) and those at high risk of exposure who do not show markers of infection (exposed uninfected). Arrows indicate level of protein expression or cellular activity (indicated by heading) compared to control group. The control groups used in each study are indicated in the appropriate column.

1.3.4 T cell mediated immunity and virus evasion of the T cell response

T cell responses to HCV develop after 8-12 weeks of infection (100). Appearance of HCV specific T cells peripherally is associated with the onset of liver inflammation (101). In the clinic, symptomatic acute infection accompanied by evidence of liver inflammation is a positive predictor of viral clearance.

Several HLA types which influence the ability of antigen presenting cells and infected cells to stimulate T cell activity have been associated with clearance or lower rates of infection. For those acutely infected with HCV genotype 1b, it has been shown that individuals with the HLA B27 alleles are better able to process and present key, conserved NS5B antigens to T cells, thus facilitating viral clearance (102). As this HLA type is commonly associated with autoimmunity, it has been proposed that HCV may mimic some 'self' proteins, thus preventing a vigorous immune response developing in most individuals. Genome wide association studies of HCV have also identified other HLA loci associated with likelihood of spontaneous HCV clearance, namely HLA-A*0301, B*5701, Cw*0102, DRB1*0101 and the region surrounding DQB1*03:01 (87).

Due to the high rate of viral turn over and rapid mutation rate, if adaptive immune responses targeting specific viral regions are required for clearance, the breadth of response is likely to be critical in determining infection outcome (74). Immune response 'breadth' has been defined in many ways but essentially relates to the ability of an individual's immune system to react to or prevent infection by a wide range of viral genotypes, sub-genotypes or within-genotype quasispecies. A broad

and sustained T cell response is related to successful virus clearance in both acute infection and chronically infected patients undergoing treatment (103-105).

In cohorts exposed to HCV who remain uninfected, T cell responses may also play an important role with evidence of adaptive HCV-specific T cell responses in up to 60% of EU(92) (99). The presence of such responses suggests the adaptive immune system is exposed to the virus but that the virus is controlled before infection can become established enough to stimulate traditional markers of infection (i.e. antibodies to core and non-structural proteins).

However, with persistent HCV antigen stimulation, T cells display an exhausted phenotype (106, 107). If HCV infection becomes chronic, this phenotype does not recover even after successful antiviral therapy(108). Experimental blockade of exhaustion associated molecules has been unsuccessful in promoting viral clearance in HCV although some boosting of adaptive responses has been seen in HIV (109), (110). This suggests that if the hosts T cell response is unable to clear virus effectively in the acute setting, it loses the ability to adequately eliminate virus. If this is the case, it is unlikely that T cells play a significant protective role in clinical outcomes once chronic infection is established.

1.3.5 B cell mediated immunity to HCV

1.3.5.1 Mechanism of B cell mediated immunity to virus

The other major component of the adaptive immune response to virus is B cell mediated, predominantly in the form of antibodies. These are produced by a specialised B cell population termed plasma cells. Prior to antigen exposure, individuals have a diverse circulating population of B cell receptors, able to recognise

different antigens. These result from recombination of different V, D and J gene segments during lymphocyte development. There are upward of 10^{14} potential combinations and the average adult has 10^{10}-10^{11} circulating B cells at any one time. On recognition of an antigen via the B cell receptor, B cells then internalise the antigen and represent it to T helper cells. On recognition of the presented antigen by $CD4^{+ve}$ T cells, co-stimulation occurs. B cells then develop into either memory B cells or antibody-secreting plasma cells. The initial antibody response to an antigen usually results in relatively non-specific binding IgM antibodies being produced. On chronic or repeat exposure to an antigen, more specific divalent IgG is generated. Over a period of chronic exposure, plasma cells divide and receptor genes undergo somatic hypermutation which over time increases the specificity and avidity of the antibodies for the antigen (Figure 1.9)

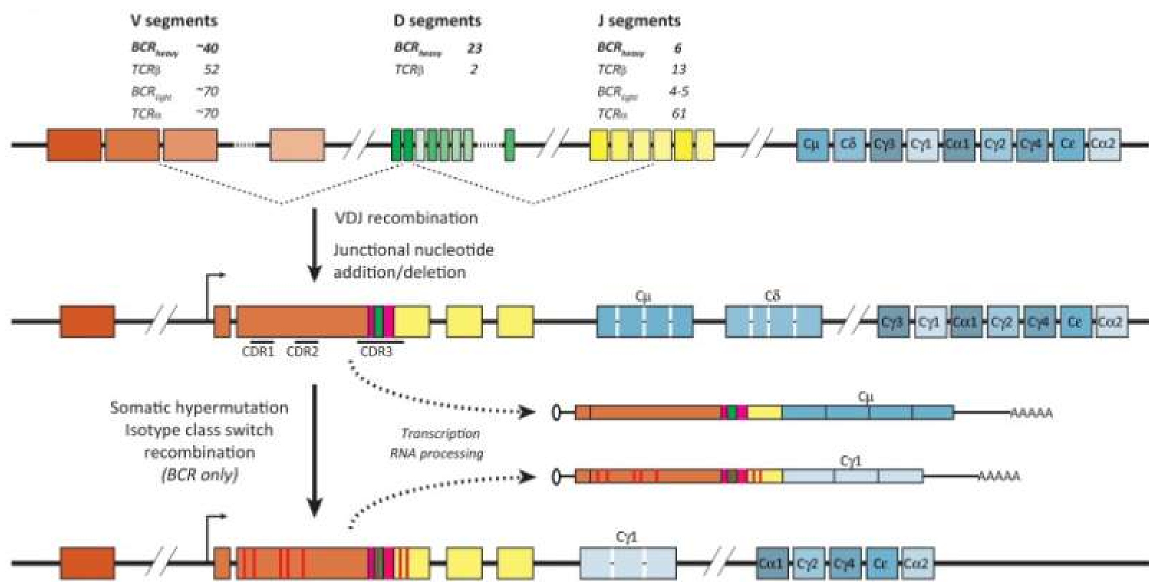


Figure 1.9 B cell antigen receptor diversification.

A schematic of the BCR heavy locus is shown; with the exception of somatic hypermutation and class-switch recombination. Antigen receptor repertoire diversity is primarily established during lymphocyte development, during which V (orange), D (green), and J (yellow) gene segments are rearranged through the process of V(D)J recombination. Numbers of distinct V, D, and J segments are shown for each antigen receptor locus. During the recombination process, nucleotides may be added or deleted at segment junctions (magenta), contributing to additional sequence diversity. Complementarity determining regions are indicated. BCR-specific secondary diversification may occur following antigen recognition. In somatic hypermutation processes, mutations (red) are introduced throughout the variable region such that modified BCRs may be selected through affinity maturation. In class-switch recombination, gene segments encoding constant regions (blue) are rearranged resulting in the production of antibodies with different isotypes and corresponding effector functions. Abbreviations: BCR, B cell receptor; TCR, T cell receptor; V, J, and D, Variable, Joining, and Diversity gene segments. [Extracted from Calis 2014(111). Permission for reuse granted through RightsLink].

Antibodies can inhibit viral infections in several ways. Neutralising antibodies are those which bind to epitopes on the virus surface which prevent interaction with host cell receptors, therefore preventing entry into target cells. Individuals also produce non-neutralising antibodies which target regions not essential for cell entry. These antibodies may promote clearance of functional virus from serum through opsonisation and subsequent phagocytosis or complement dependant lysis but would not directly inhibit target cell entry. Indeed, there is evidence that non-neutralising antibodies may enhance target cell infection in other diseases(112).

It is not known which concentration of envelope specific antibodies is required to prevent *de novo* or recurrent infection with HCV. Acutely infected individuals in the process of clearing the infection tend to have higher concentrations of total anti-HCV antibody as a whole than those who have cleared the infection some time ago(113).

General stoichiometry studies examining antibody binding have concluded that there is a relationship between virion size and the number of antibody molecules needed to neutralise the virion, preventing it from infecting its target cell. The HCV virion has been shown to vary in size between 40 and 100nm in diameter (114). Papilloma viruses, which are a similar size to the HCV virion at 40-55nm, require 38 antibody particles to neutralise each virion (115, 116). Interestingly antibodies are relatively large in comparison to these viruses at 10nm and effective neutralisation can occur without saturation of binding sites with antibody particles. However, the variation in the size of the HCV virion suggest some incorporate more lipid than others. This flexibility in particle structure may contribute to masking key viral proteins from the immune system.

1.3.5.2 The anti-envelope antibody response to HCV

In HCV, antibodies directed at different viral components appear from around 7 weeks following infection although in some individuals this can be delayed up to 6 months or longer in HIV co-infection (117). Phage display methods and purified monoclonal antibody (mAb) isolates have been used to map distinct immunogenic domains of E1E2 targeted by patient derived antibodies (118-123). Some of these are involved in cell entry and therefore elicit neutralising antibodies which prevent infection (NAb), others elicit non-neutralising antibodies. Perhaps the best characterised antibody epitope regions are the conformational immunodomains A to E described by Steven Foung (121, 123, 124). These are listed in Table 1.2 along with the names of isolated mAbs which have been shown to bind in these regions. Additionally, a separate research group have identified an alternative collection of HCV "antigenic regions" and characterised antibodies targeting these domains. These include antibodies directed at a region overlapping domains B and E in addition to domains encompassing the E1 – E2 interface (125-127). The key antibodies identified by this group are also shown on Table 1.2. Recent alanine scanning data has located the immunodomains and their key residues on the core E2 structure (See Figs. 1.10 and 1.11). Note that while Domain B and D were initially thought to be separate (124), in fact they share common residues. In addition, animal immunization studies have yielded antibodies to linear epitopes that are essential for infection (32, 128-130).

1.3.5.3 The antibody/B cell repertoire

As with the T cell response, it is conceivable that an initially broader repertoire of B cell receptors targeting HCV may limit its ability to escape from these responses. As,

until recently, sequencing the B cell receptor population could only be achieved through a relatively laborious process, much of the work on B cell repertoire to date has focused on the functional breadth of antibody response. With the advent of next generation sequencing, there is more scope to relate this to the genetic diversity and evolution of B cell populations.

1.3.5.4 Broadly neutralising antibody responses

In acute HCV infection, there is growing evidence that a robust and 'broad' antibody response can contribute significantly to viral clearance (113, 131). Previous observational studies have shown rapid onset of anti-HCV antibodies to be associated with higher likelihood of clearance (132). More recently, it has been proposed that developing a broad antibody profile capable of preventing infection by diverse strains of HCV virus predict acute clearance in a cohort infected with genotype 1a HCV(113, 133).

While there is no universally accepted definition of 'breadth' of antibody response, this is widely accepted as the functional ability of a mAb or the polyclonal antibodies derived from a single individual to bind to and prevent infection by a range of viral subspecies. Such antibodies are generally termed "broadly neutralising antibodies" (bNAbs). In the HCV field, some groups have tested against viral subspecies within one genotype to determine breadth(133) whereas others have used a panel of viruses from differing genotypes(129). As mAbs have the ability to neutralise broadly in such studies, this definition does not distinguish between polyclonal responses which target a single neutralising epitope and those which target multiple neutralising epitopes. However, as the virus can evolve to escape from some of the most potent

bNAbs(121) , the ability to target multiple epitopes may be advantageous in overcoming such escape mutations to achieve successful clearance. Little is known about which areas of the HCV envelope proteins are preferentially targeted by the humoral response in human hosts at population level as most studies analysing broadly neutralising activity examine only small numbers of individuals. However, a study screening for the prevalence of antibodies to a highly conserved linear region of E2, targeted by the mouse monoclonal bNAb AP33, found that only 2-15% of HCV infected individuals raise immunoglobulins to this region (134, 135).

Why some individuals develop a broad neutralising response and others do not is incompletely understood. Recent work examining antibody responses to HIV have suggested that viral evolution is key in activating and developing B cell receptors to produce neutralising antibodies(136), however unlike HIV, spontaneous resolution of HCV infection associated with bNAbs occurs in a significant proportion of individuals(133) suggesting that breadth of antibody response is not solely the product of a rapidly evolving virus repeatedly escaping the immune response.

1.3.5.5 Escape from the antibody/B cell response

HCV has several mechanisms by which it can evade adaptive humoral responses to establish chronic infection. Firstly, its error prone polymerase enables genetically diverse quasispecies of virus to be generated rapidly leading to the emergence of dominant NAb-escape mutants(15), (137). Figure 1.11 illustrates the regions important for binding of known bNAbs, this can be compared with the most variable sites in the amino acid sequence of the genotype 1 envelope protein, shown in

Figure 1.12. In considering vaccine design it is important to note that variability of up to 30% can be observed between genotypes(15).

Secondly, important epitopes in the virus are subject to glycan shielding. In addition, HVR1 and HVR2 are thought to play a role in shielding regions of the virus important for cell entry (138),(139). Studies of E2 structure have shown the N-terminal regions of E2 from HVR1 to HVR2 to be flexible suggesting they may be able to undergo conformational change on binding to cell surface receptors such as SRB-1 subsequently exposing CD81 binding regions. (38)

Furthermore, HVR1 and other regions of the virus may act as a decoy to misdirect the immune response. In acute infection, a predominantly HVR1 targeted response is associated with progression to chronic infection rather than acute resolution (140). Another decoy mechanism may be through secretion of nucleic acid free, envelope protein coated subviral particles as described by Scholtes (2012). Such particles have the potential to bind antiviral antibodies reducing their impact on infectious virions (141). There is also evidence that some envelope molecules may mimic self-proteins such as IgG preventing the development of a robust immune response(142).

Finally, the virus is able to transmit directly from cell to cell which typically evades humoral responses, however one group has shown antibodies directed at SR-BI blockade are effective in preventing cell-cell transmission suggesting that an antibody based approach may not be completely ineffective in preventing this mode of transmission(143).

1.3.5.6 Clinical relevance of antibody responses in chronic infection

In addition to contributing to acute clearance, there is some preliminary evidence that antibody responses may influence chronic disease outcomes. In vitro and in vivo animal studies have suggested that broadly neutralising antibodies may be able to control levels of virus and contribute to clearance even once infection has become established (144) . In a case of a chronically infected patient who spontaneously cleared HCV, a broad neutralising antibody response was generated with subsequent restoration of T cell activity(131). However, it is not yet clear whether neutralising antibodies have an overall protective role in chronic infection. Indeed, the antibody response to HCV can have pathological consequences as seen in cryoglobulinaemic vasculitis(6). If large scale vaccination is being considered, it is important to determine that stimulating such a response will not be harmful in the event of an infection developing. Studying clinical associations in patients with broadly neutralising antibodies can reveal associations with any adverse outcomes and can yield insights into patient factors associated with neutralising antibody production.

Table 1.2 Key HCV envelope epitopes targeted by monoclonal antibodies derived from patients.

Immunodomain	Key Antibodies	Key binding residues in E1E2 glycoproteins	Reference
A	CBH-4B, CBH-4D, CBH-4G	540, 542, 556,627	(120)
B	HC-1, HC-11, CBH-5, AR1B	425, 427,438, 530, 535	(120) (125)
C	CBH-7	544, 547, 549, 550	(145)
D	HC-84 (including HC-84.26, HC-84.20 and HC-84.24)	441,442,443,616 (NB also overlaps with some domain B residues)	(145)
E	AP33 AR1A	413,415,418,420	(146) (125)
B and E overlap	AR3B,	412,416,418,423,424,523, 525,530,535,540	(125)
E1 /E2 interface	AR4A	201,204,205,206, 487, 657,658,692,698	(127)
	AR5A	201,204,205,206,639,657, 658,665,692	(127)

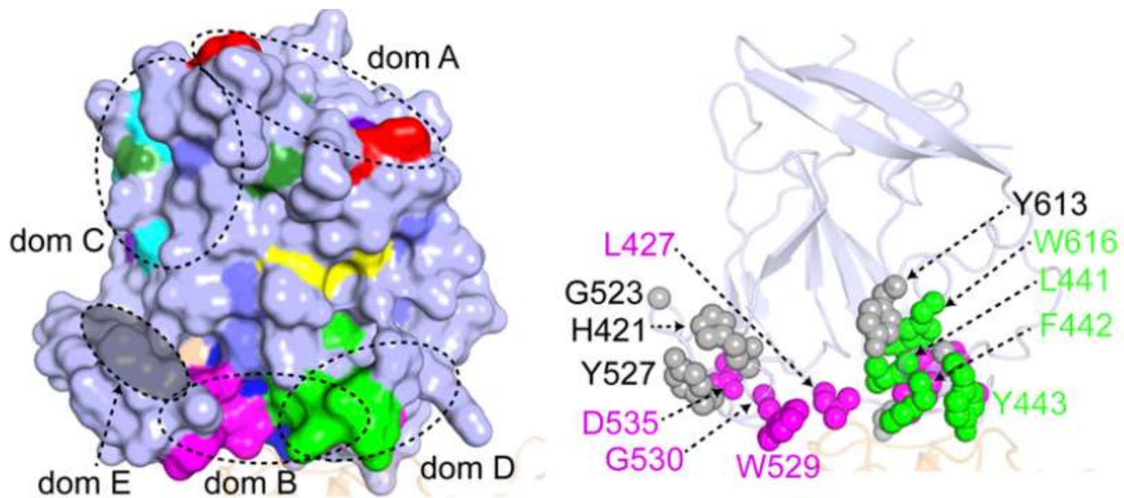


Figure 1.10 Immunodomains of the E2 protein on the core E2 structure

Binding regions of antibodies confirmed as belonging to separate immunodomains from alanine scanning experiments. These are displayed on a model of the E2 core structure as generated by Kong et al (2013) (37). Different colours notify regions which cluster together according to patterns of monoclonal antibody binding when analysed in a heatmap. Note, while Domain B and Domain D have some separate residues, there are also regions which overlap. Domain C is relatively poorly characterised compared to the other domains. Domain A represents the AP33 antibody binding region which is not included in the core E2 structure but is shown in a region where it would most likely appear on the complete E2 structure. Individual amino acid residues thought to be important in CD81 binding are shown alongside the core structure with Domain B clustering residues coloured pink and Domain D clustering residues coloured bright green. [Figure and legend adapted from Pierce et al, 2016(145) as permitted by the National Academy of Sciences].

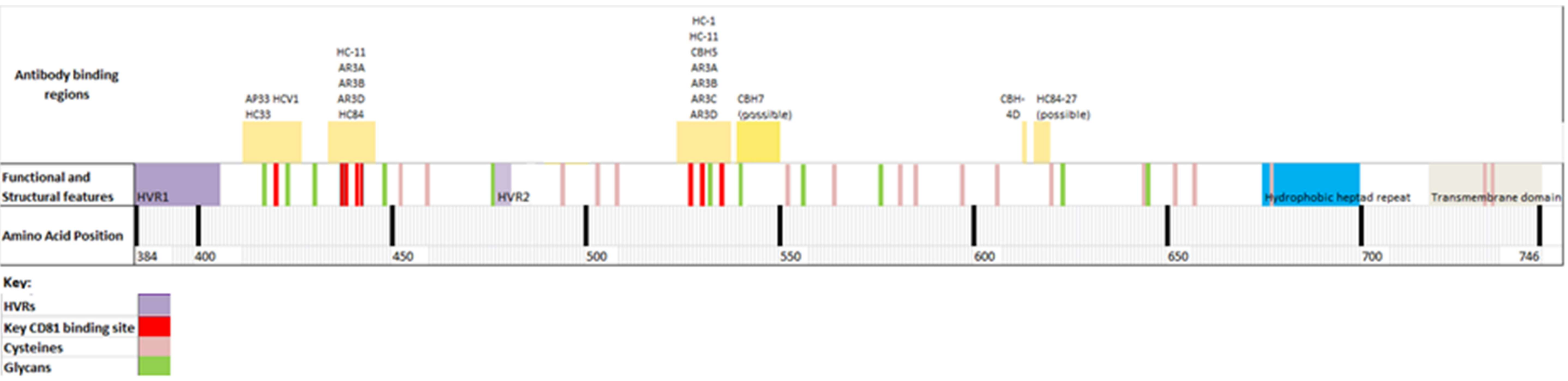


Figure 1.11 Diagram showing key structural features of E2 and binding sites of well characterised antibodies.

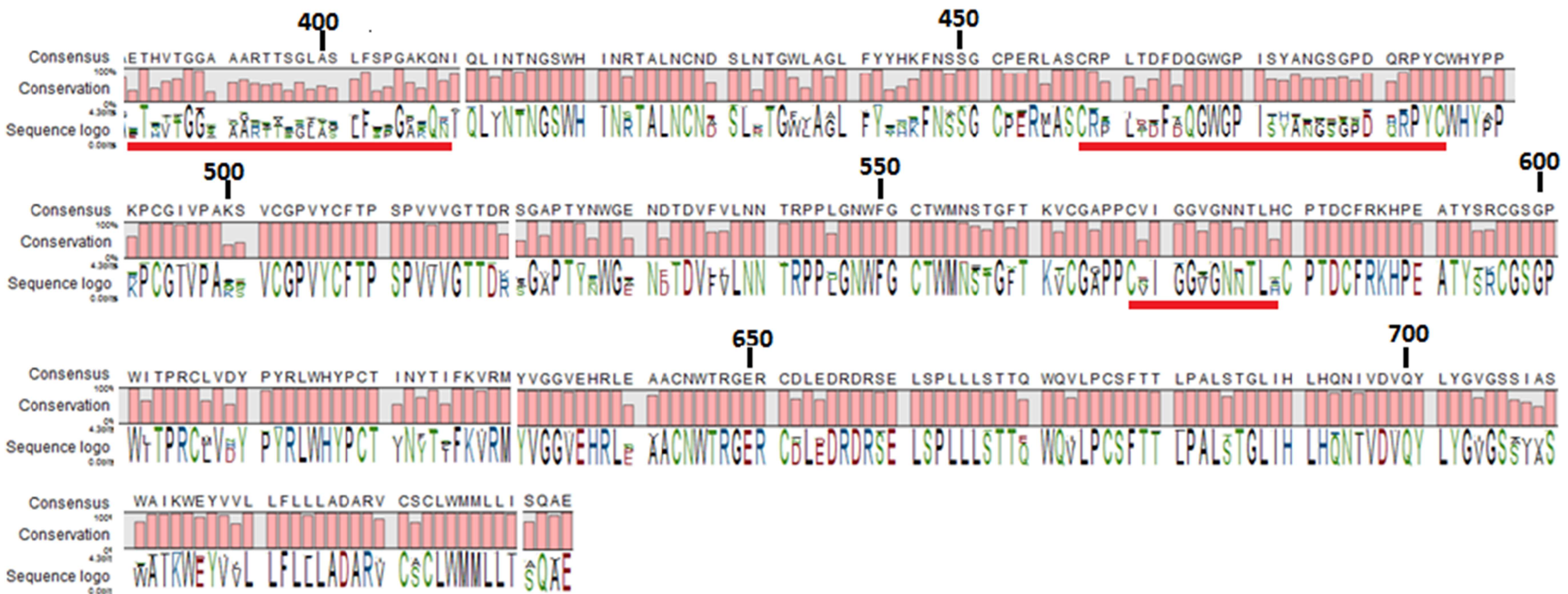


Figure 1.12 Variability of E2 within genotype 1 sequences.

All 3872 full E1E2 sequences available for gt1 in the Los Alamos HCV database were downloaded, aligned in Mega 6 and translated. This translated alignment was used to generate this consensus amino acid sequence in Genomics Workbench v 7. Consensus amino acid and percentage conservation is shown. The areas underlined in red show the HVR1, HVR2 and iGVR.

1.4 Methods of studying humoral responses to HCV

Following the discovery of HCV, initial research was limited due to a lack of in vitro and in vivo models replicating the process of infection and cell entry.

Few animals are naturally susceptible to HCV infection, despite a number of species recently discovered as carrying the related Pegivirus species. Primates can be infected, although experimentation on these animals is often not practical. Several studies inoculating primates with live virus and assessing responses to basic vaccines have been conducted (147, 148). More recently chimeric mammal models, in particular mouse models with human hepatocytes grafted onto their liver, have been generated to study effects of HCV infection in vivo(149). However, the immune responses identified in mice are not directly comparable to those in humans.

In vitro, the main challenge was identifying a liver cell line which was permissible to viral infection. Initially, this was overcome by creation of a sub-genomic replicon which mimics viral replication in Huh7 hepatoma cells(150). However, replicons do not contain envelope genes and do not undergo cell entry. Subsequently, the isolation of the JFH-1 strain of HCV, which can infect Huh7 cells and undergo a complete viral lifecycle, allowed further investigation of the effects of HCV including the cell entry step (151). Additional aids to studying replication and infection were the introduction of reporter genes into the virus such as luciferase(152). Other groups have identified different cell lines which are permissive to infection by different genotypes (153) and the JFH-1 virus can be modified to contain sequences from other genotypes(154). Another challenge with the hepatoma cell model is such cells do not secrete VLDL when grown in standard tissue culture media. As HCV virions

are typically coated in VLDL, this limits the conclusions which can be drawn from work with this cell line. Interestingly, the addition of human serum to the culture medium has been shown to restore the VLDL content of virions(155). Alternative cell models, such as the use of human liver progenitor cells (the HepaRG cell line) are also able to produce virions incorporating VLDL and can be used in studies of cell entry inhibition(156). Recently, an infection model using hepatocyte stem cells has been described which allows infection by wild type HCV from patient serum (157, 158).

For the study of cell entry alone however, a fully infective virus was not necessary. Therefore, the pseudoparticle system (HCVpp) was developed (159-161) which used transfection of HEK-293T cells with plasmids constructed from a phCMV-7a expression vector. These three plasmids contained 1) a whole E1E2 sequence derived from patient samples, with flanking regions containing start and stop codons, 2) a non-HCV polymerase and structural protein (e.g. retroviral or lentiviral gag-pol, initially Murine Leukaemia Virus was used) and 3) a reporter gene such as green fluorescent protein or luciferase. Following transfection, the HEK-293T cells produce particles coated in envelope protein containing a reporter gene (HCVpp), which are released into the media following transfection and can be harvested to ‘infect’ naive Huh7 cells. HCVpp have been widely used as a model for virus entry as they use a similar cell entry processes to live virus, largely being dependant on the CD81 protein. The level of HCVpp entry can be quantitated by looking for the strength of the reporter signal and inhibition of entry can also be inferred by measuring reduction in this signal. In addition, the lysate from the HEK-293T cells producing HCVpps contains E1E2 protein which can be used downstream in ELISA assays.

However, as with HCVcc produced in HUH7 cells, lipids are not incorporated into pseudoparticles as they would be with live virus. This is important as predominantly lipid mediated entry pathways have been identified, such as using Syndecan 4 which interacts with apolipoprotein E to mediate cell entry(162). Changes in ApoE availability are likely to influence infectivity and susceptibility to antibody mediated neutralisation(163). The VLDL receptor has also been shown to act as an HCV receptor independent of CD81(164, 165). Therefore while the HCVpp model is more practical to use than live virus by not requiring containment in a category 3 laboratory, and offers the potential to explore the behaviour of a wide range of envelope sequences, it is not an entirely faithful model of cell entry of live virus studied in vivo.

1.5 Clinical Impact of HCV

Within the UK, HCV contributes to a significant proportion of morbidity and mortality. While death rates from other major causes show a downward trend, those from end stage liver disease due to HCV have doubled in the past decade(166). While much of the measurable burden of HCV is on liver health, individuals chronically infected with HCV can report a multitude of symptoms which can be attributed to the virus and it can significantly reduce quality of life even in the absence of end stage cirrhosis(8).

1.5.1 Liver morbidity

1.5.1.1 HCV induced cirrhosis

HCV infection is not hepatocytolytic and therefore much of its pathogenic effects on the liver are secondary to activation of the host’s inflammatory responses within the

liver. Over years to decades, these processes lead to liver cell death, deposition of scar tissue and disorganised regeneration of liver. Once there is irreversible scarring and alteration of liver microarchitecture this is termed liver cirrhosis (167). HCV has several molecular mechanisms thought to contribute to liver fibrosis and ultimately cirrhosis. In particular infection has been shown to increase levels of fibrogenic TGF-β(168), HCV core and non-structural proteins can also directly interact with fibrin producing stellate cells within the liver(169).

In the modern era, diagnosis of liver fibrosis and cirrhosis in HCV can now be achieved using a non-invasive technique called transient elastography which uses shear waves to measure liver stiffness. Correlation of transient elastography values in patients with HCV liver disease with histological diagnosis of cirrhosis has been extensively validated (170). .

In the presence of ongoing liver inflammation or additional liver insults, such as alcohol use, decompensated cirrhosis can result. As the liver's vascular architecture is disrupted, the venous pressure in the portal vein can increase, leading to opening of small vessels which bypass the liver and directly connect the portal system to the systemic venous system. This has several consequences including an increase in the renin and aldosterone hormones which promote retention of salt and water ultimately leading to oedema and ascites (low protein fluid collecting in the abdomen). This can lead to infection, or kidney failure which both carry a high mortality rate (171).

Porto-systemic collaterals bypassing the liver can cause ammonia based waste products from the gut to enter the systemic circulation, rather than being metabolised

by the liver. This can cause cerebral dysfunction known as encephalopathy which can significantly impair the individual's ability to care for themselves as well as being associated with increased mortality(172).

Increased portal pressure can also result in bleeding from dilated porto-systemic collaterals termed 'varices', most commonly gastric or oesophageal. Bleeding from these vessels is associated with a 14% in-hospital mortality (173).

While cirrhosis is generally thought to be irreversible in HCV, following successful viral treatment, improvement in histological and non-invasive measurements of liver fibrosis have been observed, suggesting that reversal of cirrhosis may be possible in some individuals (174, 175). However, the majority of those who have developed cirrhosis remain at risk of these complications for life. There is also some evidence that those who clear HCV without developing cirrhosis may still be at higher risk of developing significant liver disease in the future (176).

Therefore, the ability to prevent infection altogether through public health measures or vaccination, or treatment strategies to target those in the early stages of infection are the only clear ways to prevent the large burden of liver disease caused by this condition. While several therapies to slow or reverse progression of liver fibrosis have been trialled, these have not yet shown a significant impact in human subjects(177).

1.5.1.2 Hepatocellular carcinoma

There is also evidence that both the metabolic liver effects induced by HCV and proto-oncogenic effects of some HCV proteins may have an additional role to play in

progression to hepatocellular carcinoma (178). This condition creates a significant morbidity and mortality burden on affected populations.

Compared to other malignancies, hepatocellular carcinoma (HCC) has one of the largest recent increases in incidence in the UK(179). Chronic HCV infection is thought to have had a significant impact on this with AASLD guidelines suggesting that HCV-infected patients with cirrhosis develop HCC at a rate of 2.5%/year. This risk is increased by additional liver comorbidities such as alcohol excess and HBV coinfection(180).

HCC predominantly develops in individuals who have already progressed to liver cirrhosis. This is thought to be due to the cumulative effect of DNA damage as a consequence of the chronic liver inflammation, cell damage and regeneration which occurs as cirrhosis develops (178). However, non-cirrhotic HCC has been reported in individuals mono-infected with HCV and some HCV proteins are thought to have direct carcinogenic effects.

Transgenic mouse models have identified a link between core protein and possibly NS5A with generation of HCC. Other molecular studies have found NS5B mediated inhibition of the “Retinoblastoma” tumour suppressor gene and core protein mediated dysregulation of the p53 pathway (181).

1.5.2 Cryoglobulinaemia and B cell lymphoma

HCV is the most common cause of the benign B cell disorder, mixed cryoglobulinaemia (MC), and is linked to the development of B cell lymphoma. MC is a clonal B cell disorder characterised by precipitation of antibody aggregates on

serum cooling which can be re-dissolved at 37°C. These precipitates consist of auto-reactive antibodies targeting the fixed (Fc) region of host IgG (also known as Rheumatoid factor-like activity)(6).

1.5.2.1 Clinical syndrome

While patients can have positive autoantibodies in the absence of any symptoms(182), the cryoglobulinaemia syndrome is typically associated with vasculitic symptoms and complications including skin rashes, joint pains, renal failure, pulmonary haemorrhage and peripheral neuropathies. The condition may be a precursor to B cell lymphoma although only a minority of cryoglobulin-positive patients progress to this.

1.5.2.2 Possible pathogenesis

The exact pathogenesis of cryoglobulinaemia in HCV is incompletely understood. The disorder has been associated with expansion of a naïve subtype of B cells(183). The B cell activating factor molecules have also been implicated (184). The presence of HCV is generally required for persistence of cryoglobulinaemia and it generally resolves on successful treatment of the virus. This has led some to speculate that HCV proteins may induce cryoglobulinaemia, either through directly activating B cells or by acting as a molecular mimic of the IgG Fc portion. One study reported a HCV-related B cell lymphoma which had gene usage similar to B cells bearing receptors targeting the E2 envelope protein which further supports this theory(185).

1.5.3 Cardiovascular morbidity

As seen in some other chronic inflammatory conditions, increased rates of cardiovascular disease have been reported in those chronically infected with HCV (186). This is of particular interest as the HCV lifecycle is so closely associated with lipid metabolism and dyslipidaemia is a well-documented risk factor for vascular disease. In one study HCV infection was associated with lower lipid levels but higher vascular mortality(187). Additionally, analysis of the Scottish Clinical HCV database revealed reduced cardiovascular mortality following SVR(188). However, at a population level, it is difficult to separate the effects of the HCV virus on vascular health with the impact of adverse lifestyle risk factors associated with HCV infection.

1.5.4 Neuropsychiatric effects

The presence of HCV infection with or without cirrhosis can itself be associated with fatigue and reduced quality of life (189). HCV has also been demonstrated to infect brain tissue (190) and subtle impairments in cognition, along with structural changes visible on MRI scanning, can be detected in individuals with minimal liver fibrosis(191).

1.5.5 Metabolic effects of HCV

HCV infection has been linked with an increased risk of developing features of the 'metabolic syndrome' (dyslipidaemia, hypertension and insulin resistance) with hepatic steatosis in up to 85% of infected individuals. Systemic metabolic disturbance , in particular insulin resistance progressing to Type II Diabetes Mellitus(192) is thought to be more common in gt 1 infection than with gt 3 infection, whereas hepatic steatosis alone is more likely to occur in gt 3. This highlights the

distinct pathophysiological pathways of the different genotypes (193). The presence of steatosis had been linked to adverse treatment outcomes using IFN based regimens (194).

There are multiple mechanisms by which the virus is thought to exert these effects. Hepatocytes infected with gt 3a upregulate synthesis of fatty acids with impaired β–oxidation(195), potentially through mitochondrial damage, and altered triglyceride export. HCV proteins have been shown to interfere with insulin signaling pathways, in particular core protein activates peroxisome proliferator-activated receptor gamma (PPARγ) which is a target for anti-diabetic therapies (196).

1.6 Strategies to control HCV infection and its impact

1.6.1 Medical therapy

Following the characterisation of HCV in 1989, IFN alpha 2b was the first effective therapy to be identified against the virus (197). However, this only resulted in normalisation of liver biochemistry in around 50% of individuals and infection tended to relapse on cessation of treatment.

In 1999, PEGylated IFN alpha was introduced along with the combination therapy with the antiviral agent ribavirin. This longer acting preparation of IFN led to better outcomes although the rate of Sustained Viral Response (SVR – defined as undetectable viral load 6 months after cessation of therapy) was still low (40%) (198) with significant problems with tolerability due to haematological and neuropsychiatric effects(199).

Since then, the development of several new direct acting antiviral agents (DAAs) in addition to a genotype tailored approach has transformed the face of therapy to the extent that the most recent EASL recommended regimens do not include IFN (Figure 1.13). The various agents used in this guideline are described in more detail below.

Patients	Treatment-naïve or -experienced	Sofosbuvir/ ledipasvir	Sofosbuvir/ velpatasvir	Ombitasvir/ paritaprevir/ ritonavir and dasabuvir	Ombitasvir/ paritaprevir/ ritonavir	Grazoprevir/ elbasvir	Sofosbuvir and daclatasvir	Sofosbuvir and simeprevir
Genotype 1a	Treatment-naïve	8-12 wk, no ribavirin	12 wk, no ribavirin	12 wk with ribavirin	No	12 wk, no ribavirin if HCV RNA ≤800,000 (5.9 log) IU/ml or 16 wk with ribavirin if HCV RNA >800,000 (5.9 log) IU/ml[b]	12 wk, no ribavirin	No
	Treatment-experienced	12 wk with ribavirin[a] or 24 wk, no ribavirin					12 wk with ribavirin[a] or 24 wk, no ribavirin	
Genotype 1b	Treatment-naïve	8-12 wk, no ribavirin	12 wk, no ribavirin	8-12 wk, no ribavirin	No	12 wk, no ribavirin	12 wk, no ribavirin	No
	Treatment-experienced	12 wk, no ribavirin		12 wk, no ribavirin				
Genotype 2	Both	No	12 wk, no ribavirin	No	No	No	12 wk, no ribavirin	No
Genotype 3	Treatment-naïve	No	12 wk, no ribavirin	No	No	No	12 wk, no ribavirin	No
	Treatment-experienced		12 wk with ribavirin[c] or 24 wk, no ribavirin				12 wk with ribavirin[c] or 24 wk, no ribavirin	
Genotype 4	Treatment-naïve	12 wk, no ribavirin	12 wk, no ribavirin	No	12 wk with ribavirin	12 wk, no ribavirin	12 wk, no ribavirin	12 wk, no ribavirin
	Treatment-experienced	12 wk with ribavirin or 24 wk, no ribavirin				12 wk, no ribavirin if HCV RNA ≤800,000 (5.9 log) IU/ml or 16 wk with ribavirin if HCV RNA >800,000 (5.9 log) IU/ml	12 wk with ribavirin or 24 wk, no ribavirin	12 wk with ribavirin or 24 wk, no ribavirin
Genotype 5 or 6	Treatment-naïve	12 wk, no ribavirin	12 wk, no ribavirin	No	No	No	12 wk, no ribavirin	No
	Treatment-experienced	12 wk with ribavirin or 24 wk, no ribavirin					12 wk with ribavirin or 24 wk, no ribavirin	

[a]Add ribavirin only in patients with RASs that confer high-level resistance to NS5A inhibitors at baseline if RAS testing available.
[b]Prolong to 16 weeks and add ribavirin only in patients with RASs that confer resistance to elbasvir at baseline if RAS testing available.
[c]Add ribavirin only in patients with NS5A RAS Y93H at baseline if RAS testing available.

Figure 1.13 European Association for the Study of the Liver treatment recommendations on treatment of Hepatitis C 2016.

[Extracted from EASL Recommendations on Treatment of Hepatitis C, Journal of Hepatology with permission granted through RightsLink]

1.6.1.1 Ribavirin

Ribavirin is a guanosine analogue which prevents RNA synthesis, therefore has been used as an anti-viral agent against several viruses including HCV. While its effects on RNA synthesis explain some of its antiviral activity, it is also thought to have wider anti-viral actions and is effective against some DNA viruses. Despite the fact that it is a non-specific antiviral agent, it still improves SVR rates in combination with some of the most recent DAA regimens in difficult to treat patients (200).

1.6.1.2 Protease inhibitors

Protease inhibitors (PI) were the first licenced DAA agents designed to target the HCV polyprotein specifically. Initially only two such agents, Boceprevir and Telaprevir were licenced, although several other agents are now available for use. These small molecules bind directly to the NS3 protein site responsible for cleaving the junctions between NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B(201). This prevents efficient formation of the replication complex therefore limiting HCV production.

First generation molecules were very genotype specific being active against gt 1, and more effective against gt 1b than gt 1a due to the higher natural occurrence of resistance mutations in the gt 1a sequence(202). This led to the introduction of routine testing for specific resistance mutations prior to therapy in the clinic. In those with a favourable HCV profile, SVR rates of up to 70% were reported in treatment naïve individuals without cirrhosis. However, these combinations still required prolonged treatment with IFN and ribavirin alongside PI therapy. In addition to the common side effects with IFN/ribavirin, there was a significantly increased risk of rash in triple therapy. In one report, patient withdrawal rates due to tolerability were

26% with an on-treatment mortality rate of 2%(203). These agents have largely been superseded by newer DAAs, however a newer PI Simeprevir is still recommended for use in the EASL guidelines.

1.6.1.3 NS5B inhibitors

The NS5B inhibitors are also small molecules specifically designed to target the replication complex, in this case the NS5B RNA polymerase.

Some of these have activity against gt 1, 2, 3 and 4 although they are considerably less effective against gt 3 when used as monotherapy (204).

Initial studies using the NS5B inhibitor Sofosbuvir reported SVR rates upward of 90% in IFN-free regimes for gt 1 infected patients (205). These medications are much better tolerated than IFN-based therapies with fewer drug interactions making them more practical to use. The cost of regimes containing these inhibitors has prevented them being universally used for all genotypes of infection, however the most recent NICE guidance has approved NS5B containing regimes for all individuals infected with gt 1 in the UK(206). The financial burden of increased levels of screening and treating HCV-infected patients with DAA containing regimes has been estimated to increase costs 5-fold in the short term, although longer term savings from prevention of complications could be expected(207).

1.6.1.4 NS5A inhibitors

The **NS5A** inhibitors impact on replication, assembly, and secretion. Some NS5A inhibitory molecules have pan-genotypic activity(53). Daclatasvir, the first of these

agents to be licenced, targets Domain I of the NS5A molecule involved in replication. While NS5A inhibitors have not been tested as monotherapy in large clinical trials, they enhance the efficacy of NS5B inhibitors when used in combination. For example, Ledipasvir is now marketed as a single tablet in combination with the NS5B inhibitor Sofosbuvir. This combination is reported to produce SVR rates of 95% after 8 weeks of therapy in gt 1 virus-infected patients without cirrhosis who had not previously received treatment (208, 209). Daclatasvir combined with Sofosbuvir is one of the few IFN-free combinations proven to be effective against gt 3 in patients with cirrhosis (210). Velpatasvir is the newest of these agents to be licensed which also has pan-genotypic activity (211). Therefore, with the addition of NS5A inhibitors to existing DAAs, effective IFN-free regimens are possible for the vast majority of patients with HCV, although their use is currently limited by cost.

1.6.1.5 Tailored therapy

Due to the cost and side effects involved in PI therapy, testing for the host IL28B CC mutation also became common in the clinic as individuals carrying this genotype were more likely to achieve SVR with IFN/RBV alone(88). Therefore, the introduction of these drugs not only heralded a new era in the types of treatments used for HCV but began a trend for tailored therapy based on both host and viral characteristics, highlighting the ability for different strains of the HCV virus to behave very differently and the influence of the host immune system on the virus' ability to sustain infection.

Due to the widespread efficacy of some of the newer DAAs, such highly tailored therapy is no longer necessary for most. In the UK, due to cost reasons, IFN containing regimes may still be used as a first line approach where patients are able to tolerate these. However, the most recent international treatment guidelines

suggest that the choice of antiviral treatment is based on HCV genotype, presence of cirrhosis and exposure to previous therapy, with testing for resistance mutations in the NS5A gene where this is available(65). In addition, interactions with several common medications have been noted which will also influence drug choice. While resistance to the newer DAAs is rare, it is likely that viral sequencing, in addition to host characteristics, will be useful in predicting the most successful second line regimes for those not achieving SVR with standard DAA treatment.

1.6.2 Risk factors for poorer outcome in HCV infection and treatment

1.6.2.1 HCV Genotype

As noted above, genotype has a significant impact on both the development of cirrhosis and the efficacy of different treatments. While gt 3 infection was associated with higher rates of cirrhosis in untreated infection, in the IFN/RBV era, genotypes 2 and 3 were easier to treat than gt 1 and 4 with SVR rates of 70% compared to 40%. With the advent of DAAs, gt 1 now has a larger selection of effective therapeutic agents, perhaps because the research effort has been concentrated on this genotype. It is therefore now easier to treat than gt 3, with SVR rates of >90% achievable for all clinical groups except those with decompensated cirrhosis.

1.6.2.2 Age

In HCV, as liver cirrhosis develops over a period of decades, typically 20% developing cirrhosis after 20 years of infection, adverse liver outcomes increase with age (212). There are few longer term follow up studies on the prevalence of cirrhosis beyond 20 years post-infection, however one long term study revealed that although Caucasian populations had relatively low rates of cirrhosis at age 61-80, 78% of the

Asian population studied had cirrhosis. As other factors were comparable between the groups this was felt to reflect longer duration of infection in Asian populations who were generally infected in childhood (i.e. 20 years earlier than the Caucasian population) (213). This suggests that as the HCV-infected population ages, prevalence of cirrhosis will increase dramatically unless effective treatment is implemented.

There is also evidence to suggest that those infected at an older age develop more rapidly progressive liver disease (214). Finally, in the IFN era of treatment, advanced age was associated with a higher risk of treatment failure. Whether this remains true in the DAA era is yet to be seen (215).

1.6.2.3 Alcohol use

In the western world, perhaps the biggest predictor of adverse liver outcomes is concomitant alcoholic liver disease. Innes *et al.* used the Scottish national HCV database to determine the impact of alcohol excess on liver cirrhosis in HCV and found that consuming more than 50 units of alcohol per week was the most influential of all risk factors, accounting for up to 50 % of cirrhosis in HCV infected individuals (216).

Alcohol use is also associated with a higher rate of decompensated liver disease and HCC (217). In addition to being a potent hepatotoxin in excess, alcohol may affect the HCV life cycle, promoting viral replication and having a synergistic effect in promoting liver damage in the context of HCV (218).

1.6.2.4 Coinfection

HIV coinfection is generally thought to lead to more rapid progression of liver disease. Indeed, as mortality rates from HIV infection itself have improved, liver disease from HCV / HBV and other causes is becoming one of the leading causes of death in the HIV infected population (219). HIV co-infection traditionally made successful treatment of HCV more difficult, although the DAA therapies now appear to be equally effective in patients with HIV coinfection (220).

HBV coinfection is also associated with poorer liver outcomes and HBV reactivation a recognised risk with DAA treatment in individuals previously infected with HBV(221).

1.6.2.5 Obesity

Increasing obesity levels have led to an epidemic of non-alcoholic steatohepatitis and associated liver morbidity in the developed world. Given that HCV infection can induce a metabolic syndrome, obese HCV-infected individuals are at even higher risk of developing this(222). Those who suffer from both HCV infection and the metabolic syndrome are at greater risk of adverse liver outcomes and weight loss is recommended in those with a high BMI.

1.6.3 Population level prevention strategies

The importance of reducing new HCV infections and treating existing ones has been recognised internationally by WHO and by individual countries. Within the UK, both Public Health England and Health Protection Scotland have been proactive in introducing measures to prevent new infections and accurately document the burden of disease (223, 224).

Before specific strategies to tackle HCV were developed, the HIV epidemic prompted the development of needle exchange and public education programmes to reduce transmission of blood borne viruses in general. These have undoubtedly had a beneficial effect in reducing HCV transmission within IDU populations.

In 2014 the WHO published specific guidance on reducing transmission of HCV in people who inject drugs (225). However, in Scotland a public health strategy for preventing infection has been in place since 2008 (224) which involved creating a database of HCV-infected patients to monitor outcomes and inform strategies to improve these and a number of outreach programmes for screening and treating high risk populations linked to addiction and prison services. Further initiatives such as dried blood spot screening of age targeted populations and opt-out testing in emergency departments have detected new cases and are being more widely adopted (226).

With the advent of the newer DAA therapies, several economic models have suggested that treating HCV infected individuals at high risk of transmission, for example PWIDs, would be cost effective in preventing further HCV infection in the IDU population(227, 228). Others have suggested that at present the costs involved would be prohibitive(207).

While these strategies work well for first world countries with a well-resourced and nationally funded health service, they would be more difficult to implement in countries with poorer healthcare infrastructure or a largely private provider model of healthcare, as those in need of such interventions are usually least able to pay.

1.6.4 Vaccine development

1.6.4.1 Types of vaccines

The principle of vaccination is to prime the immune system with exposure to an antigen so the next time this antigen is encountered (on a live pathogen) a rapid and robust immune response is produced, preventing or limiting the consequences of infection. The very earliest trials of HCV vaccines involved injecting live virus, or virus like particles into primate test subjects who generally cleared the infection. These trials revealed more rapid clearance of infection in "vaccinated" subjects on rechallenge (229). Analysis of the immune response generated by inoculation showed both cellular and humoral adaptive responses. Passive immunisation through exposure to serum from infected individuals, infusion of pooled immunoglobulins or monoclonal, anti-HCV antibody has also been shown to reduce the risk of infection in primates and humans (12, 130, 230). Further studies using modified whole virus produced in cultured cells (HCVcc) demonstrated induction of broadly neutralising antibodies in mice (231).

Attempts to identify specific HCV proteins which would give the broadest and most potent protection have involved trials of numerous combinations of structural and non-structural proteins. One barrier to a specific protein based approach is difficulty in generating an immune response which is broad enough to prevent infection by the various strains of virus present. In HCV, this is particularly relevant given the huge diversity of sequence seen both within and between genotypes. One approach to combat this is to identify proteins which are relatively conserved across strains which should elicit a 'broad' immune response, effective against different genotypes and subgenotypes. In vaccines for other viruses various strategies are used to achieve

this. One method is to use envelope or surface antigens which induce a ‘sterilising’ humoral response i.e. preventing cellular infection, such as the recombinant surface antigen used in Hepatitis B vaccination(232) . In others, for example the recently designed Multimeric-001 influenza vaccine (233), a number of conserved proteins are included which induce both antibody and cellular responses. The choice of proteins included in vaccines depend on specific characteristics of the virus, immunogenicity of the proteins and whether the aim is to prevent infection altogether or simply ensure that infection follows a more benign course. One potential target in HCV is the conserved E2 epitope corresponding to the broadly neutralising antibody AP33, however this appears to be poorly immunogenic in humans (134).

To combat lack of immunogenicity, a protein of interest is often expressed on the backbone of an immunogenic protein or whole virus and an immunostimulant adjuvant may be used (234). In initial chimpanzee trials of using whole envelope protein as an immunogen, the addition of the oil emulsion agent MF59 improved clearance of subsequent infections suggesting that a more vigorous immune response was generated(235). This agent has also been used in human trials of HCV vaccination(236). Other work exploring use of organic compounds such as archaeosomes or m-ADP have also shown increased immunogenicity in animal studies(237). Using an immunogenic viral vector to deliver the vaccine has also been explored, one anti-HCV vaccine using this strategy is based on an Adenovirus vaccine and has been trialled in primates and healthy subjects (238).

Additional considerations in vaccine development are the mode and timing of vaccines delivery. Some, such as polio, can be delivered orally or intranasally, but traditionally, the majority of human vaccines have been delivered intramuscularly.

Recently it has been shown that influenza vaccines delivered more superficially into the skin using a microneedle patches may be more effective that using the intramuscular route (239). While novel delivery systems may have cost implications, if they improve uptake and effectiveness this may justify such cost(240).
As the immune response to an antigen tends to wane over time, some vaccines require a ‘booster’ vaccination to be given after a period of years has elapsed, for example that against tetanus toxoid. For other viruses, rapid evolution requires more frequent vaccination such as in influenza. In addition, some vaccines are more effective following a course of multiple exposures rather than a single immunisation such as the Hepatitis B vaccine. Finally, from a practical perspective, storage conditions of vaccine preparations differ; with some requiring cold storage wheras others remain stable at room temperature for prolonged periods of time. The ideal vaccination for the populations who would most benefit from HCV immunisation would be a single or compressed course of vaccinations with a preparation not requiring continuous cold storage. This would enable delivery of the vaccine to areas with less developed healthcare systems and to more transient populations such as people who inject drugs (PWIDs) as has been demonstrated for other viral vaccines (241).

While B and T cell responses to many HCV viral proteins have been recognised, T cell targeted vaccination strategies have tended to use non-structural protein antigens whereas B cell targeted strategies often concentrate on responses to the envelope proteins. It is unfortunate that the vaccines at the later stages of development have not aimed to elicit a strong B cell and T cell response in

combination as experts in the field have suggested this strategy is likely to be necessary for a broadly effective vaccine (242).

1.6.4.2 T cell targeting vaccines

The most promising T-cell focused vaccine to enter clinical trials has involved the HCV non-structural proteins expressed on an adenoviral backbone. This has been trialled on 30 healthy subjects eliciting robust T cell responses, however efficacy of preventing infection in individuals at high risk of HCV transmission has yet to be seen (238). Another study of a non-structural peptide vaccine elicited a 60% CD4 response rate in healthy subjects (243). While this is encouraging, this is a much lower response rate than found in most anti-viral vaccines which have been adopted in clinical practice.

A novel approach to T cell stimulation in harnessing and modifying host antigen presenting cells has also been trialled (244). While this has proved effective in eliciting a strong T cell response, it is difficult to see such an approach being a practical and cost effective solution for the populations who would most benefit from an effective vaccine.

1.6.4.3 B cell targeting vaccines

Several researchers have sought to induce bNAbs through vaccination, predominantly by using part or all of the E1E2 molecules. Stamataki *et al* (2007) showed that immunisation of rodents with E1E2 had the ability to induce strain specific NAbs with some cross-strain neutralising activity (245).

The pharmaceutical company Chiron have been developing a commercial vaccine based on recombinant gt1a E1E2 proteins which, when administered with an oil:water adjuvant, produced sterilizing immunity in some chimpanzees against gt 1a and 1b viruses and shorter duration of infection in others. This vaccine has also undergone Phase 1 trials in human subjects where pan-genotypic neutralising antibodies were observed in one subject(246). Interestingly this vaccine has also been shown to induce significant CD4 T cell responses suggesting that any protection conferred by envelope protein based vaccines may be due to a combination of adaptive responses(236). However, in one trial, only 5/16 vaccinated subjects' serum were able to reduce infectivity of gt 1a virus by 50%, while some individuals produced non-neutralising antibodies which interfered with the neutralising response (247), this suggests that a vaccine based on specific epitopes rather than the whole E1E2 protein may produce more effective protection.

It is interesting to note that reinfection in individuals possessing neutralising antibodies has been described suggesting that protection is incomplete or that, in these cases, it may have been too 'strain specific' as evidenced by the fact that few of those who progressed to chronic infection possessed 'cross-reactive' antibodies (113). Therefore, the ultimate aim for those producing an envelope protein based vaccine is to produce one that generates an antibody response effective against a broad range of HCV strains.

1.7 Hypotheses and scope of thesis

I aimed to explore the following hypotheses in the work described in this thesis:

- There are a wide range of B cell responses to HCV infection with some individuals better able to produce neutralising antibodies which prevent infection by diverse strains of the virus. This is influenced by host characteristics.

- In those who are exposed to HCV but do not develop or resolve infection, anti-envelope antibody responses play a significant role.

- In chronic HCV infection, the anti-envelope B cell response plays an important role in HCV pathogenesis or prevention of this.

- Further characterising the host and antibody characteristics of HCV infected individuals displaying a broadly neutralising response and viral mechanisms of bNAb evasion may reveal important targets for vaccine design.

- In those who develop chronic HCV infection, HCV has a detrimental effect on the B cell population as another mechanism for resisting viral clearance by the immune system.

I addressed these hypotheses through laboratory based work using blood samples from cohorts of clinically characterised individuals infected with or exposed to HCV.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals/Reagents

1 kb DNA Ladder (Invitrogen)

100 bp DNA Ladder (Invitrogen)

2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) (BDH)

3,3’5,5’-tetramethylbenzidine (TMB) substrate (Sigma)

Absolute ethanol (Bamford Laboratories)

Agarose (Melford)

Ampicillin (Melford)

β-mercaptoethanol (Sigma)

Bromophenol Blue (BDH)

Ethanol (ThermoFisher Scientific)

Glutathione –S – Transferase (GST) (produced in house)

Hanks Balanced Salt Solution (HBSS) (Sigma)

Histopaque (Sigma)

IgG (human) (Sigma)

IgM (human) (Sigma)

Isopropanol (ThermoFisher Scientific)

Kanamycin (ThermoFisher Scientific)

Methanol (ThermoFisher Scientific)

NEBNext® Multiplex Oligos for Illumina® (New England Biolabs)

Phosphate Buffered Saline (Tissue Culture grade) (Sigma)

Proteinase K (ThermoFisher Scientific)

Random Hexamers (Roche)

RLT buffer (Qiagen)

RNAse free DNAse (Qiagen)

Sodium Chloride (NaCl) (BDH)

Streptavidin (Sigma)

Sucrose (BDH)

Triton X-100 (Sigma)

Tween-20 (Bio-Rad Laboratories)

2.1.2 Kits

300 cycle MiSeq version 2 reagent kit (Illumina)

Agencourt® Ampure® XP beads (Beckman Coulter)

Bright-Glo Luciferase Assay System (Promega)

CloneJet PCR cloning kit (ThermoFisher Scientific)

Gateway® Vector Conversion System (ThermoFisher Scientific)

HotStarTaq DNA Polymerase Kit, (Qiagen)

Hydragel 4IF dynamic protein electrophoresis kit (Sebia)

KAPA HiFi Real-Time PCR Library Amplification kit for Illumina libraries (KAPA Biosystems)

KAPA SYBR® FAST ABI Prism qPCR library quantification kit (KAPA Biosystems)

LR Clonase II Enzyme Mix (ThermoFisher Scientific)

NAb™ Protein G Spin Kit 0.2mL (ThermoFisher Scientific)

pENTR/D-TOPO directional cloning kit (ThermoFisher Scientific)

Phusion HighFidelity DNA Polymerase Kit (New England Biolabs)

QIAamp Viral RNA Kit (Qiagen)

QiAMP DNA Mini Kit (Qiagen)

Qiagen Plasmid Maxi Kit (Qiagen)

QIAprep Spin Miniprep Kit (Qiagen)

QIAquick Gel Extraction Kit (Qiagen)

Qubit® dsDNA High Sensitivity kit (Invitrogen)

Restriction enzymes and buffers (assorted) (New England Biolabs)

RhF latex agglutination assay (Siemens)

RNAse H (ThermoFisher Scientific)

RNeasy Plus Mini Kit (Qiagen)

Superscript III Reverse Transcriptase Kit (ThermoFisher Scientific)

TaqMan ® SNP Genotyping Assay (Life Technologies)

Vilo Reverse Transcriptase Kit (Invitrogen)

2.1.3 Equipment

2200 TapeStation (Agilent Technologies)

7500HT Fast Real-Time PCR system (Applied Biosystems)

Chameleon II plate reader (Hidex)

GeneAmp PCR Machine (Applied Biosystems).

MiSeq System Desktop Sequencer (Illumina)

Nanodrop 1000 spectrophotometer (Thermo Scientific, UK)

Qubit® 2.0 Fluorometer (Thermo Fisher Scientific)

Varioskan microplate reader (Thermo Fisher Scientific)

2.1.4 Cell lines

Cells	**Description**	**Source**
Huh7	Human Hepatoma cell line	Jean Dubuisson (CNRS, Institut de Biologie de Lille, Lille, France)
HEK-293T	Human Embryonic Kidney cell line	American Type Culture Collection

2.1.5 Antibodies

2.1.5.1 Primary Antibodies

Table 2.1 List of primary antibodies used

Antibody Name	Description	Type	Reference
Anti-E2 AP33	Monoclonal (biotinylated and non-biotinylated)	Mouse	(129, 248)
Humanised AP33 (hAP33)	Monoclonal Also known as MRCT10	Humanised mouse	(249)
Anti-E2 CBH-4B*	Monoclonal Biotinylated	Human	(120)
Anti-E2 CBH-5*	Monoclonal Biotinylated	Human	(119)
Anti-E2 CBH-7*	Monoclonal Biotinylated	Human	(119)
Anti-E2 HC-1*	Monoclonal Biotinylated	Human	(250)
Anti-E2 HC-11*	Monoclonal Biotinylated	Human	(121)
Anti-E2 HC-84.1*	Monoclonal	Mouse human chimeric	(124)
Purified IgG from patient samples	Polyclonal IgG	Human (purified using NAb™ Protein G Spin Kit ThermoFisher Scientific)	(251) / this project
Anti-SRB1 mAb151-NP1	Monoclonal	Human-mouse chimera	(252)
Anti-mu(human IgM)	Polyclonal (Sigma)	Goat	
Anti-CD81 (clone JS-81)	Monoclonal. (BD Biosciences)	Mouse	

*Kindly provided by Professor Steven Foung

2.1.5.2 Secondary Antibodies

Anti-streptavidin-HRP conjugate (Sigma)

Anti-mouse-HRP conjugate (Sigma)

Anti-human IgG-HRP conjugate (Sigma)

Anti-mu (human IgM)-HRP conjugate (Abcam)

2.1.6 Solutions

2.1.6.1 Bacterial propagation

Luria Broth (LB)

170 mM NaCl, 10 g/l Bactopeptone, 5 g/l yeast extract

LB-agar

LB plus 1.5 % (w/v) agar

2.1.6.2 DNA Manipulation

DNA loading dye

30 % glycerol, 0.25 % bromophenol blue, 0.25 % xylene blue

TBE (10x)

8.9 M Tris-borate, 8.9 M boric acid, 0.02 M EDTA (pH 8.0)

TE buffer (supplied by Qiagen)

0.01M Tris-HCl (pH 8.0), 0.001M EDTA

ddH_2O water

pH adjusted to 8.0

2.1.6.3 Cell Lysis

Cell Lysis Buffer (LB2)

20 mM Tris-HCl pH 7.4, 20 mM iodoacetamide, 150 mM NaCl, 1 mM EDTA, 0.5 % Triton X-100

2.1.6.4 Tissue Culture

Trypsin solution

0.25 % (w/v) Trypsin dissolved in phosphate buffered saline (PBS)

Versene

0.6 mM EDTA in PBS, 0.002 % (w/v) phenol red

DMEM complete

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal calf serum (FCS) (heat inactivated at 56oC for 30 min), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM non-essential amino acids and 2 mM glutamine.

2.1.7 Oligonucleotide Synthesis

Oligonucleotides were purchased from Sigma unless otherwise stated in the text.

2.1.8 Patient-derived E1E2 mammalian expression plasmids

Table 2.2 shows all patient-derived E1E2 sequence clones discussed in this thesis, some of which were expressed in the HCVpp system and used for downstream assays. Where the sequence has been used as one of the panels of HCVpp developed to test for breadth of neutralisation (cross-genotype or intragenotype 1) this is indicated under neutralisation panel.

Table 2.2 Table of patient derived E1E2 sequences inserted in phCMV plasmids and used in the HCVpp system in this thesis.

Name of clone	Location	Infects as HCVpp	Panel	Accession number	Additional description	Ref
H77c (1a)	USA	Y	IG1 XG	AF011751	gt 1a also referred to as gt 1a control or wt gt 1a	(253)
UKN1B5.23 (1b)	Nott, UK	Y	IG1 XG	AY734976	gt 1b also referred to as gt 1b control or wt 1b	(254)
JFH-1 (2a)	Japan	Y	XG	AB047639	gt 2a referred to as gt 2a in methods	(151)
UKN 2B1.1 (2b)	Nott, UK	Y	XG	AY734982	gt 2b referred to as gt 2b in methods	(254)
UKN3A13.6 (3)	Nott, UK	Y	XG	AY894683	gt 3a referred to as gt 3a or gt 3 in methods	(254)
UKN4.11.1 (4)	Nott, UK	Y	XG	AY734986	gt 4a referred to as gt 4 in methods	(254)
UKN1a14.38 (1av93)	Nott, UK	Y	IG1	AY734971.1	gt 1a	(254)
UKN1a14.43 (UKN96)	Nott, UK	Y	IG1	AY734968.1	gt 1a	(254)
UKN1A20.8 (1av220)	Nott, UK	Y	IG1	EU155192.1	gt 1a	(254)
UKN1B14.818 (UKN181)	Nott, UK	Y	IG1	-	gt 1b	(254)
1012-01	Gla, UK	Y	-	-	gt 1a from CHCV patient 1012	
1012-02	Gla, UK	Y	IG1	KU645403	gt 1a from CHCV patient 1012	(251)
1012-03	Gla, UK	Y	-	-	gt 1a from CHCV patient 1012 (similar to 12-02)	
1012-04	Gla, UK	Y	-	-	gt 1a from CHCV patient 1012 (similar to 12-02)	
1012-06	Gla, UK	Y	-	-	gt 1a from CHCV patient 1012	
1012-08	Gla, UK	N	-	-	gt 1a from CHCV patient 1012	
1012-10	Gla, UK	Y	-	-	gt 1a from CHCV patient 1012	
1012-11	Gla, UK	N	-	-	gt 1a from CHCV patient 1012	
1012-20	Gla, UK	Y/N	-	-	gt 1a from CHCV patient 1012	
1012-22	Gla, UK	Y	-	-	gt 1a from CHCV patient 1012	
1047-10	Gla, UK	Y			gt 1 from CHCV patient 1047	

1047-16	Gla, UK	Y			gt 1 from CHCV patient 1047	
1013-01	Gla, UK	Y	IG1	KU645404	gt 1a from CHCV patient 1013	(251)
1013-04	Gla, UK	N	-	-	gt 1a from CHCV patient 1013	
1013-04	Gla, UK	Y	-	-	gt 1a from CHCV patient 1013	
1013-07	Gla, UK	N	-	-	gt 1a from CHCV patient 1013	
1034-11	Gla, UK	Y	IG1	KU645405	gt 1a from CHCV patient 1034	(251)
1034-12	Gla, UK	N			gt 1a from CHCV patient 1034	
1034-14	Gla, UK	Y/N			gt 1a from CHCV patient 1034	
1034-15	Gla, UK	N			gt 1a from CHCV patient 1034	
1037-02	Gla, UK	Y/N			gt 1a from CHCV patient 1037	
1037-04	Gla, UK	Y	IG1	KU645406	gt 1a from CHCV patient 1037	(251)
1037-12	Gla, UK	Y/N			gt 1a from CHCV patient 1037	
1112-01	Gla, UK	N			gt 1a from CHCV patient 1112	
1112-02	Gla, UK	N			gt 1a from CHCV patient 1112	
1112-03	Gla, UK	N			gt 1a from CHCV patient 1112	
1112-04	Gla, UK	N			gt 1a from CHCV patient 1112	
1112-05	Gla, UK	N			gt 1a from CHCV patient 1112	
ET10 (TPC in Chapter 6)	Lon, UK	Y	IG1	KU645407	gt 1a sample taken from patient ET in 2010	
ET08 (TPA in Chapter 6)	Lon, UK	Y			gt 1a sample taken from patient ET in 2008.	

Abbreviations used in the table: Nott (Nottingham), Gla (Glasgow), Lon (London). Infectivity was tested as described in Methods and recorded as Y if the luciferase signal given using the plasmid was 10 times higher than background. Y/N indicates a reading twice background levels but not 10 times higher, N indicates a reading lower than 2x background (i.e. luciferase signal from HCVpp not containing envelope sequences). Where a term other than the standard name is used to refer to the sequences in the text (e.g. gt 2a for JFH-1) this is indicated in additional information. Where HCVpps bearing the sequence have been used in neutralisation panels described in Methods, this is indicated with IG1 for intragenotype 1 panel and XG for cross-genotype panel.

2.2 Clinical cohorts

2.2.1 Cohort of individuals chronically infected with HCV (CHCV)

Individuals with either gt 1 or gt 3a chronic HCV infection were prospectively recruited from three HCV clinic sites in Glasgow. Patients were excluded if they were on treatment at the time of enrolment, had a history of other liver comorbidities or hepatocellular carcinoma or had a BMI >31. These exclusions were included to ensure that any liver pathology detected in the individuals was likely to be secondary to their HCV. In addition, no patients had a prior history of clinical cryoglobulinaemia. A cohort of 24 healthy controls (HC) with no history of liver disease, IVDU or other systemic pathologies was also recruited from adjacent clinics and staff volunteers. Recruitment of these cohorts and subsequent experiments using their samples and analysis using anonymised clinical data were approved by the West of Scotland Research Ethics committee. Informed consent was obtained according to the declaration of Helsinki.

All individuals completed a clinical questionnaire and additional clinical details were obtained from patient notes. All individuals had blood tests for routine clinical parameters including liver function tests (LFTs) and viral load in addition to IL28B status. They also had experimental whole blood and serum samples taken. For all patients recruited at one site, facilities were available to test for cryoglobulinaemia, therefore these individuals gave an additional serum sample collected in a serum tube pre-warmed to 37°C. Transient elastography measurement of liver stiffness was performed in all HCV positive individuals using a Fibroscan® (Echosens).

Recruitment of patients and collecting clinical samples and patient data for this cohort was conducted by the research nurse team from the Glasgow Research Centre. Further processing of patient samples and analysis of these and

anonymised patient data was performed jointly by me and my colleague, Dr Mark Robinson (MRC University of Glasgow Centre for Virus Research).

2.2.2 Historical cohort

This cohort consisted of individuals recruited from 1999-2005 by Professor Peter Mills, Consultant Gastroenterologist, Gartnavel General Hospital, Glasgow. They were originally recruited as part of a study of liver biopsies in HCV. Liver biopsies were taken at the point of recruitment which were analysed by local pathologists with liver disease stage and key features recorded. A snap frozen liver biopsy and serum samples were also taken at the point of recruitment which were stored at -70°C. Clinical information for the majority of the patients was recorded at baseline and maintained in a database with clinical outcome data (progression of liver disease / date of death/date of last follow up) being available for 98 patients within the database. Recent clinical data had been entered by medical students Jeeva John and Mark White and Harriet Tan. Informed consent was obtained from subjects at the time of recruitment according to the declaration of Helsinki. Local ethical approval was granted for recruitment to the original study and an amendment granted to me by the West of Scotland Regional Ethics Service (trial registration number 12/WS/0231) to enable use of the serum samples from these patients taken at the time of recruitment and linked, anonymised clinical data in my research project.

2.2.3 Exposed uninfected (EU) cohort

A cohort of current IDU who were not known to have ever been infected with HCV infection or other blood borne viruses was recruited between 2003 and 2014 by Professor Matthew Cramp and his research team at the University of Plymouth from a variety of locations in Plymouth, UK as previously described (92, 93).

These individuals were screened for evidence of current or previous HCV infection using diagnostic tests for antibodies to core and non-structural proteins (third generation ELISA by Abbot IMx) and HCV RNA by qualitative PCR (Amplicor, Roche Diagnostics). A confidential interview using a structured questionnaire was conducted to collect demographic data and a detailed injecting history. This included age at first injection, duration of injecting behaviour, frequency of injecting episodes, current injecting behaviour, frequency of sharing intravenous paraphernalia (needles, syringes, filters, spoons and water), frequency of sharing with a contact known to have HCV infection, and risk of non-IDU HCV exposure. The EU cohort was derived from a subgroup of this group of IDUs who were negative for both tests for HCV infection and included those judged to be at substantial risk of HCV exposure based on a >1 year history of injecting drug use and regular sharing of needles and related paraphernalia. All individuals had serum and whole blood samples drawn for laboratory analysis and clinical details entered in respective databases. Where possible, follow up clinical information and clinical samples from recruited individuals were obtained. Two individuals who were found to have developed HCV infection on further testing were excluded from the cohort. Dr Matthew Cramp kindly provided the samples from this cohort for testing. Drs Paraskevi Mandalou and Megghie Ow in his group provided the clinical data and assisted me with some of the laboratory assays on these samples.

2.2.4 The St Mary's Acute Hepatitis C Cohort

The St Mary's Acute Hepatitis C Study (104), is a well-characterised cohort of HIV-infected individuals with acute HCV infection recruited by Dr Emma Thomson. On recruitment to the study following acute HCV infection, clinical information, viral

and biochemical parameters and residual serum and whole blood samples were taken at serial time points (TP) during the course of infection.
Informed consent in writing was obtained and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki with ethical approval granted by the Riverside Research Ethics Committee. I was given permission by Dr Thomson (MRC University of Glasgow Centre for Virus Research) to use selected samples from this cohort in my research.

2.3 Cell Culture

All components of cell culture media were supplied by Invitrogen. Huh7 and HEK-293Tcells were grown in DMEMcomplete and maintained at 37°C in an atmosphere of 5 % CO_2.

Cell lines were typically grown in 80 cm^2 or 175 cm^2 tissue culture flasks (Nunc). Cells were passaged at 90 % confluency by washing gently with PBS followed by their removal with trypsin (Sigma) diluted 1:100 in versene (TV). Cells were then resuspended in 10 ml of DMEMcomplete before re-seeding or use in experiments.

2.4 Generation of HCV Pseudoparticles and lysate of cells expressing E1E2 glycoproteins

2.4.1 Amplification of patient-derived viral sequences

2.4.1.1 Viral RNA extraction and cDNA synthesis

Viral RNA was extracted from 140 µl of patient serum using the QuiAMP Viral RNA mini kit (Qiagen) as per protocol and eluted in 60 µl of buffer AVE (Qiagen). This was converted to cDNA using the Superscript III First Strand cDNA synthesis kit (ThermoFisher Scientific) using random hexamers (Roche), with an RNA digestion

step. 8 µl of eluted RNA was mixed with 1 µl of random hexamers (Roche) 1µl dNTPs (0.25mM), and RNase free water added to make a total volume of 11µl. This was incubated for 5 mins at 65°C before cooling on ice. Subsequently, 4µl First strand buffer, 1µl DTT (0.1M), 1µl RNaseOUT™ Recombinant Ribonuclease Inhibitor and 1µl SuperScript™ III reverse transcriptase (200units/µl) were added. RT was performed at 50°C for 60 minutes before heat-inactivation at 70°C for 15 mins. Following cooling, 2 U of RNAse H (ThermoFisher Scientific) was added and the mixture incubated at 37 ^{o}C for 20 mins before storing at -20 ^{o}C.

2.4.1.2 Design of primers for cloning patient derived E1E2 sequences

Degenerate nested primers were designed to flank E1E2 (Table 2.3). These were adapted from primers previously described (160) with degenerate positions based on the most common variable nucleotides from an alignment of all gt 1a and gt 1b sequences stored on the Los Alamos database (www.hcv.lanl.gov accessed 24/11/2012). This alignment was generated using CLC Genomics Workbench 7 (see Fig. 1.12). As with the original primers, stop and start codons were added along with a tag to allow directional cloning. The length of the primers was also adjusted to optimise PCR. As a positive control to confirm the presence of HCV-specific cDNA, primers for NS5B were also used (a gift from Dr Carol Leitch within the MRC University of Glasgow Centre for Virus Research).

Table 2.3 Primers used in PCR amplification of genotype 1 E1E2

Genotype 1 E1E2 degenerate primers			
Primer	**Sequence 5' – 3'**	**GC content (%)**	**Tm (^{O}C)**
Gt 1a/b outer sense	GTG AAY TAY GCR ACA GGG AA	40-55	49-53
Gt 1a outer antisense	GCA AAG CAG AAR AAC ACG AG	45-50	52-53
Gt 1 a/b inner sense	*CACC* ATG GGT TGC TCY TTY TCT ATC TTC	38-48	47-49
Gt 1a inner antisense	AAAGTTTCTAGA**TTA** CYG CCT CYG CYT GGG AKA	56-78	49-56
Gt 1b Outer Antisense	AGGCRGCRCARAAGAACACRA	52	63-70
Gt 1b Inner Antisense	AAAGTTTCTAGA**TTA**RGCCTCRGCYTGRGCTA	44	67-72
Control NS5B gt 1primers*			
HCV1/3_OS	CRT ATG AYA CCC GCT GYT TTG AC	50	64.8
HCV 1 IS	CTCCACAGTCACTGAGAGCGAYAT	52	67.6
HCV 1 IAS	AATGCGCTRAGRCCATGGAGTC	54	70.1
HCV 1 OAS	CCT GGA GAG TAA CTR TGG AGT G	50	53.3

*Kindly supplied by Dr Carol Leitch. Start codon is underlined, stop codons are highlighted in bold and directional tag is indicated in italics. Regions indicated in red text are additional nucleotides added to optimise the PCR reaction

2.4.1.3 PCR amplification of E1E2-encoding sequences and gel purification

cDNA was amplified using a nested PCR with the degenerate primer pools described above and HotStarTaq polymerase enzyme kit (Invitrogen) according to protocols for a 20 µl reaction using 2 µl of cDNA. 1 µl from the outer primer PCR was used as DNA template for the reaction using the inner primers. PCR was performed in a GeneAmp PCR Machine (Applied Biosystems). Cycling conditions for PCR were as shown in Table 2.4.

Table 2.4 PCR cycling conditions for amplification of E1E2-encoding sequences

Step		E1E2 Outer Primers		E1E2 Inner Primers		NS5B Outer Primers		NS5B Inner Primers	
		Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	Temp (°C)	Time (sec)
Initial denaturation / activation		95	300	95	300	95	300	95	300
Denaturation	**X 35 cycles**	94	15	94	15	94	15	94	15
Annealing		50	60	67	60	53	30	55	30
Extension		72	90	72	90	72	90	72	90
Final extension		72	600	72	600	72	600	72	600
Hold		4	∞	4	∞	4	∞	4	∞

PCR products were purified by running on a 0.8% agarose gel in 0.5% TBE using DNA loading gel alongside a 1 kb DNA ladder (Invitrogen). These were visualised using a UV lamp and bands at an appropriate size (1.8 kb) were excised. DNA was then extracted using the Qiagen Gel Extraction kit (Qiagen). DNA was eluted using TE buffer (Qiagen). NS5B primers to conserved regions within this sequence were used as a positive control in optimising the PCR protocol, subsequently samples known to have E1E2 amenable to amplification by the primers were used as positive controls. The inner gt 1a primers were also used to amplify the HCV gt 1a H77 sequence using the existing functional E1E2 expression vector as a template. This was used as a positive control in the downstream cloning and expression experiments.

2.4.2 Cloning into directional TOPO Gateway^{TM} entry vector

Purified products arising from successful PCR reactions were subcloned into the pENTR D-TOPO directional cloning vector (ThermoFisher Scientific) according to the product protocol. Briefly 4 µl of purified DNA was added to 1 µl of kit salt solution and 1 µl of linearised pENTR D-TOPO vector (Fig. 2.1). After incubating at room temperature for 30 mins, the solution was chilled on ice and 2 µl was added to competent OneShot ®*E.coli* cells (ThermoFisher Scientific) and incubated for 30 mins. These were then subjected to heat shock at 42°C for 30 seconds, chilled on ice for 5 mins, and incubated in 250µl of S.O.C. Medium at 37°C for 1 hour with shaking. Subsequently, 50 µl of bacterial culture was spread on selective LB Agar plates containing 50 µg/ml Kanamycin. Following incubation overnight at 37°C, 20 (or maximum number of discrete colonies where less than 20 had formed) were selected and cultured in 5 ml LB Broth containing 50 µg/ml Kanamycin. Vector DNA was then extracted from the cultures using the QIAprep Spin Miniprep Kit (Qiagen) according to protocol.

The presence of inserts was screened for by performing a digestion reaction. Due to internal digestion sites in the E1E2 protein, digestion using the AscI and NotI restriction site flanking the insert did not enable easy differentiation between plasmids with inserts and those without. Therefore PvuII was used which gave 3 bands at 2.5kb, 1.5kb and 1.1kb and smaller fragments <400bp with no insert and 4 bands at 2.5kb 1.8kb 1.3kb and 1.0kb insert alongside smaller fragments <400bp when insert was present. 1 µl eluted plasmid DNA was digested using the PvuII enzyme (NEB) incubated at 37°C for 1h (Fig. 2.2). Samples containing inserts were submitted to GATC Biotech AG (Germany) for Sanger sequencing to confirm that they carried the desired E1E2-encoding sequences. Sequences which were complete without internal stop codons were selected for insertion into the

mammalian expression vector phCMV(161) that had been specifically adapted to act as a Gateway®-compatible destination vector (phCMVGateway®) by Dr Vanessa Cowton in our lab (see section 2.4.4 for details).

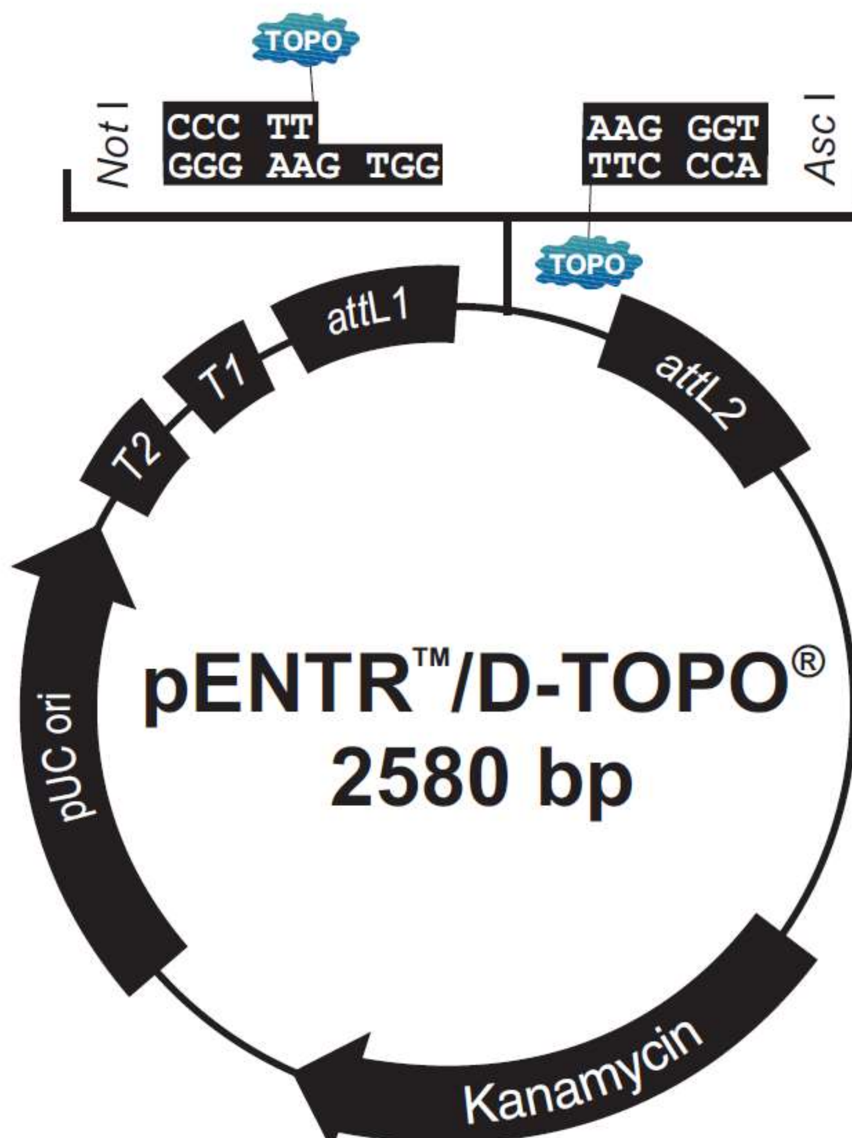


Figure 2.1 pENTR D –TOPO directional cloning vector

This diagram shows the layout of the vector with directional insertion sites, kanamycin resistance enzyme and digestion restriction sites for digestion enzymes NotI and AscI. Image obtained from thermofisher website.[https://tools.thermofisher.com/content/sfs/manuals/pentr_dtopo_man.pdf accessed 16/11/2016]

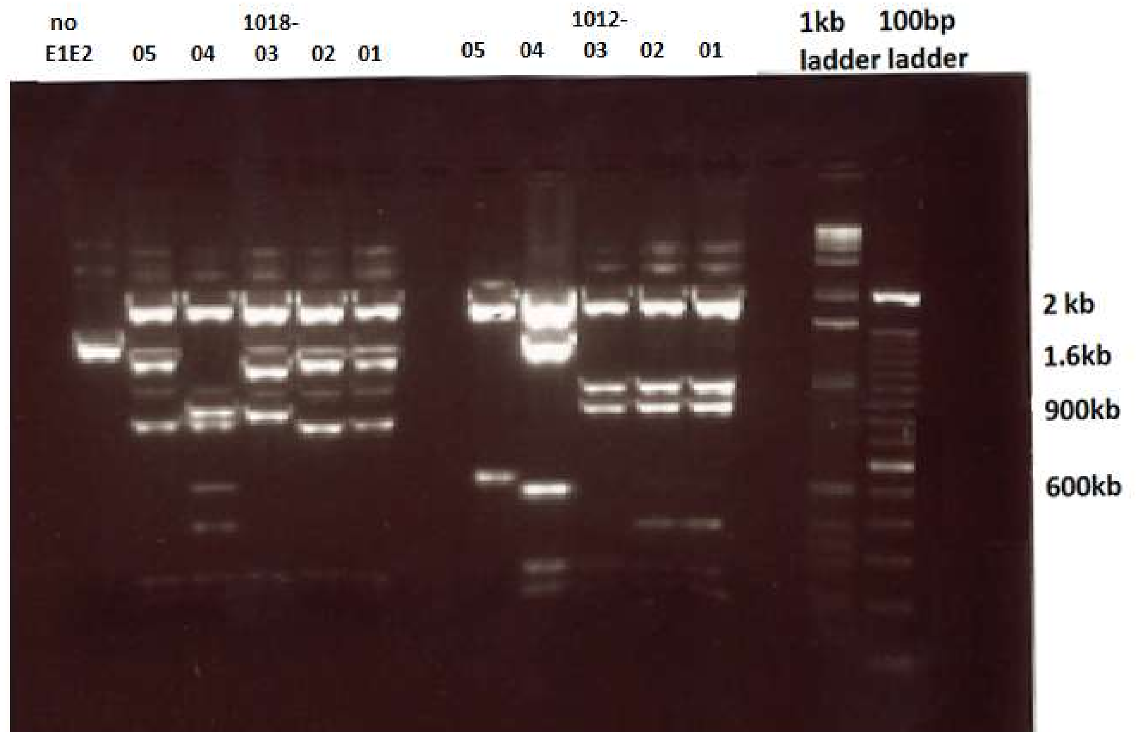


Figure 2.2 PuvII digestion of pENTR-D TOPO vectors to determine presence of an E1E2 insert.

Vector DNA was obtained using the QIAprep Spin Miniprep Kit and eluted in water. 1µl of the elutate was added to a PuvII enzyme solution (NEB)in appropriate NEB buffer and incubated at 37°C for 60 mins. The resulting digestion mix was run on an 0.8% Agarose gel for 1 hour at 70V. The presence of a single band at the leftmost well is undigested DNA from a phCMV plasmid not containing E1E2. Presence of a single band at 1.6kb and at 600kb suggested no insert. Presence of multiple smaller bands suggested an insert. Due to the presence of internal restriction sites in some E1E2 sequences the pattern of smaller bands was not the same for all inserts. Those samples with evidence of an insert were sent for confirmatory Sanger sequencing.

2.4.3 Calculating dN/dS ratios for E1E2 sequences from single patients

For individual patients where more than 3 unique sequences were identified through amplification and subcloning as above, the dN/dS ratio was calculated.

This can reveal regions under positive and negative selection due to host and viral fitness pressures.

Sequences from each patient were aligned initially in CLC Genomics Workbench v. 7.0.3. and these alignments transferred to the MEGA 6.0 program for further analysis (16). Nucleotide substitutions resulting in an amino acid change compared to conservation of an amino acid were identified by HyPhy diversity analysis and dN/dS ratios were generated. The HyPhy software also generated probability values for conservation or non-conservation of each amino acid position.

2.4.4 Transferring E1E2-encoding sequences into the phCMVGateway® expression vector

pENTR vectors containing envelope sequences of interest were used to transfer the E1E2 sequence to a phCMVGateway® expression vector. This vector had been generated using the Gateway ® Vector Conversion System (ThermoFisher Scientific) by Dr Vanessa Cowton. To perform the reaction, 150 ng of entry clone DNA was mixed with 300 ng of phCMVGateway® expression vector and TE buffer added to give a final volume of 8 µl. Subsequently 2 µl of LR Clonase II enzyme mix (ThermoFisher Scientific) was added to the solution, mixed by pipetting and incubated at 25°C for 1 hour. Next, 1 µl of Proteinase K was added and incubated for 10 mins at 37°C. 2 µl of the reaction mix was used to transform chemically competent *E. coli* by heat shocking at 42°C for 30 seconds then returning to ice for 2 mins followed by incubating at 37°C in 250 µl of LB for 1 hour. 50 µl of this mixture was plated onto LB agar plates containing 10 µg/ml ampicillin overnight. Colonies which grew were then incubated in 5 ml of LB containing 10 µg/ml ampicillin for 16 hours and plasmids extracted from the resulting bacterial culture using the Qiagen Miniprep Kit as per protocol. Quantity of the plasmid DNA

obtained was measured using a Nanodrop spectrophotometer (ThermoFisher Scientific). Functionality of the sequences in the expression vector was tested by generating HCVpps.

2.4.5 Generating HCVpps by HEK-293T cell transfection

HEK-293T cells in subconfluent 10 cm tissue culture dishes were co-transfected with the retrovirus packaging vector pMLV gag – pol, the transfer vector pMLV-Luc and a HCV E1E2-expressing vector phCMVcE1E2 using a Calcium chloride precipitation method as described by Tarr *et al* (160). Some of the phCMV vectors had been created as part of a previous project, others were generated during the course of this work using methods described above. 24 hours after co-transfection, medium was replaced with 6 mls of fresh DMEM containing 10% FCS and 10 mM HEPES, pH7.0. After 72 hours, the supernatant medium was harvested and passed through a 0.45 μM filter and used as a source of HCVpp.

2.4.6 Infectivity testing of HCVpps

HCVpps were tested for their ability to infect Huh7 cells as follows: Huh7 cells were seeded at 4×10^3 in a 96 well Immulon-II plate (Nunc) and incubated for 24 hrs. HCVpps from individual patient sequences were generated and 40 μl of HCVpp containing supernatant added to Huh7 cells, after 3 hrs the medium was changed and the cells incubated for a further 72 hrs before lysing and testing for infectivity using the Light Glo Luciferase assay (Sigma).

Infectivity was confirmed in HCVpp generating a light unit signal 10x the background signal from uninfected Huh7 cells (approximately 1000 RLU in most assays). Indeterminate infectivity was defined as a RLU signal between 2 and 10

times background levels. For use in downstream neutralisation assays, only HCVpps with a signal 10 times above background were selected.

2.4.7 HEK-293T cell lysis and extraction of lysate

Media was removed from 10 cm tissue culture dishes containing previously transfected HEK-293T cells. 1ml of Lysis Buffer 2 (LB2) was added and incubated while rocking for 10 mins. Following lysis, the lysate was clarified by spinning at 13,000 revolutions per minute for 1 min. The supernatant was removed and either used directly or stored at -20°C for future use.

2.4.8 Testing HEK-293T cell lysate for E1E2 production

For some HCV E1E2-expressing vectors not producing infective pseudoparticles, transfected HEK-293T cells were lysed in Lysis Buffer 2 using 1ml per plate. Lysates were centrifuged for 5 min to pellet cell debris and the supernatant lysate was tested for the presence of E2 protein in a capture ELISA. This used apreviously described method whereby ELISA plates coated with *Galanthus nivalis* agglutinin (GNA) lectin were used to capture E1E2 glycoproteins from HEK-293T cell lysates which were then probed using anti E1E2 antibodies(123). Lysate from HEK-293T cells transfected with a non-E1E2 containing plasmid were used as a negative control. Test lysates were diluted 1:3 in PBS with 0.05%Tween and 2%Milk added (PBSTM) and 100 µl/well added in triplicate to 2 separate GNA plates. Following incubation and wash steps, one plate was probed with the antibody AP33 at (at 0.1 µg/ml in PBST) the other with CBH-4B (at 2 µg/ml) (255). These recognise a linear and a conformational epitope on HCV E2 glycoprotein respectively. Following incubation and washing, HRP-conjugated anti-mouse or anti-human IgG (Sigma, cat no A4416 or A0170, respectively) was added at a concentration of 1:1000 (A4416, approximately 1 µg/ml) or 1:5000 (A0170,

approximately 2 µg/ml) in PBST. Following incubation for 1 hr, the plates were washed with PBST and then the bound antibodies detected using TMB (3,3', 5, 5'-tetramethylbenzidine, Sigma) substrate. Presence of E2 was confirmed by an absorbance level higher than the mean negative control plus 2 standard deviations in the AP33 assay. Correct folding of E2 was confirmed by an absorbance level higher than the mean negative control plus 2 standard deviations in the CBH-4B assay as this antibody is conformation sensitive. For individuals where several E1E2 sequences had been isolated, some producing infective HCVpp and others resulting in E1E2 expression but non-infective HCVpps, sequences were aligned using Mega 6.0 molecular genetics software (16) to identify the variable regions between functional and non-functional sequences.

The majority of the experiments in this section (2.4.8) were performed under my supervision by medical students Ruairi Wilson and Jane Hamilton. I designed the experiments and analysed and interpreted the results in conjunction with the students.

2.5 Generating panels of HCVpps bearing a diverse range of E1E2 sequences for testing breadth of antibody binding and virus neutralisation

2.5.1 The Cross Genotype (XG) panel

To determine reactivity and neutralisation of patient-derived IgG across different genotypes, a panel of HCV pseudoparticles bearing test envelope proteins from the main infecting genotypes in the UK was used. This was termed the Cross Genotype (XG) panel and comprised E1E2 from 6 HCV sub-genotypes which had been generated prior to this project and are described elsewhere (129). The sequences incorporated into this panel were: gt1a H77c (Accession number:

AF011751), gt1b UKN1B5.23 (AY734976); gt2a JFH-1 (AB047639); gt2b, UKN 2B1.1 (AY734982); gt3a UKN3A13.6 (AY894683); gt4 UKN4.11.1 (AY734986). While it would have been useful to test against gt 5 and gt 6, the E1E2 sequences which were available in the HCVpp system for these genotypes proved to have low infectivity (<1000 RLUs) and therefore could not be used reliably in neutralisation assays.

2.5.2 The intragenotype 1 (IG1) panel

To allow investigation of antibody reactivity against a diverse range of envelope variations within a single genotype, as might arise during a natural infection, I developed the intragenotype 1 (IG1) panel.

All gt 1 phCMVcE1E2 vectors held in the lab were tested for functionality in the HCVpp system as described previously. This included phCMVcE1E2 vectors bearing E1E2 sequences derived from gt1 HCV-infected patients from the CHCV cohort and the St Mary's acute HCV cohort (256) generated as detailed above. In addition, it included a bank of phCMVcE1E2 vectors generated as part of a previous project using patients from the Trent Cohort (17).

An alignment of all sequences was generated in CLC Genomics Workbench 7. Gt 1a sequence H77 was included as a standard reference sequence and ten additional sequences were selected by phylogenetic analysis (Fig. 2.3) on the basis of overall genetic difference (*p* distance) and representation of amino acid variability found in all available gt1 E1E2 sequences registered with the Los Alamos HCV sequence database (see Fig. 1.12).

Legend for Figure 2.3 on following page

Figure 2.3 Molecular phylogenetic analysis of HCV gt1 E1E2 amino acid sequences to determine the IG1 panel.

A phylogenetic tree was generated from protein alignments of all gt 1 E1E2 sequences inserted into phCMV vectors held in the lab. This was generated by the maximum likelihood method using the JTT matrix-based model(257). A discrete gamma distribution was used to model differences in the evolutionary rates among sites (5 categories [gamma distribution parameter = 0.3680]). The tree with the highest log likelihood (−7,055.2655) is shown. The tree is drawn to scale, and the genetic distance for each branch length is indicated by the scale bar. Bootstrap analysis with 1,000 replicates was performed. The percent support for branches with >70% bootstrap support is indicated. Sequences are indicated by coloured symbols as follows: blue, sequences classified to be functional in the HCVpp system; red, non-functional sequences; green, functional sequences included in the gt1 panel. Evolutionary analyses were conducted with the MEGA (v.6) program(16) Molecular Phylogenetic analysis by Maximum Likelihood method. [Figure and Legend reproduced from Swann et al, J. Virol, 2016 (251) as permitted by the American Society for Microbiology for authors of papers published in their journals].

2.6 ELISA Assays

2.6.1 Serum IgG Purification

Serum was incubated with Triton X-100 (Sigma, UK) at a final concentration of 0.05% to deactivate any virus particles present. Disinfected serum in 250 µl aliquots was then added to a Protein G IgG purification spin column (Thermo Scientific, UK), washed to remove all detergent and IgG eluted, as per manufacturer's protocol, in 1.2 ml of elution buffer and the eluate neutralised. The final concentration of IgG was determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific, UK).

2.6.2 GNA ELISA to detect human IgG binding to E1E2 proteins

Briefly, Immulon II ELISA plates (Dynal) coated with *Galanthus nivalis* agglutinin (GNA) were used to capture E1E2 glycoproteins from lysates of HEK-293T cells

transfected with plasmids expressing E1E2 glycoproteins. In general these were diluted 1:3 in PBST prior to use. IgG for each test subject was subsequently added at a concentration of 200 μg/ml in PBS containing 0.05% Tween-20 and 2% Skimmed Milk Powder (PBSTM). Plates were incubated for 1 hour and washed 6 times in PBST. The bound human IgGs were detected using an HRP conjugated anti-human IgG antibody (Sigma A0170) diluted 1:5000 (2μg/ml), incubated for 1 hour and washed as previously. Presence of secondary antibody was determined using TMB (3,3', 5, 5'-tetramethylbenzidine, Sigma) substrate incubated for a maximum of 10 mins or until a clear colour reaction was visible in the positive control. The reaction was stopped with 0.5 M sulphuric acid. Absorbance values were measured at 450 nm using a Varioskan microplate reader (Thermo Fisher Scientific). Occasionally variations in secondary antibody were used – where applicable this has been stated clearly in the text.

2.6.3 Purified sE2 ELISAs

2.6.3.1 Detection of Human IgG binding to sE2

A soluble form of gt 1a E2 (H77) protein (sE2) was generated following expression in High Five insect cells (Life Technologies Ltd) and subsequently purified by Dr Ania Owsianka in our lab. Immulon II ELISA plates were coated with 100 μl per well of sE2 at concentration of 1 μg/mL. Plates were washed and 50 μl of serum diluted 1:50 in PBSTM was added to each well in duplicate. After washing, binding of human IgG was detected as described for the GNA ELISAs.

2.6.3.2 Detection of Human IgM binding to sE2

To detect IgM, Immulon II ELISA plates (Dynal) were coated with sE2 as described above. Subject serum was diluted 1:50 with PBSTM and pre-incubated three times on a glutathione-S-transferase coated plate to remove non-specific

antibodies, then added to the E2-coated plate. After incubation and wash steps using high tween PBSTM (0.5% Tween-20), IgM binding was detected using HRP-conjugated anti-Mu antibody (Abcam) at a 1:500 dilution(2µg/ml). The remainder of the ELISA protocol was as for the E1E2 ELISA described above.

2.6.3.3 E2 epitope competition ELISA

Competition ELISAs were conducted using purified patient IgG samples to determine if IgG targeted specific CD81-binding sites where a characterized set of antibodies are known to bind (see Table 2.5).

Immulon II plates were coated with sE2 as above and incubated with purified patient IgG at 200 µg/ml in PBSTM for 1h. Subsequently, monoclonal antibodies (mAbs) (listed in Table 2.5) to discrete E2 immunodomains (119, 120, 145) were added at a concentration close to their EC_{50} (determined by producing dilution curves of these antibodies on sE2 coated plates Fig. 2.4). All antibodies were biotinylated except HC-84.1 which was a mouse-human chimera. Streptavidin-HRP was added to detect biotinylated antibodies and anti-mouse IgG (Sigma A4416) to detect HC-84.1 (1:10000 dilution, approximately 0.1µg/ml for both reagents) and binding measured as above. The reduction in relative binding of each mAb (calculated as percentage reduction in absorbance) on addition of patient IgG compared to PBSTM control was determined.

Table 2.5 Antibodies used in E2 epitope competition ELISA

Immuno domain	Antibody	EC50 (µg/ml)	Key binding residues in E1E2 glycoproteins	Reference
A	CBH-4B (non-neutralising)	0.004	540, 542, 556,627	(120)
B	HC-1	0.05	529, 530, 535	(120) (125)
B	HC-11	0.003	425, 427,436, 437,438, 442,443, 529,530, 535	(120)
C	CBH-7	0.01	544, 547, 549, 550	(145)
D	HC-84.1	9.6	441,442,443,616 (NB also overlaps with some domain B residues)	(145)
E	AP33	0.01	413,415,418,420	(146) (129)

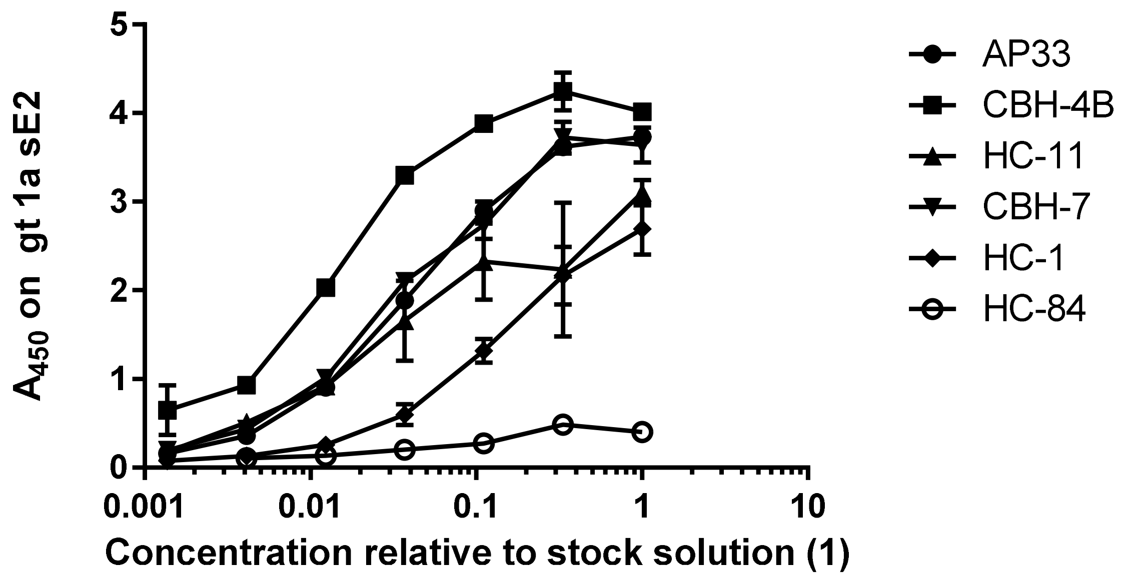


Figure 2.4 Concentration curves of ELISA absorbance values for antibodies to known epitopes on sE2

Antibodies as listed above were diluted from a stock concentration, which was 1 µg/ml in PBST for all except HC-84 where the stock concentration was 250 µg/ml. 50 µl/well of stock solution and serial 1:3 dilutions in PBST were added to wells on a sE2 coated Immulon plate and binding detected using either streptavidin-HRP conjugate or anti-mouse monoclonal antibody A4416 (Sigma) as described in the text. Absorbance values were plotted and EC50 values calculated using GraphPad Prism 6.0 software and are listed in Table 2.5. Mean and SEM values are shown.

2.7 Assays to determine HCVpp infectivity in the presence of antibodies and cell entry protein blockade

2.7.1 HCVpp neutralisation assay

Neutralisation assays were conducted to determine the ability of test IgG to prevent infection of Huh7 cells by HCVpps bearing various envelope sequences. The IgG and HCVpps used for each experiment are described fully in the relevant chapters. These assays were performed as previously described (119). Briefly, Huh7 cells were seeded at 4×10^3 cells per ml in DMEMcomplete with 200 µl added to each well in a 96-well flat bottomed plate (Nunc) and incubated overnight. The following day, test subject IgG was added to 40 µl of HCVpp-containing medium prepared from transfection of HEK-293T cells as described above. In general IgG was added at a concentration of 100 µg/ml, which represents an approximate serum dilution of 1:50-1:100. For some samples where IgG could not be purified, a dilution of heat-treated serum was used. In addition, in some cases a dose/response curve was generated by testing at serial dilutions of IgG. This is described fully in the relevant sections. The mouse monoclonal anti-E2 antibody MAb AP33 (258), (259) and healthy control IgG were included as positive and negative controls, respectively. After incubation for 1 h the IgG-HCVpp mixture was added to the Huh-7 cells (following removal of medium). After incubation for 3 h, the inoculum was replaced with fresh medium and following a further 72 h incubation luciferase activity in infected cells was detected as a marker of HCVpp infectivity using the Bright-Glo Luciferase kit (Promega, UK) and relative luminosity measured in relative light units (RLU) using a Chameleon II plate reader. Virus neutralisation was defined as 50% reduction in luminosity compared to wells where HCVpps were added without IgG.

2.7.2 HCVpp Susceptibility to SR-BI and CD81 blockade

To determine if differential use of entry proteins was a factor in susceptibility to neutralisation, selected HCVpp were tested for sensitivity to blockade of the cell surface receptors SR-BI and CD81.

HCVpp were generated as described previously. Prior to adding these to Huh7 cells, the cells were incubated with 40 µl modified DMEM containing anti-CD81 antibody (clone JS-81, Sigma) at 1 µg/ml or anti-SR-BI MAb151-NP1(252, 260) at decreasing concentrations from 10 µg/ml to 0.03 µg/ml. After 1 h the DMEM was removed and 40 µl of test HCVpp were added or DMEM added to control wells. These were incubated for 4 h and then medium changed. Infectivity of HCVpp in the presence of cell receptor blockade was determined using a Glo Luciferase Assay as described above at 72 h post-infection.

2.8 Testing for the ability of purified IgG to neutralise cell culture infectious HCV (HCVcc)

These experiments were conducted by Dr Vanessa Cowton and Mrs Sarah Cole with purified IgG from the CHCV cohort after discussion with me.

Infectious virus was produced in Huh7 cells by electroporation of RNA encoding full-length JFH-1 or chimeric JFH-1. The chimeric strains 2B.1.1/JFH1 and gt1a H77/JFH1 (HQL) have been described previously (123, 261). The neutralization of cell culture infectious HCV (HCVcc) infection was tested in Huh7-J20 cells, a stable cell line expressing green fluorescent protein fused in-frame to alkaline phosphatase with a cleavage site containing the recognition site for the HCV

NS3/NS4 protease. Infection and replication of virus results in secretion of alkaline phosphatase into the extracellular culture medium(261).

Huh7-J20 cells were seeded at 1 × 10^4 cells/well in a 48-well tissue culture dish and incubated at 37 °C. Supernatant media from electroporated cells containing secreted virus was incubated with test IgG at a concentration of 100µg/ml for 1 h at 37 °C before adding to Huh-J20 cells for 3 hours. This was then replaced with 400 µl of fresh medium and the cells incubated at 37 °C for 72 hours.

To measure the extracellular SEAP activity (therefore relative infectivity), 90 µl of culture medium was collected and mixed with 10 µl of 10-times concentrated LB2 to inactivate the virus. The SEAP assay was performed using the Phospha-Light assay system (Thermo Fisher) to measure secretory alkaline phosphatase (SEAP) levels.

2.9 Cryoglobulin detection and analyses

2.9.1 Cryoglobulin detection

Only one of the two hospital sites used to recruit the CHCV cohort and corresponding HCs had facilities available for testing for cryoglobulins according to accepted standards (262). Therefore, all CHCV cohort patients and HC recruited at this site had an additional sample of serum taken for cryoglobulin testing. A small number of individuals at the second site also had samples taken and transferred in a pre-warmed container for testing. For each individual, a 9 ml whole blood sample was collected in pre-warmed serum tubes and maintained at 37°C until the sample had been spun in a centrifuge and the supernatant serum transferred to a universal container. This serum was stored at 4°C for 7 days and observed for precipitate development. A patient was recorded as cryoglobulin-

positive if a precipitate formed, disappeared on re-warming to 37°C and reappeared on further cooling for 7 days (Fig. 2.5). The presence of immunoglobulin was subsequently confirmed by cryoglobulin typing where the volume of precipitate allowed. Preparing samples and testing for the presence of a cryoglobulin precipitate was performed by me and Dr Mark Robinson.

Figure 2.5 Image of cryoglobulin precipitate

Image of 2 samples after chilling at 4°C for 7 days showing no precipitate on left hand sample while clear precipitate is visible on the right. While a small amount of red cell contamination is visible in the right hand sample, the precipitate disappeared on warming to 37°C for 30 min and reappeared on re-cooling for 7 days.

2.9.2 Cryoglobulin isolation and typing

Following re-precipitation, cryoglobulins were washed 3 times in ice cold PBS and, if sufficient sample remained, were re-suspended for typing using 250 µl PBS by warming to 37°C. 10 µl of cryoprecipitate solution was added to 6 wells of a Hydragel 4IF dynamic protein electrophoresis kit (Sebia). Following

electrophoresis, the gel was stained using the secondary antibodies provided for IgG, IgM, IgA, Igk, Igλ followed by a destaining step. Presence of a polyclonal band in one lane and a mono- or oligo-clonal band in another indicated a Type 2 MC (Fig. 2.6). Presence of 2 polyclonal bands indicated Type 3 MC. Typing was performed by the Immunology Laboratory, Gartnavel Hospital, Glasgow.

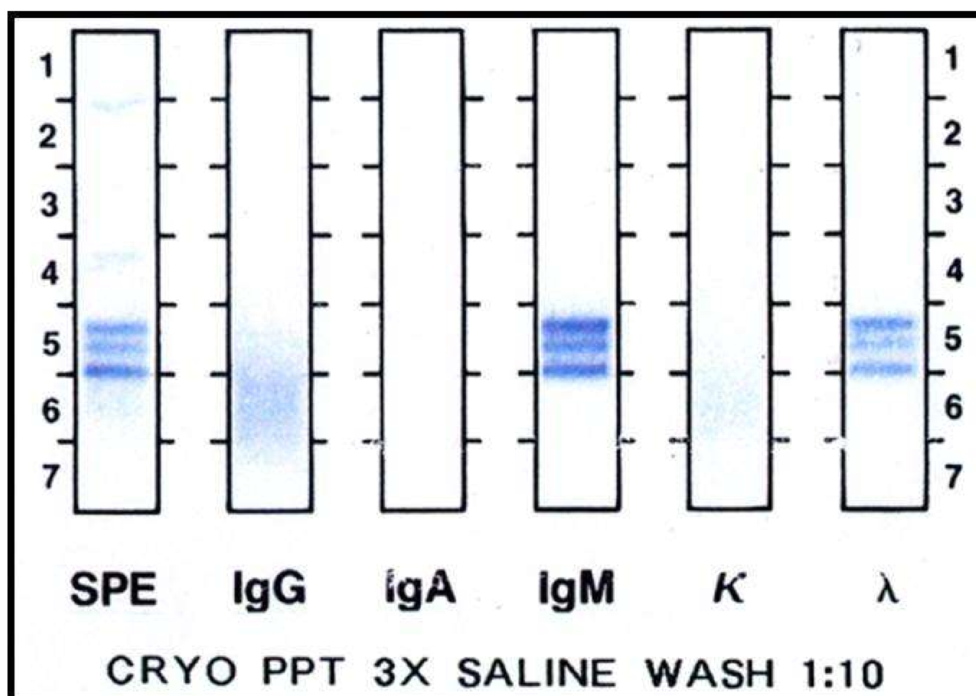


Figure 2.6 Protein electrophoresis of a cryoglobulin with monoclonal IgM bands.

[Image reproduced from https://www.studyblue.com (accessed 10/10/16)] Example of an electrophoresis gel of a cryoglobulin precipitate prepared as described above with staining indicating binding of secondary antibodies to the antibody classes indicated below each lane. This particular example shows monoclonal bands in IgM and polyclonal IgG / light chains indicating a Type 2 MC. NB this image is shown to illustrate a positive result and is not an image from samples collected during this study.

2.9.3 Rheumatoid factor testing

Where sufficient sample remained following cryoglobulin testing, 10 µl of the remaining resuspended cryoprecipitate was tested for Rheumatoid factor (RhF)

activity using the Siemens RhF latex agglutination assay. This was performed by staff at the Immunology Laboratory, Gartnavel Hospital, Glasgow.

2.9.4 Testing cryoglobulins for binding to sE2

2.9.4.1 IgM concentration ELISA

IgM from the resuspended cryoglobulin precipitate was tested for its ability to bind to sE2 compared to IgM from serum samples taken from the same individual. Cryoglobulins from 4 patients with G1 HCV were heated to 37°C and re-suspended in saline as above. Serum from the same patients, cryoprecipitate solution and serum from HC were diluted to 1:50 in PBST and tested for concentration of IgM using a standard IgM ELISA – all steps prior to addition of the secondary antibody were conducted at 37°C to prevent precipitation of the cryoglobulin. Briefly, Immulon plates were coated with a goat anti-human IgM (Sigma) at a 1:10000 dilution then blocked with PBSTM. Test serum and cryoprecipitate were diluted 1:100 and 1:1000 in PBSTM and 100 µl added in duplicate alongside serial dilutions of human IgM of known concentration (Sigma). Purified IgG was used as a negative control. IgM binding was detected using HRP-conjugated anti-Mu antibody (Abcam) at a 1:500 dilution. After a further incubation and wash step, TMB substrate was then added and the reaction allowed to continue for 7 mins before stopping with 50 µl of 0.5 M H_2SO_4. The strength of the colour signal was read using a Varioskan plate reader to determine the A_{450}. Absorbance readings for the standard curve of IgM were used to calculate the concentration of IgM in cryoprecipitates and serum.

2.9.4.2 Testing cryoglobulins for binding to sE2

Immulon 96-well plates were coated with sE2 in PBS (1 µg/ml, 100 µl/well) as previously described. Reprecipitated cryoglobulin samples, corresponding serum

samples and HC serum samples were diluted in high tween (0.5%) PBSTM (PBS with 0.5% Tween-20 and 2% skimmed milk powder) and heated to 37 °C. Sample concentrations were adjusted to ensure equal concentrations of IgM. 100 μl of each sample were pre-incubated with GST coated plates for 20 min to pre-adsorb non-specific antibodies, this was repeated a total of 3 times. The pre-adsorbed samples were subsequently added in duplicate to the gt1a sE2-coated plate. These initial steps were conducted at 37°C to prevent precipitation. Subsequently, the plates were washed in high Tween PBSTM and HRP-conjugated anti-Mu was added (Abcam). After a further incubation and wash step, TMB substrate was then added and the reaction allowed to continue for 7 mins before stopping with 50 μl of 0.5 M H_2SO_4. The strength of the colour signal was read using a Varioskan plate reader to determine the A_{450}.

2.10 B cell receptor (BCR) gene amplification

2.10.1 PBMC separation and RNA extraction

An 18 ml whole blood sample was obtained from each patient in the CHCV cohort and associated HCs. Peripheral blood mononuclear cells (PBMCs) were isolated from this by centrifugation over a Histopaque gradient and washing with Hanks Balanced Salt solution (HBSS). Two aliquots of PBMCs suspended in HBSS were removed and the PBMCs pelleted by further centrifugation. To obtain RNA, the supernatant was removed with a pipette and the PBMC pellet lysed using 1 ml buffer RLT containing ß- mercaptoethanol. Subsequently total RNA was isolated using the RNA-Easy kit (Qiagen) following an on-column DNase-digestion (Qiagen) step according to manufacturer's protocol. RNA quality and quantity were assessed on a 2200 TapeStation (Agilent Technologies).

2.10.2 cDNA synthesis and PCR for BCR gene sequencing

Sequencing of the BCR of selected patients was conducted in collaboration with Dr Mark Robinson and Dr Rachael Bashford Rogers (RBR) from the Sanger Centre, Cambridge. PBMCs from 6 HCV positive, cryoglobulinaemia positive patients, 6 HCV positive, cryoglobulin negative patients and 4 healthy controls (from the CHCV cohort) were prepared for BCR diversity analysis. Total RNA was extracted as described above. cDNA libraries were prepared using an Ig heavy chain specific antisense primer and selected samples also had libraries prepared using IgG and IgM specific primers as published by RBR and reused with her permission (Table 2.6) (263).

Reverse transcription was performed using the VILO kit (Invitrogen). Briefly, for each reaction 500 ng total RNA was mixed with 4 µl 5x VILO Reaction Mix, 2 µl 10x Superscript ® Enzyme Mix and DEPC-treated water up to a total reaction volume of 20 µl. Tube contents were incubated at 25°C for 10 mins then at 42°C for 60 mins. The reaction was terminated by heating to 85°C for 5 mins.

2.10.3 BCR gene amplification and analysis.

Subsequently the BCR cDNA libraries were amplified using a pool of B cell receptor primers as published by RBR (Table 2.6) (263). Each reaction contained 0.5 µl each of sense and antisense primers of a 5 µM working stock, 0.5 µl HotStarHiFi® High-Fidelity DNA Polymerase (Qiagen), 4 µl 5x HotStarHiFi PCR buffer and 2 µl cDNA per 20 µl reaction. The following PCR program was used: 5 mins at 95°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 65°C and 1 min at 72 ^{o}C, with a final extension cycle of 7 mins at 72 ^{o}C on a GeneAmp (ThermoScientific) thermocycler.

Table 2.6 Primers used for amplification of BCR genes

Pimer (5' to 3')	Region	Sense/Antisense
CTTACCTGAGGAGACGGTGACC	J_H consensus	Antisense
GGCCTCAGTGAAGGTCTCCGCAAG	VH1-FR1	Sense
GTCTGGTCCTACGCTGGTGAAACCC	VH2-FR1	Sense
CTGGGGGGTCCCTGAGACTCTCCTG	VH3-FR1	Sense
CTTCGGAGACCCTGTCCCTCACCTG	VH4-FR1	Sense
CGGGGAGTCTCTGAACATCTCCTGT	VH5-FR1	Sense
TCGCAGACCCTCTCACTCACCTGTG	VH6-FR1	Sense
CAGGAGACGAGGGGGAA	BCR IgM	Antisense
CACCGTCACCGGTTCGG	BCR IgG	Antisense

These primers are as designed and published by RBR (263) and reused with her permission.

2.10.4 NGS library preparation and sequencing

PCR products were purified using Agencourt® Ampure® XP beads (Beckman Coulter) and checked for purity using the TapeStation; for all heavy chain samples, clear bands were seen at approximately 315 bp in length and quantified using a Qubit® 2.0 Fluorometer with the Qubit® dsDNA High Sensitivity kit (Invitrogen) using the Qubit High Sensitivity dsDNA assay. 16 µl of PCR product was end-repaired, A-tailed and adaptor-ligated using the Kappa Real-Time Library Amplification kit and NEBNext® Multiplex Oligos primers for Illumina (NEB) as per

protocol. All amplified libraries were checked on a 2200 TapeStation (Agilent Technologies) to ensure specific products and were quantified using the Kappa SYBR FAST Illumina library quantification kit. Purification and library preparation was performed predominantly by Mark Robinson with some assistance from me. The prepared library samples were sequenced using a MiSeq System Desktop Sequencer (Illumina) with a 300 cycle MiSeq version 2 reagent kit (Illumina) and sequences sent to RBR for further analysis using an in-house phylogenetics programme. Further details of the analyses performed using this programme are given in Chapter 8.

2.11 Host DNA isolation and single nucleotide polymorphism (SNP) analysis for HLA genes rs9275224 and rs2395522

In the CHCV cohort DNA was extracted from 200 μl of whole blood using the QIAmp DNA Mini Kit (Qiagen) as per protocol. As no whole blood sample was available for the Historical cohort individuals, the QIAmp Viral RNA kit was used without a DNAase step to extract both RNA and DNA simultaneously from 140 μl of serum stored at -70°C. Custom primers for SNPs rs9275224 and rs2395522 were obtained from ThermoFisher Scientific. 1 μl of DNA from CHCV samples or 5 μl of RNA/DNA mix from historical samples were included in a qPCR reaction using a TaqMan® Assay kit to test each sample for the presence of SNPs in both genes. The qPCR reaction was performed on a 7500HT Fast Real-Time PCR system (Applied Biosystems). Samples were assigned as A/G heterozygotes or AA or GG homozygotes according to the colour signal generated.

2.12 Statistical analysis and bioinformatics software

Statistical analysis was conducted using GraphPad Prism 6 Software (GraphPad Software, California) and SPSS v. 19.09 (IBM, New York) for the most part of this project. Where other programmes were used, this has been stated in the individual chapters.

Molecular genomics analysis was conducted using CLC Genomics Workbench 7 (Qiagen Bioinformatics) and MEGA 6.0 (MEGA Software (16)) software unless stated otherwise in the text.

3 Broad anti-HCV antibody responses and their association with improved clinical disease parameters in chronic HCV infection

[The work described in this chapter has been published in Swann *et al., J. Virol, 2016 (251)* - the American Society of Microbiology copyright licence allows authors to reproduce their work as part of academic theses]

3.1 Introduction

Antibodies targeting the HCV envelope glycoproteins E1 and E2 can contribute significantly to viral clearance in acute HCV infection (113, 131). A recent study on individuals acutely infected with gt 1 HCV found that those developing a broad neutralising antibody (bNAb) response, able to prevent infection by a diverse range of HCV envelope strains *in vitro*, were more likely to clear infection(133).There is evidence that bNAbs may contribute to viral suppression and even clearance once infection has become established in some cases (144) (131).

It is likely that host genetics play a significant part in the ability to mount a bNAb response as genetic polymorphisms have been associated with a bNAb profile in acute clearance (133). Similarly, host HLA type is known to be important in T cell mediated clearance of some HCV genotypes(264). In HIV it has also been postulated that it is viral evolution which drives a bNAb response (136) with the development of matured affinity responses. However, it is difficult to prove whether co-evolution of broadly neutralising antibodies is predominantly dependent on viral characteristics, host physiology and genetics or an equal contribution from both.

Although there is evidence that bNAbs have clinical relevance in acute infection, during chronic HCV infection neutralising antibody (NAb) responses, targeting E1E2 envelope glycoproteins and capable of preventing infection by some strains of HCV, are generated in many individuals. It is unclear if these antibodies play a protective role. Indeed, humoral immune responses induced by HCV can have pathological consequences as seen in cryoglobulinaemic vasculitis (6). Therefore I aimed to investigate the presence of polyclonal broadly neutralising antibody (bNAb) responses in chronically infected HCV (CHCV) patients in order to determine any association with clinical outcomes and host factors. I also aimed to explore the relationship between overall anti-E1E2 antibody binding and functional neutralising ability of the polyclonal responses.

3.2 The CHCV and healthy control cohorts

Subjects with either gt1 or gt3 CHCV were prospectively recruited from 3 local liver clinics as described in Materials and Methods. Individuals with co-existing liver pathologies or with BMI >31 were excluded. Healthy controls with no liver pathologies or significant co-morbidities were also recruited and their samples used as negative controls in subsequent experiments. All subjects completed a symptom questionnaire, clinical details were recorded and baseline biochemistry, virology and host IL28B genotype profiles were determined. Liver stiffness of CHCV individuals was measured by transient elastography using a Fibroscan ® (Echosens). Serum and whole blood samples were obtained from all participants. Samples from 8 of the recruited healthy controls and 51 of the chronically infected cohort were available at the time this part of my research was conducted and therefore were used in the

following experiments. Their demographics are summarised below (Table 3.1 and 3.2). Comparisons of categorical variables were made using Chi Squared tests, ordinal variables were compared using Wilcoxon Rank Sum tests. Gt 3 individuals were more likely to be non-Caucasian (*P*=0.04) as would be expected given that gt 3 is most prevalent in Asia.

Table 3.1: Demographics of CHCV and Healthy Control cohorts

Demographics	CHCV (n=51)		Healthy Controls (n=8)	
	Number	Percentage /Range	Number	Percentage /Range
Age **Median (range)**	46	(28-68)	50	(30-69)
Gender (M/F) **No. male (%)**	35	(68.6)	4	(50)
Ethnicity **Caucasian (%)**	47	(92.2)	8	(100)
Source of infection **No. IVDU (%)**	32	(62.7)		
Estimated duration of infection (years)	25	(2-58)		
IL28B CC genotype (%)	17	(35.4)*		
Anti-HBc positive (%)	11	(24.4)**		
BMI (kg/m^2) **Mean (range)**	26	(19-31.5)		
Diabetes **Present (%)**	2	(3.92)	0	(0)
Previously treated$ (%)	21	(46.7)**		
HCV RNA load pre-treatment IU/ml **Median (range)**	$6.8x10^5$	(2272 - $1.1x10^7$)		
Cirrhosis **Present (%)**	18	(36)		
Transient elastography (kPa) **Median (range)**	9	(4-75)		

*3 subjects not tested **no information available for 6 subjects. $All individuals had HCV infection at the time of testing; those previously exposed to interferon were either relapsers or null responders. No individuals were on therapy at the time of sampling. There were no significant differences between healthy controls and CHCV in the parameters measured using Chi squared test for categorical data and Wilcoxon rank sum test for ordinal data.

Table 3.2 : Comparison of demographics and clinical parameters between CHCV gt 1 and gt 3 infected patients.

Demographics	Gt 1 (n=27)		Gt 3 (n=24)		P value
	Number	Percentage /Range	Number	Percentage /Range	
Age **Median (Range)**	47	(35-66)	45.5	(28-68)	0.49
Gender (M/F) No. male (%)	18	(67)	17	(71)	0.77
Ethnicity Caucasian (%)	27	(100)	20	(83)	**0.04**
Source of infection No. IVDU (%)	19	(70)	13	(54)	0.26
Estimated duration of infection (years) Median (range)	20	(5-40)	26	(2-58)	0.50
IL28B CC genotype (%)	8	(30)	9	(38)	0.56
Anti-HBc positive (%)	3	(11)	8	(33)	0.09
BMI kg/m^2 **Mean (Range)**	24.9	(19.8-29.7)	25.6	(19.4-31.5)	0.94
Diabetes Present (%)	1	(4)	1	(4)	1.00
Previously treated (%)	14	(52)	7	(29)	0.15
HCV RNA load pre-treatment IU/ml **Median (Range)**	498520	(2272-11300000)	788327	(7687-7030000)	0.59
Cirrhosis **Present (%)**	9	(33)	10	(42)	0.57
Transient elastography (kPa) **Median (Range)**	8.3	(3.5-69.1)	9.25	(4-75)	0.60

P values were calculated using Chi squared test for categorical data and Wilcoxon rank sum test for ordinal data.

3.3 The cross genotype (XG) and Intragenotype 1 (IG1) panels

To determine reactivity and neutralisation of patient-derived IgG across a diverse range of envelope sequences, two panels of HCV pseudoparticles bearing test envelope proteins were used. The first, the XG panel, enabled analysis of antibody reactivity with E1E2 proteins of different viral genotypes. It is based on a previously described panel (see Materials and Methods).

The second, termed the IG1 panel was designed as part of my project to allow investigation of antibody reactivity within a single genotype as might arise during a natural infection. The E1E2 sequences from gt1 infected patients from the CHCV cohort were PCR-amplified and cloned into the mammalian expression vector phCMV. This process is described briefly below with further detail in Materials and Methods.

3.3.1 Generating HCVpps for use in the IG1 panel

Gt 1 infected samples in the CHCV cohort with higher viral loads were selected for amplification. Viral RNA was extracted from CHCV patient serum using the QIAamp Viral RNA mini kit (Qiagen) and converted to cDNA using the Superscript III First Strand cDNA synthesis kit (ThermoFisher Scientific).

3.3.1.1 Amplifying E1E2 from patient serum

Initially previously published primers (160) were used to attempt to amplify the whole of E1E2 from cDNA. NS5B primers donated by Dr Carol Leitch were used as a positive control to confirm presence of virus (see Materials and Methods for

sequences). A product would be expected at 1.8kb for the E1E2 primers and a 600bp for the NS5B primers. Unfortunately, the original E1E2 primers failed to yield any PCR product despite confirmation of the presence of virus using the NS5B primers (Fig. 3.1). The only product shown in the E1E2 primer group is likely to represent a primer dimer complex. While there is evidence of possible contamination in the negative control for the NS5B primers in this PCR there may be non-specific binding of primer to human genetic material creating some faint non-specific bands. This band is different to the clear single positive band shown in those patients with gt 1 HCV infection.

As several attempts at amplification of E1E2 with the previously published primer set did not produce a product of the correct size, a modified set of degenerate nested primers flanking E1E2 were designed as described in Materials and Methods with a directional cloning tag added to the inner sense primer. Cycling conditions were as described in Materials and Methods.

PCR products of the correct size were obtained for 10/15 samples tested with an example of positive products shown in Fig. 3.2. In this figure products of the correct size (1.8kb) are clearly demonstrated for pts 1013, 1032 and 1034 with a smaller (incomplete E1E2) product for 1021 and primer dimers only for the remainder of samples tested. Further PCR reactions generated for sample 1012,1018,1029,1047 and 1112. Altering the annealing temperature was required to obtain PCR products for some of the samples (1010 and 1037), however full length E1E2 could not be amplified from others (1002,1003,1021,1031 and1046) despite prior confirmation of presence of HCV cDNA using NS5B primers to conserved regions. As the product generated from these primers had to produce a functional, correctly folded protein in

frame while including minimal extra genetic material from core protein and p7 there were limited options to alter the sequence of these primers to optimise PCR conditions.

For those samples where PCR products of the correct molecular weight were generated, these were purified by running on a 0.8% Agarose gel, excising bands of an appropriate size (1.8kb) and extracting DNA using the Qiagen Gel Extraction kit.

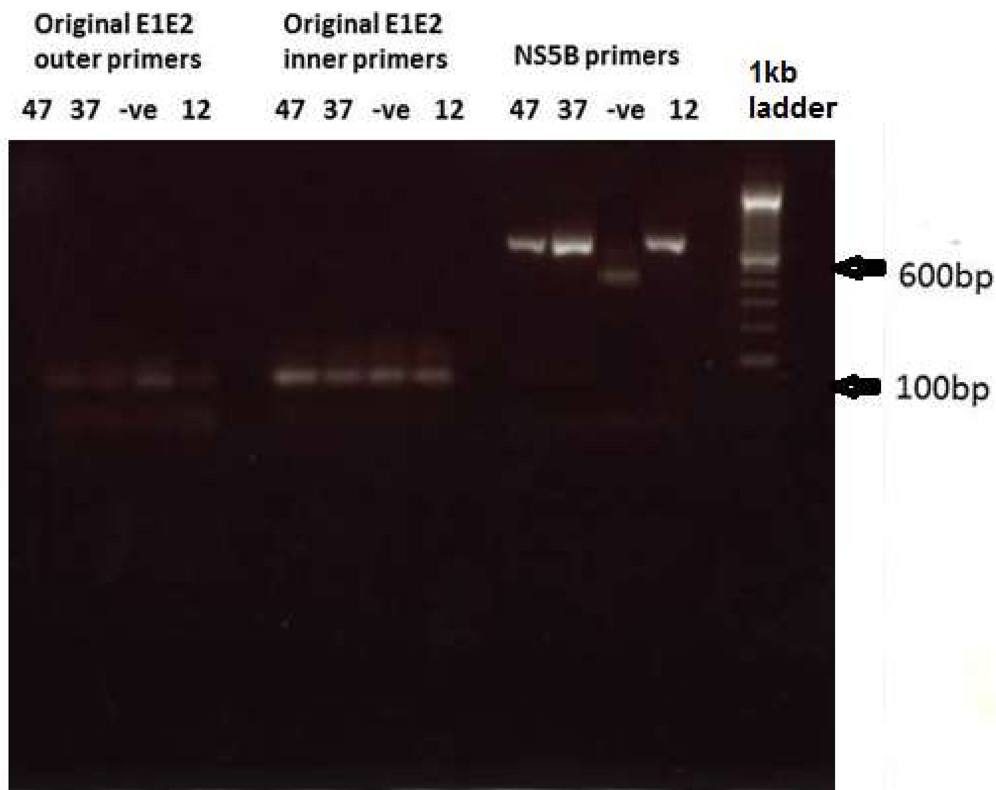
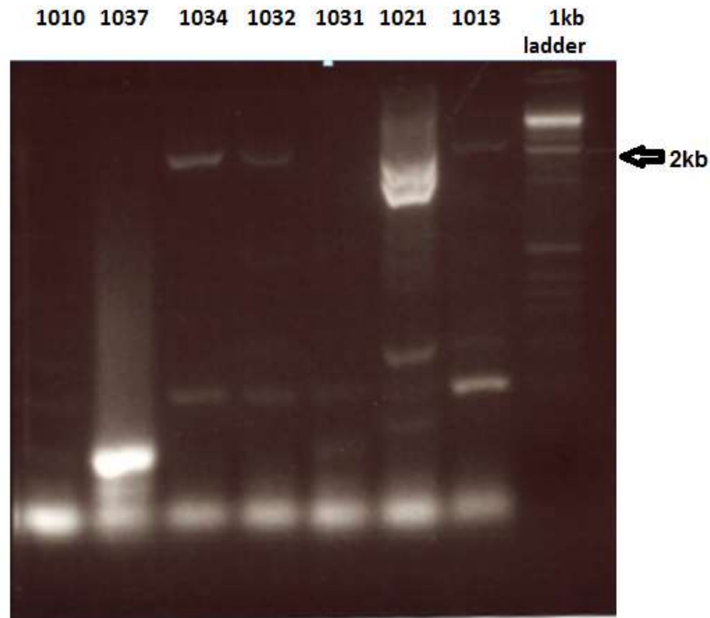


Figure 3.1 PCR of cDNA from patient serum samples with published E1E2 primers and validated NS5B primers for HCV gt1.

Three gt 1 infected samples from the CHCV cohort with higher viral load were selected for amplification of E1E2 sequences (1012, 1037 and 1047 labelled 12,37 and 47 respectively) alongside a healthy control sample (labelled –ve). A cDNA library was created from HCV RNA isolated from patient serum using the QiaAMP Viral RNA mini kit (Qiagen). The complete E1E2-encoding region was amplified using previously described nested primers(160) using a HotStarTaq PCR kit. In addition, a separate PCR was performed on the same samples using previously validated NS5B region primers as described in Materials and Methods. The PCR product was run on a 0.8% agarose gel alongside an Invitrogen 1kb DNA ladder. The expected product size for E1E2 is 1.8kb and 600bp for NS5B.



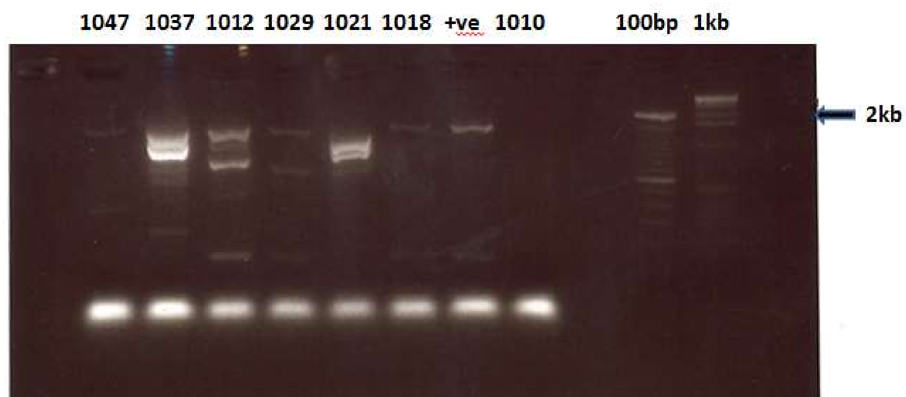


Figure 3.2 Two gels displaying products from PCR of cDNA from patient serum samples with modified E1E2 primers.

Gt 1 infected samples from the CHCV cohort with higher viral load were selected for amplification of E1E2 sequences. A cDNA library was created from HCV RNA isolated from patient serum using the QiaAMP Viral RNA mini kit (Qiagen). The complete E1E2-encoding region was amplified using degenerate nested primers as described in Materials and Methods using HotStarTaq PCR kit. The positive control is from including the standard gt 1a H77 E1E2 expression vector as a template in the second step of the nested PCR. The PCR product was run on a 0.8% agarose gel alongside an Invitrogen 1kb DNA ladder. The expected product size for E1E2 is 1.8kb.

3.3.1.2 Identifying E1E2 sequences of interest to test in the HCVpp system

The purified PCR products were sub-cloned using the pENTR D-TOPO directional cloning kit (ThermoFisher Scientific, see Materials and Methods). Colonies from each subcloning reaction were cultured in selective media and plasmid DNA extracted and screened for vector inserts by digesting with *PVUII*. Those plasmids shown to have inserts were then sequenced by GATC-Biotech to confirm the presence of E1E2-encoding sequences.

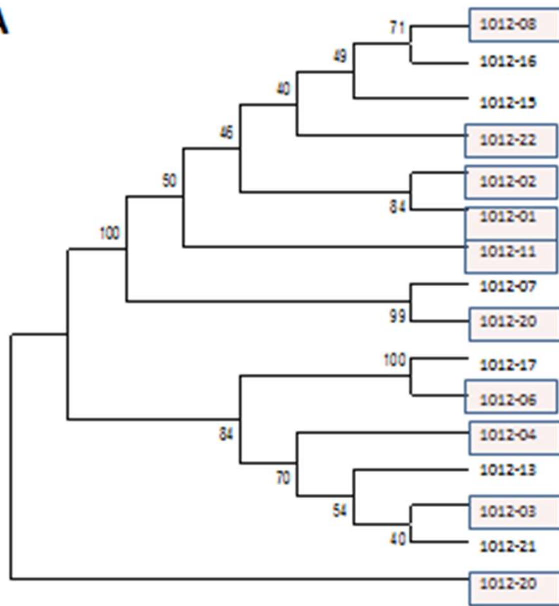
At least one E1E2 sequence was obtained from each of these 10 individuals. For some, over 20 sequences were obtained, in others, fewer than 5 successful sub-cloning reactions were generated despite repeated attempts. A selection of E1E2 sequences were chosen for insertion into phCMV expression vectors (as described in materials and Methods) from each of the patients with multiple sequences. For those 5 individuals with 5 or more unique sequences cloned a phylogenetic tree was constructed and sequences selected on the basis of overall genetic distance from other sequences and variability at key amino acid residues at known neutralising epitopes. For the remaining 5 individuals where fewer than 5 sequences, an attempt to transfer all unique sequences to an expression vector was made. Overall a total of 39 sequences were transferred into vectors for testing in the HCVpp system.

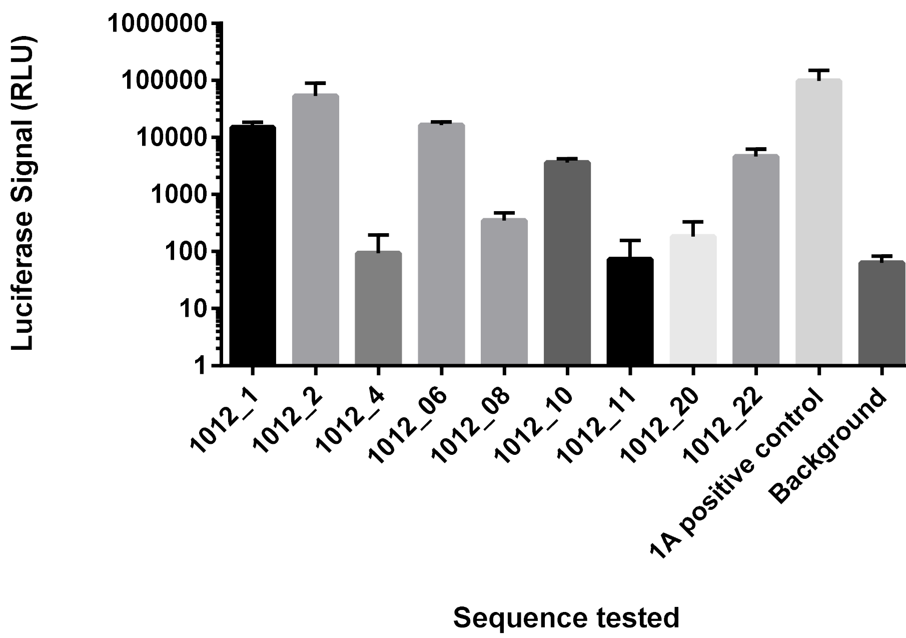
These expression vectors were used to generate HCVpp which were then tested for their ability to infect Huh7 cells as described in Materials and Methods. Infectivity was determined by assessing the luciferase signal 72 hours post-infection. Those HCVpp generating a signal of > 1000 RLU (approximately 10x the background level from uninfected Huh7 cells) were deemed functional, those with signals below this level were deemed non-functional as they were inappropriate for use in downstream

neutralisation assays because natural variability of the assay would make IC50s difficult to interpret at a signal value so close to background. However,Some of theseHCVpps had evidence of weak infective activity displaying a signal between 2 and 10 times background level. The process of generationg a phylogenetic tree and determining HCVpp infectivity data for sequences from patient 1012 is shown in Fig. 3.3.

3.3.1.3 Selecting E1E2 sequences to use in the Intragenotype 1 (IG1) panel

All sequences functional in the HCVpp system from the CHCV cohort (generated as above), and others historically generated functional sequences were aligned and a phylogenetic tree generated as described in Materials and Methods section 2.5. From these analyses, 11 sequences giving the greatest level of diversity were selected to form the IG1 panel. The selected panel provided variability at 146 of the 156 amino acid residues identified as variable in >10% of gt1 E1E2 sequences from the Los Alamos database and an additional eleven sites that represent more minor variations (see Figure 1.12).





Legend for Figure 3.3 on following page

Figure 3.3 A) Phylogenetic tree of sequences derived from pt 1012 and B) Infectivity of HCVpps bearing those sequences.

A) Sequences were obtained from subcloning the E1E2 PCR product from patient 1012 generated as described in materials and methods were aligned using MEGA 6 software and a Maximum Likelihood phylogenetic tree constructed with bootstrap values based on 1000 replicates. The pink boxes represent sequences chosen to transfer to the expression vector based on genetic distance from other sequences and variation at key aa residues. B) Huh7 cells were infected with HCVpp bearing E1E2 sequences from different patients. At 72 h post infection, cells were lysed and luciferase activity determined. The bars show mean RLU with error bars representing the SEM. Functionality was determined by a RLU value >10x background (i.e. uninfected Huh7 cells). The positive control was HCVpps generated using the standard gt 1a expression vector bearing the H77 sequence. HCVpps were tested in quadruplicate over 2 separate experiments.Mean and SEM are shown.

3.4 Cross-genotypic antibody reactivity is associated with lower viral load.

Patient IgG reactivity to HEK-293T-cell expressed Panel XG E1E2 was tested by GNA-capture ELISA as described in Materials and Methods. IgGs from healthy control (HC) samples were included as a negative control and a standard curve of the broadly binding linear anti-E2 antibody AP33 was included as a positive control and to allow comparison of results across ELISA plates.

In order to compare clinical parameters between those patient IgGs with broader binding characteristics and those who were poor binders or only bound to one or two genotypes, it was necessary to determine which patients to include in broad and narrow binding categories. Unlike neutralisation assays, there is no accepted level of absorbance indicating positive binding in this assay; indeed across genotypes the

absorbance levels given by AP33 was extremely variable at the same concentration of antibody. Moreover, while the lysates were all prepared in the same way, it is likely that levels of expression varied between the constructs. As all of these individuals have been exposed to HCV it was difficult to validate an appropriate ‘cut off’ for a positive ELISA result, with the majority of individuals showing absorbance levels >2x the HC samples used over several genotypes. At the time of optimising this assay, recruitment of healthy control samples had not completed, however if a larger pool of these samples had been available an alternative method would have been to set an arbitrary cut off based on HC values.

Despite these limitations there were individuals who clearly had stronger and broader antibody binding than others displaying absorbance levels of over 10-fold that of HCs (Table 3.3). Therefore, to allow comparison of binding strength and breadth within the cohort, I chose to rank each sample according to its relative binding strength based on absorbance readings for each genotype. Samples were ranked from 1 (highest) to 51 (lowest) for each gt. I then added all gt ranks for each individual. From this sum of ranks, a final rank position was assigned, which gave an overall indication of the relative binding strength and breadth for each patient.

Table 3.3: Relative absorbance of samples (compared to 1µg/ml AP33) against E1E2 in the XG panel and ranking of samples according to overall binding.

Sample	Gt 1a	Gt 1b	Gt 2a	Gt 2b	Gt 3	Gt 4	Rank 1a	Rank 1b	Rank 2a	Rank 2b	Rank 3	Rank 4	Sum of ranks	**Overall Rank**
1001/00	6.7	33.1	1.4	2.3	7.7	5.9	19	2	11	8	19	19	78	**6**
1002/00	0.4	0.4	0.1	0.0	3.6	0.0	51	51	51	42	33	48	276	**50**
1003/00	13.4	18.7	2.8	0.6	61.9	67.6	12	9	6	15	1	2	45	**1**
1006/00	4.9	6.0	0.3	0.1	4.9	1.8	24	31	31	29	29	37	181	**34**
1008/00	1.5	10.8	0.4	0.2	57.2	0.6	39	21	27	21	2	46	156	**28**
1009/00	1.5	7.4	0.4	0.1	27.3	0.6	41	27	25	36	4	47	180	**33**
1010/00	86.8	5.2	0.4	0.1	2.1	4.2	1	34	22	25	41	24	147	**27**
1012/00	31.4	13.8	0.5	0.4	23.1	50.3	7	15	17	17	6	3	65	**4**
1013/00	3.6	6.0	0.2	0	3.2	2.7	28	32	45	49	35	34	223	**42**
1015/00	0.9	9.2	0.4	0.2	34.6	4.0	47	25	21	23	3	27	146	**26**
1016/00	7.9	13.1	0.7	0.2	19.6	17.7	17	19	15	18	9	7	85	**9**
1018/00	8.2	14.5	0.4	0.2	7.1	6.6	16	13	28	19	22	17	115	**15**
1020/00	2.4	3.0	0.3	0.1	22.1	0.7	33	43	33	35	8	45	197	**36**
1021/00	26.6	13.2	1.2	3.1	3.4	27.5	8	18	12	5	34	4	81	**7**

1022/ 00	39.1	4.5	0.3	0.1	2.4	9.8	4	37	40	39	40	11	171	**29**
1023/ 00	14.3	13.5	0.1	0.1	16.8	11.2	10	16	49	28	10	9	122	**19**
1024/ 00	3.0	2.9	0.3	0.1	3.8	1.1	29	45	32	32	31	41	210	**40**
1029/ 00	6.4	12.8	0.4	0.1	22.7	9.3	21	20	20	37	7	13	118	**18**
1030/ 00	6.7	4.9	0.3	0.1	0.8	1.6	20	35	29	31	50	38	203	**38**
1031/ 00	34.5	7.0	0.8	0.1	2.5	10.7	5	29	14	27	38	10	123	**20**
1032/ 00	8.4	10.1	0.4	0.1	6.0	22.9	15	23	19	38	26	5	126	**21**
1033/ 00	0.7	3.7	0.2	0	2.4	0	48	40	44	47	39	49	267	**49**
1034/ 00	6.4	13.4	0.4	0.1	25.7	18.9	22	17	26	26	5	6	102	**11**
1035/ 00	76.2	8.2	0.3	0.1	2.1	13.3	2	26	35	30	42	8	143	**24**
1036/ 00	1.7	20.8	1.5	2.3	3.8	5.6	36	6	9	9	32	20	112	**14**
1037/ 00	2.7	7.1	0.1	0.2	1.5	3.6	31	28	50	22	44	28	203	**38**
1038/ 00	1.9	9.7	0.3	0.0	1.3	2.9	35	24	39	44	47	33	222	**41**
1040/ 00	12.3	28.0	4.5	4.3	3.0	9.8	14	3	3	4	36	12	72	**5**
1041/ 00	1.0	5.5	0.2	0	8.5	0.8	45	33	43	46	16	44	227	**43**
1042/ 00	41.3	6.0	0.3	0.2	7.4	9.1	3	30	30	20	20	14	117	**17**
1043/ 00	1.1	20.6	1.9	3.0	7.1	4.5	44	7	7	6	23	23	110	**12**
1045/	1.6	3.3	0.3	0.0	0.3	1.5	38	42	37	45	51	39	252	**47**

00													
1046/ 00	14.0	23.8	1.5	1.7	5.7	6.9	11	5	10	12	27	16	81	**7**
1047/ 00	32.2	3.5	0.3	0.1	1.5	1.9	6	41	38	34	45	36	200	**37**
1049/ 00	2.7	1.5	0.3	0.1	10.3	2.9	32	49	36	24	13	32	186	**35**
1050/ 00	18.8	4.3	0.2	0.1	10.5	95.3	9	38	41	33	12	1	134	**23**
1052/ 00	0.7	1.6	0.2	0	7.2	0	49	48	48	48	21	50	264	**48**
1054/ 00	5.8	2.9	0.3	0.1	15.6	4.6	23	44	34	40	11	22	174	**30**
1055/ 00	2.7	2.6	0.2	0.1	2.9	1.0	30	46	42	41	37	42	238	**46**
1056/ 00	3.9	2.2	0.2	0.0	4.8	1.4	27	47	46	43	30	40	233	**45**
1057/ 00	1.1	1.5	4.0	16.4	1.4	2.5	43	50	4	1	46	35	179	**32**
1060/ 00	1.5	4.8	21.8	0	0.2	0	40	36	1	51	52	51	231	**44**
1061/ 00	7.0	34.0	3.9	6.4	9.9	8.1	18	1	5	2	14	15	55	**2**
1062/ 00	4.7	19.2	1.8	2.5	9.1	3.1	26	8	8	7	15	31	95	**10**
1063/ 00	0.5	13.8	0.6	1.1	6.3	4.1	50	14	16	13	25	26	144	**25**
1064/ 00	0.1	4.2	0.2	-0.7	1.2	1.0	52	39	47	50	48	43	279	**51**
1072/ 00	1.2	10.1	0.4	0.7	2.0	3.2	42	22	24	14	43	29	174	**30**
1074/ 00	1.6	16.4	0.9	2.0	7.9	5.5	37	10	13	11	18	21	110	**12**
1089/ 00	2.0	16.2	0.4	2.1	8.0	4.1	34	12	18	10	17	25	116	**16**

1112/00	13.3	25.4	5.2	5.5	6.3	6.0	13	4	2	3	24	18	64	**3**
1128/00	4.8	16.3	0.4	0.5	5.6	3.1	25	11	23	16	28	30	133	**22**
HC 1025	0.9	0	0.03	0	1.0	0	-	-	-	-	-	-	-	**-**
HC 1027	0.4	0.8	0.02	0.00	0.1	0.04	-	-	-	-	-	-	-	**-**

The table shows adjusted absorbance values compared to Healthy Control samples for E1E2 GNA ELISAs using purified IgG from CHCV patients at 200µg/ml. To allow comparison between separate ELISA plates, absorbance readings were standardised according to absorbance values on a standard concentration curve of AP33, a linear antibody known to bind to most genotypes of HCV E2. The values shown in the table is the concentration of AP33 (in µg/ml) giving an equivalent Absorbance value to the sample. For healthy control samples (denoted by HC) and some CHCV samples, 0 has been used where the ratio was lower than 0.01. Values are the average obtained over 3 experiments with samples tested in duplicate Samples were ranked from strongest binding (1) to weakest binding (51) for each subgenotype tested against. These ranks were totalled for each individual and this total was used to give an overall rank. .

The simplest way to compare those with broader and narrower binding characteristics was to divide the cohort in half based on binding rank and to compare the top ranked half with the lower half to determine any clinical associations with breadth of antibody binding. Those in the top ranked half (i.e. with broader ELISA reactivity profiles) had a significantly lower viral load (*P*=0.03, Fig. 3.4). Individuals with a broader breadth of binding were more often infected with gt 1 HCV and conversely individuals with a narrower breadth of binding were more often infected with gt 3 HCV (*P*=0.04, Table 3.4). These associations appear to be independent as there was no relationship between gt of infection and viral load (*P*=0.59, Table 3.2).

The association of cross-genotype breadth and infection with gt 1 may simply reflect a closer sequence homology between gt 1 and the other genotypes tested i.e. gt 1a is more closely related to gt 2 and gt 1b is closely related to gt 4a(14). Another explanation is that gt 3 envelope sequences may be less variable, therefore generating a less diverse pool of antibodies. However, if this was the case gt 3 virus-infected individuals would be expected to have a higher rate of reactivity against the standard laboratory gt 3 sequence. In fact the number of individuals with a low level of reactivity to their own genotype was similar between gt 1 and gt 3 infected individuals. 9 gt 3-infected individuals had an adjusted absorbance <5 x mean HC absorbance to gt 3 lysate, compared with 8 gt 1 infected individuals with an adjusted absorbance <5 x HC mean absorbance when tested on the gt 1a lysate. It is also possible that gt 3 infection fails to induce bNAb through other, unknown mechanisms.

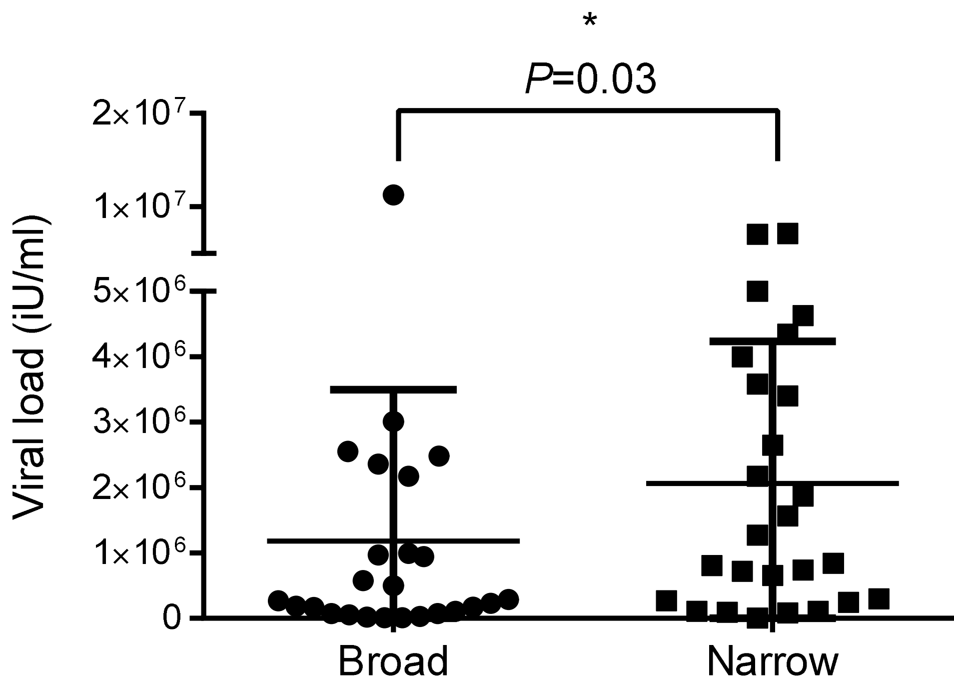


Figure 3.4 Association of viral load with cross-genotypic E1E2 binding.

The relative binding of CHCV cohort IgG to E1E2 from 6 subgenotypes of HCV was determined by ELISA. The IgG samples were ranked from 1 to 51 according to their binding signal for each subgenotype. The sum of these ranks was used to order the samples from highest cross-genotypic binding to lowest. The binding of the highest ranked of the cohort was regarded as “Broad” and that of the lower half as “Narrow”. The viral loads of the two groups were compared using the Mann Whitney U test. Mean and SD are shown.

Table 3.4 Associations of IgG binding of panel XG by E1E2 ELISA with clinical features.

	XG ELISA broad (n=25)	XG ELISA narrow (n=26)	P value
Age Median (range)	46 (29-66)	46 (28-68)	0.63
Gender (M/F) No. male (%)	19 (76)	16 (62)	0.26
Ethnicity No. Caucasian (%)	1 (4)	3 (12)	0.32
Source of infection No. IVDU (%)	17 (68)	14 (54)	0.39
Estimated duration of infection (years) Median (range)	19 (2-40)	31 (5-58)	0.29
IL28B CC genotype (%)	9 (36)	8 (31)	0.77
Anti-HBc positive (%)	6 (24)	5 (19)	0.74
BMI kg/m^2 Mean (range)	25.9 (21-31.5)	25 (19.8-30)	0.69
Diabetes Present (%)	1 (4)	1 (4)	1.00
Previously treated (%)	9 (36)	12 (46)	0.57
HCV RNA load pre-treatment IU/ml Median (Range)	265234 (7687- 3010000)	1054634 (2272-7140000)	**0.03**
Cirrhosis Present (%)	9 (36)	10 (38)	0.57
Transient elastography (kPa) Median (Range)	8.85 (3.5 - 45)	9 (4 -75)	0.99
Genotype 1 (%)	17 (68)	10 (38)	**0.04**
Genotype 3 (%)	7 (28)	16 (64)	**0.04**

Statistical comparisons were made using non parametric tests (Fisher's exact test for categorical data, Wilcoxon Rank Sum for ordinal or numeric data). Significant *P* values are shown in bold.

3.5 XG neutralisation does not have an association with clinical factors

Purified IgGs derived from the CHCV cohort were tested for their ability to neutralise (reduce infectivity by 50% or more) HCVpp bearing envelope proteins from Panel XG (Table 3.5). Breadth of neutralisation, defined as broad (neutralisation of >3/6 genotypes) or narrow (<4/6 genotypes), was analysed for association with clinical factors (Table 3.6). Twenty (39%) of the 51 individuals tested had broad cross-genotypic neutralising IgGs.

Table 3.5 Percentage reduction in infectivity (neutralisation) of HCVpp in the XG panel by CHCV patient purified IgG

Sample ID	Gt 1a	Gt1b	Gt 2a	Gt 2b	Gt 3	Gt 4	Number of gt neutralised (/6)
1001/00	73.9	48.2	45.8	5.1	47.8	67.3	2
1002/00	-6.7	-51.7	20.9	-4.2	34.2	15.9	0
1003/00	76.7	71.9	84.4	30.2	66.3	89.6	5
1006/00	61.2	16.1	54.0	18.3	36.2	77.4	3
1008/00	54.6	42.5	65.0	23.7	77.5	73.5	4
1009/00	50.8	39.3	67.3	20.9	48.4	71.4	3
1010/00	73.6	60.5	73.1	37.0	45.7	86.1	4
1012/00	74.6	56.1	84.9	45.4	76.2	86.7	5
1013/00	84.8	11.1	39.4	30.8	48.9	70.0	2
1015/00	68.0	37.3	66.7	26.8	48.7	82.9	3
1016/00	75.7	79.8	88.9	7.4	74.9	89.1	5
1018/00	76.8	50.8	40.4	-6.2	49.6	79.6	3
1020/00	56.1	44.7	38.8	5.0	53.0	83.3	3
1021/00	62.7	34.7	55.4	25.7	46.2	88.1	3
1022/00	73.1	55.1	63.3	32.5	51.6	81.3	5
1023/00	58.7	54.4	67.1	-6.8	73.5	90.1	5
1024/00	55.4	65.6	72.5	29.0	40.2	76.8	4
1029/00	46.4	30.9	83.2	13.4	67.1	87.8	3
1030/00	18.4	13.7	54.9	26.4	27.9	79.6	2
1031/00	41.0	1.2	11.0	38.2	31.6	75.3	1
1032/00	64.5	-8.8	81.0	40.3	79.1	86.0	4
1033/00	23.5	68.3	34.4	13.7	27.8	61.9	2
1034/00	47.7	42.7	47.3	16.6	26.4	59.1	1
1035/00	82.4	65.7	66.7	45.1	48.3	87.5	4
1036/00	65.3	46.5	52.2	23.7	22.5	65.1	3
1037/00	74.6	54.5	65.5	16.1	61.6	90.9	5
1038/00	38.6	40.6	77.1	29.4	51.7	79.0	3
1040/00	75.3	53.4	34.4	29.6	29.4	63.6	3
1041/00	66.6	-29.2	43.7	25.8	71.5	71.9	3
1042/00	67.9	57.0	57.4	39.6	80.2	89.9	5
1043/00	44.4	45.5	31.7	-4.1	32.9	60.7	1
1045/00	61.0	22.5	37.2	36.3	-22.6	65.0	2
1046/00	79.4	60.0	62.5	50.2	64.0	82.9	6
1047/00	52.5	5.4	40.3	45.6	62.9	71.4	3
1049/00	58.1	24.0	66.8	45.0	50.3	82.0	4
1050/00	84.4	49.5	51.5	45.5	54.3	76.2	4
1052/00	62.0	-29.6	34.5	14.3	19.7	54.2	2
1054/00	13.6	-5.0	39.1	5.5	37.1	82.7	1
1055/00	61.5	0.2	32.1	1.7	53.7	79.2	3
1056/00	73.8	0.3	53.1	21.4	50.1	73.3	4
1057/00	41.3	49.1	60.4	29.4	56.8	70.0	3
1060/00	41.9	45.1	49.7	31.2	45.9	65.8	1

1061/00	65.2	55.5	46.0	31.9	52.8	75.8	4
1062/00	46.3	60.5	10.2	0.3	41.2	60.2	2
1063/00	27.8	32.4	39.1	15.2	43.5	72.2	1
1064/00	24.6	37.9	31.8	12.6	34.1	48.2	0
1072/00	61.9	36.3	39.2	21.8	28.8	60.2	2
1074/00	61.3	37.4	41.0	34.1	37.2	76.0	2
1089/00	28.4	23.9	23.6	26.4	44.9	60.5	1
1112/00	70.9	35.9	63.5	38.9	50.3	69.8	4
1128/00	81.7	70.3	48.1	39.0	65.9	81.9	4

This table shows mean percentage reduction in infectivity (or percentage neutralisation) of HCVpp of the relevant genotype on Huh7 cells in the presence of 100µg/ml of purified IgG as described in Materials and Methods. Values are averaged over 3 experiments with samples tested in duplicate. Infectivity was determined using the signal from a BrightGlo Luciferase assay of infected Huh7 cell lysate. The percentage reduction in infectivity is taken as the percentage reduction in RLU signal in test wells compared to control wells where no IgG was added. Individuals were classed as 'neutralising' an HCVpp genotype if the reduction in infectivity was 50% or greater. Negative values indicate infectivity was enhanced by the test IgG.

Association of clinical parameters with neutralisation group was determined using Fisher's exact test for categorical variables and Wilcoxon Rank Sum testing for ordinal data. There were no significant associations of breadth of cross-genotypic neutralisation with viral load or other clinical factors (Table 3.6). However, a trend to association of broader inter-genotypic neutralisation with anti-hepatitis B virus (HBV) core (anti-HBc) antibody status was seen (P=0.07). This may reflect a cohort who participated in higher risk injecting behaviour experiencing both exposure to HBV and also a wider range of HCV viral strains. It is also interesting to note that clearance of HCV in chronically infected patients has been reported in those exposed to HBV suggesting this may stimulate an immune response against HCV(265, 266) .

Table 3.6 Associations of cross-genotypic neutralisation breadth with demographics and clinical parameters.

	XG panel		
	Broad (n=20)	**Narrow (n=31)**	**P value**
Age Median (Range)	47 (35-66)	45 (28-68)	0.51
Gender (M/F) No. male (%)	13 (65)	22 (71)	1.00
Ethnicity Caucasian (%)	18 (90)	29 (93)	1.00
IVDU (%)	14 (70)	18 (58)	0.55
Duration of infection Median years (range)	27.5 (12-55)	18 (2-58)	0.38
IL28B CC genotype (%)	6 (30)	11 (35)	0.77
anti-HBc positive (%)	7 (35)	4 (13)	0.07
BMI mean kg/m^2	25.5 (19.8-31.5)	25.6 (19.4 -30.3)	0.47
Diabetes (%)	0 (0)	2 (6)	0.51
Previously treated (%)	6 (30)	15 (48)	0.39
HCV RNA pre-treatment IU/ml Median (Range)	966647 (7687-5000000)	615833 (2272 -11300000)	0.76
Cirrhosis Present (%)	7 (35)	12 (39)	1.00
Fibroscan kPa Median (Range)	9.0 (3.5-45)	7.3 (4-75)	0.72
Genotype 1 (%)	12 (60)	15 (48)	0.26

Statistical comparisons were made using non parametric tests (Fisher's exact test for categorical data, Wilcoxon Rank Sum for ordinal or numeric data).

3.6 Intra- genotype 1 (IG1) panel neutralisation breadth is associated with degree of liver fibrosis, age and body mass index (BMI) but not with viral load.

Similarly, IgG from 20 CHCV individuals with gt 1 infection were tested for neutralising activity against the intragenotype panel (IG1) and the number of pseudoparticles neutralised to the 50% level by 100µg/ml purified IgG from each individual calculated (Table 3.7). Neutralisation of >7/11 strains was defined as ‘broad’ while ‘narrow’ neutralisation was defined as <8/11 strains.

Analysis of the demographics of broad and narrow neutralising individuals in this subgroup was conducted as above (Table 3.8). Interestingly, this revealed an association between breadth of neutralisation and age, with the broadly neutralising group being significantly younger (*P*=0.024; Table 3.8). In addition, for this subgroup of gt 1-infected individuals there was a striking association between breadth of neutralisation activity and liver fibrosis. The broadly neutralising group had lower levels of liver fibrosis as determined by transient elastography (*P*=0.009; Table 3.8, Fig. 3.5a) and significantly fewer cirrhotic individuals (*P*=0.02; Fig. 3.5b) than the narrowly neutralising group. To determine if age was a confounding factor in the link between neutralisation breadth and fibrosis I analysed the impact of both variables together using the Generalised Linear Model (SPSS V.19.0). This showed the association between neutralisation breadth and Fibroscan readings remained significant when corrected for age (*P*=0.025, Generalised Linear Model, SPSS V. 19.0).

There is also an association of broader neutralisation and lower BMI which just reaches statistical significance (Table 3.8, Fig. 3.6a *P*=0.048). This may simply reflect the effect of confounding , although there was no correlation between BMI and Fibroscan readings in this cohort (Fig. 3.6b Pearson's r=0.07, *P*=0.77) an association between obesity and cirrhosis in larger populations is well documented (222). There is some evidence that obesity can impact on the humoral response in influenza, therefore it is an intriguing possibility that obesity may also play a role in breadth of antibody mediated neutralisation in HCV (267). As with Panel XG, there was no association between breadth of neutralisation of Panel IG1 and viral load.

Table 3.7 Percentage neutralisation of individual HCVpps in intra-genotype 1 panel by purified IgG from individual patients.

Anonymous code	1012-02	1a	1b	UKN96	1034-11	1037-04	1013-01	ET10	1A v 93	UKN181	1Av220	Intragenotypic breadth
1001/00	29.3	69.3	14.1	60.6	52.4	53.4	44.6	72.9	72.0	80.5	10.6	7
1002/00	-17.1	0.2	-13.2	-22.0	-7.6	1.6	-24.1	-13.1	-13.1	0.1	-8.8	0
1003/00	57.2	88.3	72.8	64.1	53.1	84.5	54.5	90.2	54.4	88.6	25.4	10
1010/00	43.7	78.1	31.0	55.8	54.8	76.6	41.4	69.5	46.1	87.0	17.7	6
1012/00	53.6	79.6	34.4	57.9	66.6	84.4	49.5	78.8	75.7	87.1	23.7	8
1013/00	61.2	86.1	17.2	72.2	89.0	80.6	58.3	88.0	66.6	80.9	37.2	9
1016/00	50.2	87.0	55.1	49.3	63.5	75.3	40.5	88.7	66.2	94.0	44.0	8
1022/00	54.7	76.6	39.3	46.4	55.1	65.7	53.1	75.6	48.9	64.7	24.1	7
1023/00	73.5	84.4	35.1	68.5	75.9	70.9	64.9	76.8	56.1	72.1	17.2	9
1030/00	23.1	57.6	45.2	20.1	21.3	47.9	20.4	59.6	27.3	62.9	16.4	3
1031/00	46.2	77.1	37.0	55.5	72.3	72.2	64.2	67.8	55.7	70.1	27.6	8
1032/00	57.2	86.1	43.8	81.7	89.2	73.7	69.5	74.4	80.6	89.7	27.6	9
1034/00	31.9	47.2	24.5	24.5	21.6	53.4	3.9	41.7	24.0	50.3	18.1	2
1035/00	66.6	78.3	42.9	61.9	70.9	67.2	64.6	82.5	61.0	76.0	44.7	9
1036/00	50.7	79.5	41.0	50.0	55.4	69.1	43.8	69.5	41.6	57.5	28.3	7
1037/00	44.6	94.1	27.6	34.3	79.2	62.1	41.5	75.0	53.7	76.8	16.2	6
1045/00	33.1	70.0	12.4	28.2	52.8	47.9	29.6	49.5	29.1	69.6	0.2	3
1047/00	51.5	80.2	35.8	57.8	50.7	62.8	35.4	76.8	56.0	83.3	3.6	8
1072/00	42.0	81.6	42.8	44.3	68.2	59.1	25.6	65.7	39.5	61.3	22.8	5
1112/00	71.3	78.6	54.2	71.3	78.7	75.0	56.7	81.6	52.3	73.2	25.7	10

HCVpp neutralisation was conducted as described above and in the Materials and Methods section. Briefly, 40μl of each HCVpp were incubated with purified IgG at 100μg/ml from patient serum before adding to pre-seeded Huh7 cells in a 96 well plate. After 72 hours infectivity was determined by measuring the luciferase activity in infected cells. Relative infectivity compared to controls where medium only had been added was determined and percentage neutralisation was calculated. The colouring of the boxes above indicates degree of neutralisation from red - <25% reduction in infectivity to dark green >70% neutralisation. In the number neutralised column, red shading indicates <8 HCVpp strains neutralised (i.e. narrow) and green indicates >7 strains (i.e. broad).

Table 3.8 Associations of inter-genotypic neutralisation breadth with demographics and clinical parameters.

	Intra-genotypic panel (IG1)		
	Broad (n=10)	**Narrow (n=10)**	**P value BvN**
Age Median (Range)	41.5 (38-66)	51.1(44-65)	**0.024**
Gender (M/F) No. male (%)	6 (60)	6 (60)0)	1.0
Ethnicity Caucasian (%)	10 (100)	10 (100)	1.0
IVDU (%)	8 (80)	6 (60)	0.63
Duration of infection Median years (range)	15.5 (12-37)	24 (5-37)	0.70
Estimated Age at infection	23.5 (4-45)	24.5 (11-50)	0.89
IL28B CC genotype (%)	3 (30)	2 (20)	1.0
anti-HBc positive (%)	2 (20)	1 (10)	1.0
BMI mean kg/m^2	24.2 (21.0-28.5)	26.6 (21.2-29.7)	**0.048**
Diabetes (%)	0 (0)	1 (10)	1.0
Previously treated (%)	3 (30)	6 (60)	0.20
HCV RNA pre-treatment IU/ml Median (Range)	218838 (2272-2359543)	498520 (88676-11300000)	0.13
Cirrhosis Present (%)	1 (9)	7(70)	**0.02**
Transient elastography kPa Median (Range)	5.4 (4.1-14)	21.1 (3.5-70)	**0.009**
Genotype 1 (%)	10 (100)	10 (100)	1.0

Statistical comparisons were made using non parametric tests (Fisher's exact test for categorical data, Wilcoxon Rank Sum for ordinal or numeric data). Significant P values are shown in bold.

A

P=0.009

Transient Elastography (kPa)

80
60
40
20
0
-20

IG1 Broad
IG1 Narrow

B

P=0.02

No. IG1 panel HCVpp neutralized >50%

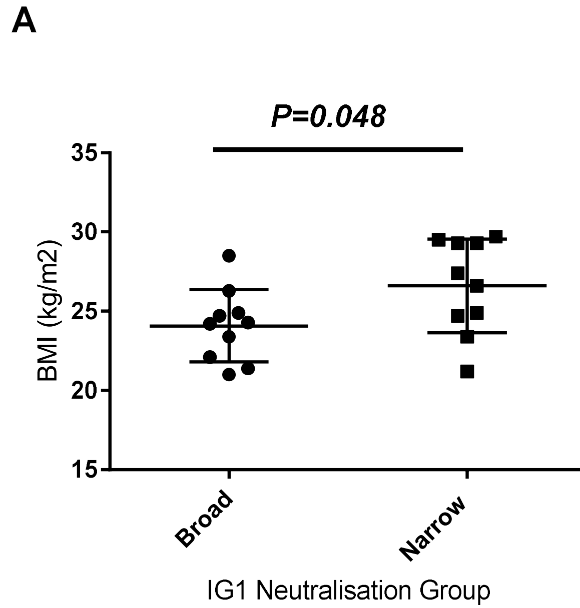
15
10
5
0

No cirrhosis
Cirrhosis

Figure 3.5 Association of intra-genotype 1 neutralisation breadth and liver fibrosis.

A) Fibrosis as quantified by Transient Elastography using Fibroscan ® was measured for each individual at the time of sampling. Individuals were divided into broad and narrow neutralising groups according to their ability to neutralise the IG1 panel as described above and Fibroscan values for individuals in each group compared using the Wilcoxon Rank Sum test. B) Those individuals tested in the IG1 panels were categorised as having cirrhosis or no cirrhosis on the basis of Fibroscan, clinical and biopsy data. The number of HCVpp neutralised to 50% in the IG1 panel by the individuals in each of these groups were compared using the Wilcoxon Rank Sum test. Mean and SD are shown.

A



B

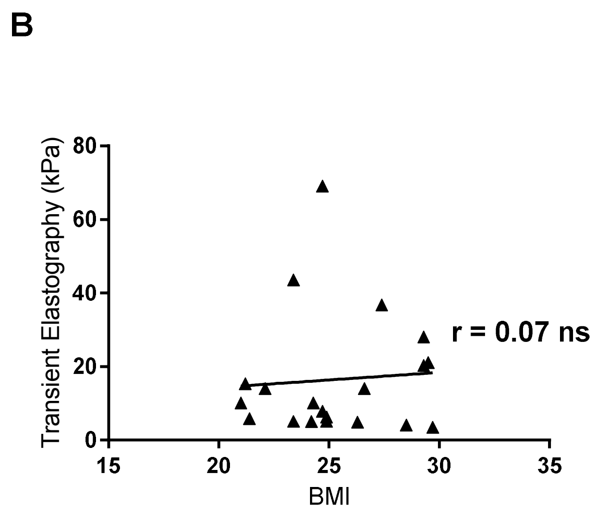


Figure 3.6 Association of BMI with A) IG1 breadth of binding and B) Fibroscan ® measurement of liver fibrosis

A) The BMI of CHCV cohort individuals was assessed in clinic at the time of sampling. This information was compared for individuals with IG1 broad and narrow neutralising profiles and compared using a Wilcoxon Rank Sum test. Mean and SD are shown. B) The Fibroscan readings for individuals used in the IG1 cohort was plotted against their BMI and Pearson's r coefficient of correlation calculated where a value of 1 indicates a perfect correlation.

3.7 Neutralisation of HCVcc and clinical parameters

While HCVpps are a good model for viral entry and generally have a good correlation with neutralisation experiments using live virus (HCVcc) bearing similar sequences , experimental results obtained with HCVpp do not always give identical results to HCVcc(154).

Therefore I aimed to test the ability of the CHCV cohort to neutralise 3 different HCVcc bearing envelope sequences for gt1a (GT1HQL), gt 2a (JFH-1 sequence) and Gt 4 (Ad4a). These experiments were performed by Dr Vanessa Cowton and Mrs Sarah Cole as they are required to be carried out in a Category 3 facility, however the analysis of results is my own.

Experiments were performed as described in Materials and Methods, briefly infectious virus was produced in Huh7 cells by electroporation of RNA encoding full-length JFH-1 or chimeric JFH-1. The neutralization of cell culture infectious HCV (HCVcc) infection was tested in Huh7-J20 cells, and infectivity was assessed at 3 days post-infection using a Phospha-Light assay system (Thermo Fisher) to measure secretory alkaline phosphatase (SEAP) levels.

Percentage neutralisation for each of the CHCV individuals is shown in Table 3.9. Only 13/51 subjects were able to neutralise one or more of the HCVcc at the 50% level. Eight of these 13 were broad neutralisers in the XG panel and 5/6 of those which had been tested in the IG1 panel were broad neutralisers.

Cohort demographics were analysed according to whether their IgG could neutralise any of the HCVcc panel at the 50% level. There were no significant differences in clinical parameters between the groups (Table 3.10). This may simply be a reflection of the small number of individuals in the neutralising group but it again suggests that cross genotypic neutralising ability is perhaps less important in chronically infected individuals than a broad intragenotypic neutralising response.

Table 3.10 Clinical characteristics of individuals by HCVcc neutralising group

	HCVcc Non-Neutralising	HCVcc Neutralising	P value
Age Median (range)	46 (28-68)	47 (37-66)	0.43
Gender No. male (%)	28 (73)	7 (54)	0.30
Ethnicity Caucasian (%)	35 (92)	12 (92)	1.00
Source of infection No. IVDU (%)	25 (66)	7 (54)	0.51
Estimated duration of infection (years) Median (range)	22 (2-51)	28 (5-55)	0.76
IL28B CC genotype (%)	11 (29)	6 (46)	0.31
Anti-HBc positive (%)	7	4	0.74
BMI kg/m^2 Mean (range)	25.7 (19.4-31.0)	25 (21.0-31.0)	0.58
Diabetes Present (%)	2 (5)	0 (0)	1.0
Previously treated (%)	14 (37)	7 (54)	0.34
HCV RNA load pre-treatment IU/ml Median (Range)	1884564 (8670-11300000)	892486 (2272-3010000)	0.13
Cirrhosis Present (%)	15 (39)	4 (31)	0.50
Transient elastography (kPa) Median (Range)	9.5 (4-69.1)	9.0 (4-28)	0.70
Genotype 1 (%)	18 (47)	9 (69)	0.31
Genotype 3 (%)	20 (53)	4 (31)	0.31

Individuals were classified as HCVcc neutralising if their IgG neutralised any of the HCVcc at the 50% level. P values were calculated using Fisher's exact test for categorical variables and Wilcoxon Rank Sum for ordinal variables

Table 3.9 CHCV individuals purified IgG ability to neutralise HCVcc virus

Patient sera	GT1 HQL	GT2a JFH1	Gt4 Ad4a	Number of cc neutralised
1001	32.5	28.7	2.6	0
1002	9.8	6.6	-11.7	0
1003	54.7	51.7	48.8	2
1006	26.5	16.8	30.2	0
1008	29.9	21.4	4.3	0
1009	24.5	28.6	18.4	0
1010	36.5	20.9	17.0	0
1012	44.0	40.0	6.1	0
1013	51.6	24.4	12.7	1
1015	37.9	47.5	7.4	0
1016	42.1	46.0	36.1	0
1018	31.3	37.7	20.2	0
1020	26.6	37.2	-42.7	0
1021	44.5	64.2	6.9	1
1022	35.6	40.5	10.5	0
1023	38.6	37.3	16.1	0
1024	35.2	42.1	2.5	0
1029	40.9	36.1	-4.4	0
1030	24.5	36.6	-17.7	0
1031	40.1	19.3	19.8	0
1032	41.8	54.2	15.2	1
1033	25.7	28.6	18.0	0
1034	30.8	27.1	12.2	0
1035	35.1	25.5	19.0	0
1036	36.0	17.0	-12.5	0
1037	34.0	19.7	9.6	0
1038	38.7	53.4	-9.8	1
1040	30.8	39.8	4.8	0
1041	33.3	39.9	-8.8	0
1042	67.0	65.2	20.0	2
1043	48.6	35.0	21.6	0
1045	51.5	47.5	-2.6	1
1046	39.9	61.8	25.1	1
1047	46.3	57.0	14.4	1
1049	42.0	48.8	26.1	0
1050	59.6	60.3	6.8	2
1052	34.5	35.8	-52.6	0
1054	41.6	38.8	-1.8	0
1055	33.8	43.0	-0.8	0
1056	45.4	54.2	-3.9	1
1057	36.4	44.4	-20.0	0
1060	43.1	39.6	3.0	0
1061	52.1	50.5	-15.2	2
1062	39.4	21.4	-21.7	0
1063	22.1	38.1	-8.7	0
1064	16.6	30.8	3.1	0
1072	37.2	34.1	23.9	0
1074	42.5	38.0	7.7	0
1089	38.4	30.9	15.6	0
1112	50.0	36.4	19.9	1
1128	32.6	39.2	-1.1	0

Percentage reduction in infectivity of HCVcc is shown when incubated with sample IgG as detailed in Materials and Methods compared to HCVcc infectivity without sample IgG. Red shading indicates low or no neutralisation. Blue shading indicates higher levels of neutralisation. The number of cc neutralised column indicates how many of the 3 HCVcc strains had infectivity reduced by 50% or greater by sample IgG.

3.8 Correlation between ELISA binding profiles and neutralisation

To determine the level of agreement between individual ELISA binding profiles and breadth of neutralisation activity, non-parametric correlation co-efficients (Spearman's rho) between the assays were calculated. For strength of ELISA binding to individual genotypes and ability to neutralise the same E1E2 sequences in the HCVpp system, there was a modest correlation for 4 of the subgenotypes but not for either of the gt 2 E1E2 sequences (Fig. 3.7 a-f). Outliers are also visible in all genotypes which have similar levels of HCV glycoprotein binding to neutralising samples, but do not neutralise in the HCVpp system.

The correlation between ELISA binding rank and the number of strains neutralised in the various panels was also calculated (Fig. 3.8). In those 20 gt 1-infected individuals who were tested against both XG and IG1HCVpp panels, there was a significant correlation between the number of strains neutralised in the two panels (Spearman's Rho correlation co-efficient =0.56 P=0.008, Fig. 3.8a). Overall for the full cohort there was a modest correlation of cross-genotypic ELISA binding rank with number of genotypes neutralised in Panel XG and a stronger correlation with Panel IG1

(Spearman's Rho correlation co-efficient -0.31, *P*=0.03 and -0.56, *P*=0.008, respectively) (Figs. 3.8b, 3.8c). The modest correlation between ELISA binding and neutralisation highlights that while an individual with strong HCV-binding antibodies tends to have potent neutralising antibodies this is not always the case. I also compared the number of HCVpps neutralised in the XG panel with the number of HCVcc neutralised, this again revealed a modest correlation (Fig. 3.8d Spearman's Rho correlation co-efficient 0.35, *P*=0.012) which goes some way to confirm that the HCVpp assay has relevance to live virus.

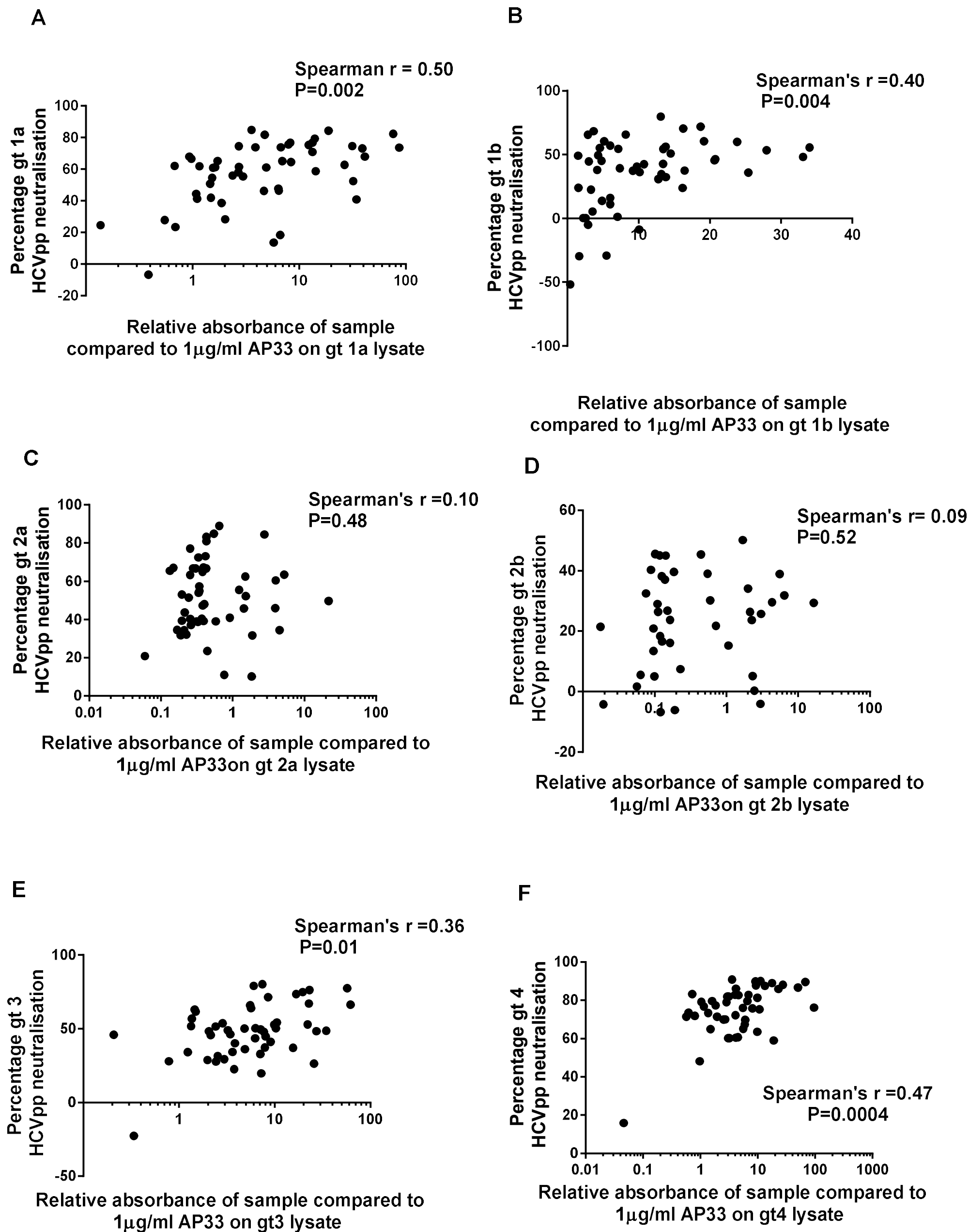


Figure 3.7 Non-parametric correlation of individual samples' absorbance on E1E2 by ELISA and percentage neutralisation of HCVpp for each subgenotype tested.

ELISA assays and HCVpp neutralisation assays were carried out with purified patient IgG out as previously described. For each subgenotype (A-1a,B -1b, C -2a, D-2b, E- 3 and F-4) individual samples' percentage neutralisation of HCVpp was plotted against relative absorbance compared to 1µg/ml AP33 calculated using a standard concentration curve. Spearman's r co-efficient and corresponding *P* value were calculated using GraphPad Prism 6.0.

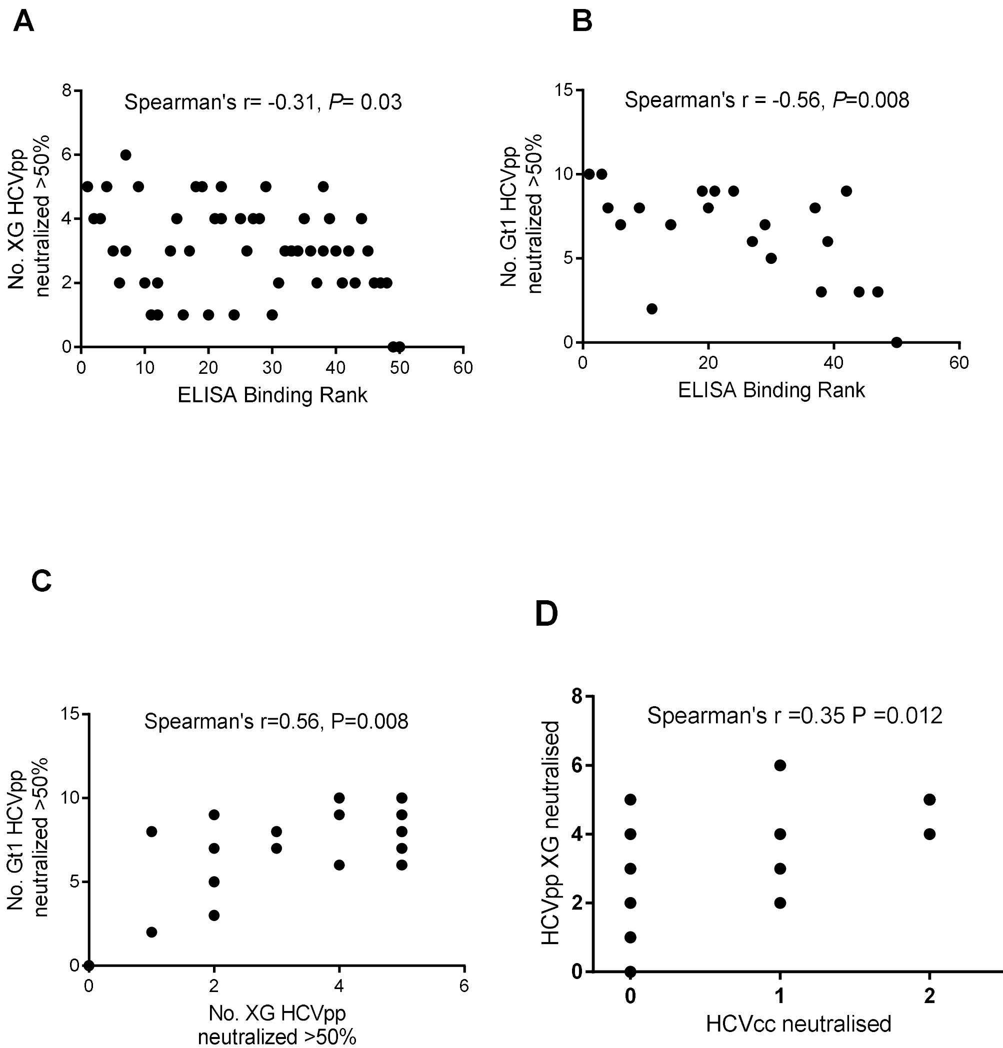


Figure 3.8 Non parametric correlations between neutralisation in the different panels and ELISA binding rank.

Nonparametric correlation between E1E2 ELISA binding and neutralization. (A, B). The number of HCVpp neutralized was plotted against the ELISA binding rank (calculated as described previously) for panel XG (A) and panel Gt1 (B). (C) For those gt1-infected individuals whose antibodies were tested for reactivity against HCVpp in both panels, the number of HCVpp isolates in panel XG neutralized was plotted against the number of HCVpp isolates in panel Gt1 neutralized at the 50% level. (D) The neutralization activity of antibodies from the full CHCV cohort against HCVpp in panel XG and the HCVcc viruses (1A-HQL, JFH-1, 2B1.1/JFH1) was analysed, and the number of strains neutralised to the 50% level plotted for each individual. Spearman's rho correlation coefficient and corresponding *P* value was calculated for all graphs.

3.9 Role of HLA-DQB1 polymorphisms in predicting antibody neutralisation breadth

Two SNPs (designated rs9275224 and rs2395522) in an MHC Class II gene, HLA DQ have recently been shown to have an association with breadth of antibody response in acute HCV infection and acute viral clearance. This region is involved in antigen presentation (133). Therefore I analysed our cohort for any association between this and the number of HCVpp neutralised in both panels.

There was no significant association between presence of these alleles and the number of HCVpp neutralised in Panel XG (*P*=0.41; Fig. 3.9a). However, the presence of the rs9275224 GG or AG allele was significantly associated with a greater number of HCVpp neutralised in Panel IG1 (*P*=0.038; Fig. 3.9b). There was no association between SNP rs9275224 and liver fibrosis *P*=0.46; Figs. 3.10). In addition, I tested another genetically linked HLA-DQ SNP rs2395522. The SNP typing in the CHCV cohort showed 100% linkage of subtype with the rs9275224 allele (i.e. all rs9275224 GG individuals had the rs2395522 AA type, AG had AT and AA had TT respectively). As expected the AA or AT allele was also significantly associated with increased neutralisation breadth in panel IG1 (*P*=0.038, data not shown as the graphs are identical due to the genetic linkage between the SNPs). These SNPs are present in the intergenic region of HLA-DQ; their functional consequences are unknown, however, they are associated with autoimmune diseases including systemic lupus erythematosus, systemic sclerosis and rheumatoid arthritis (268, 269). They have also been identified as being associated with spontaneous HCV clearance in GWAS studies (87). Interestingly, while only

20% of the European population carry the unfavourable alleles (NCBI Genbank data https://www.ncbi.nlm.nih.gov/projects/SNP/snp_viewTable.cgi?pop=1409 , accessed 11/04/2017), 40% of our CHCV population had the rs9275224 AA genotype adding further weight to the hypothesis that these genes are involved in acute viral clearance or resistance.

A

P=0.41
No. XG HCVpp neutralised >50%
0
2
4
6
8
GG/AG
AA

B

P=0.038
No. IG1 HCVpp neutralised >50%
0
5
10
15
GG/AG
AA

Legend for Figure 3.9 on following page

Figure 3.9 Association between HLA DQ SNP rs9275224 and number of genotypes neutralised in the (A) XG panel and (B) IG1 panel.

Number of genotypes neutralised in the XG and IG1 panels were determined as described previously. SNP typing for each individual in the CHCV cohort was conducted using the TaqMan ® SNP Genotyping Assay (Life Technologies) assay as described in Materials and methods. For individuals in the favourable (GG/AG) and unfavourable (AA) genotypes number of HCVpps neutralised at the 50% level in each panel was plotted. P values were calculated using the Wilcoxon Rank Sum test. Median and interquartile range are shown.

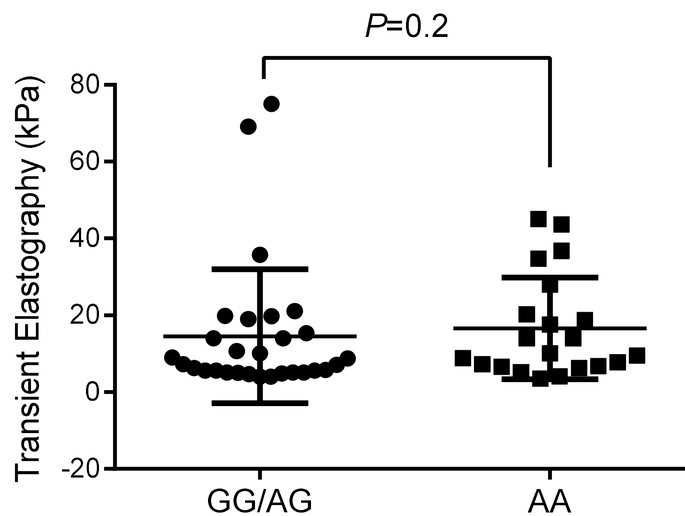


Figure 3.10 Comparison of and levels of fibrosis as measured by transient elastography by Fibroscan ® across the two groups HLA DQ SNP rs9275224 genotypes.

Number of genotypes neutralised in the XG and IG1 panels were determined as described previously. SNP typing for each individual in the CHCV cohort was conducted using the TaqMan ® SNP Genotyping Assay (Life Technologies) assay as described in Materials and Methods. For individuals in the favourable (GG/AG) and unfavourable (AA) genotype groups, liver stiffness by transient elastography measured using a Fibroscan ® machine (Echosens) was plotted. *P* values were calculated using the Wilcoxon Rank Sum test. Mean and SD are shown.

3.10 Chapter 3 Discussion

3.10.1 Cross genotypic glycoprotein binding by ELISA and viral load

This analysis of a clinical cohort chronically infected with HCV has yielded several new insights into host factors associated with functional breadth of the antibody response. A broad cross-genotype HCV-binding antibody response as measured by ELISA was significantly associated with reduced viral load while no association was evident between viral load and breadth of neutralisation activity. This suggests distinct biological roles for non-neutralising and neutralising anti-HCV antibodies. The link seen between broader ELISA binding and lower viral loads could reflect the presence of antibodies binding to conserved non-neutralising regions which may promote opsonisation and phagocytosis, thus helping clear bound virus from serum but not effectively preventing hepatocyte infection. Conversely, viral loads may be lowered artefactually in some individuals with cryoglobulinaemia which may confound this association.

3.10.2 Association of neutralisation breadth with clinical features

Although no clinical features were significantly associated with cross-genotype neutralisation of HCVpp or HCVcc, significant associations were observed using a larger intra-genotype 1 panel. Most importantly, it has shown that individuals infected with HCV gt 1 who are better able to neutralise an HCVpp panel incorporating different gt 1 E1E2 sequences are less likely to have cirrhosis or significant liver fibrosis. As this panel encompasses the majority of variable aa positions observed

within gt 1 envelope sequences it is likely to represent common aa substitutions that may occur within host virus in attempting to escape the adaptive immune response.

This association is not sufficient evidence to conclude that a bNAb profile protects from liver fibrosis. However, it corresponds with case studies documenting more rapid progression of liver injury and fibrosis in individuals with genetic or iatrogenic antibody suppression (270, 271). It is biologically plausible that those individuals with a diverse array of antibodies are better able to prevent spread of new HCV variants within the liver, and therefore could have slower progression of disease. Of note, the ‘broadly neutralising’ group was also significantly younger with lower BMIs. There is already evidence that those infected with HCV in older age have more rapid disease progression (212) and the cellular aging process is linked to progression of liver disease with biomarkers of ageing shown to have an association with liver disease progression in HCV infection(272, 273). From an immune perspective the breadth of the B cell repertoire narrows with age (274). In addition, those with a higher BMI have been found to have shorter telomere length (a marker of ageing) within immune cells(275). Therefore a narrower breadth of neutralisation may reflect an ageing immune system in this population.

It is also important to consider other possible confounding factors. If older patients were infected with an earlier strain of HCV virus, their antibody response may be more adapted to this older virus, whereas the viral sequences used in the IG1 panel were mainly derived from samples collected in the past decade. As HCV is constantly evolving in response to immune pressures placed on it by different hosts, these sequences may contain escape mutations from common responses to older

version of virus. This process of immune evasion has been documented within individuals by Dowd *et al*. (137).

It is not clear why these associations were not observed from testing for antibody breadth against panel XG. It may be these associations are gt 1-specific or simply caused by limitations due to smaller numbers of genotypes used to determine 'breadth' in the cross-genotypic panel. It is also important to remember that genotype is not a good predictor of ease or difficulty of neutralisation (276). Some individuals who had narrow XG neutralisation profiles showed broad neutralisation activity against the IG1 panel. As the XG panel was selected solely based on genotype it may not have provided an adequate array of variation at key epitopes (e.g. aa 412 to 445, which contains key binding regions for monoclonal bNAbs such as HC-11, see Fig. 3.11) to identify individuals with a broad antibody response. Conversely, it could be that some bNAbs may be gt1 specific.

No association between levels of E1E2 binding and fibrosis was found. An earlier study showed an association between absorbance values against gt 1 envelope and fibrosis levels in chronically infected individuals, (277). I did not find any relationship between antibody binding to the autologous genotype and clinical outcomes although there was a trend towards cirrhosis in gt 1-infected individuals with poor binding to gt 1a E1E2 (P=0.10, Fig. 3.12). In light of the significant association between breadth of neutralisation and lower levels of fibrosis, this suggests that if anti-envelope antibodies do have a protective effect, this is most marked where the antibodies target regions necessary for virus entry.

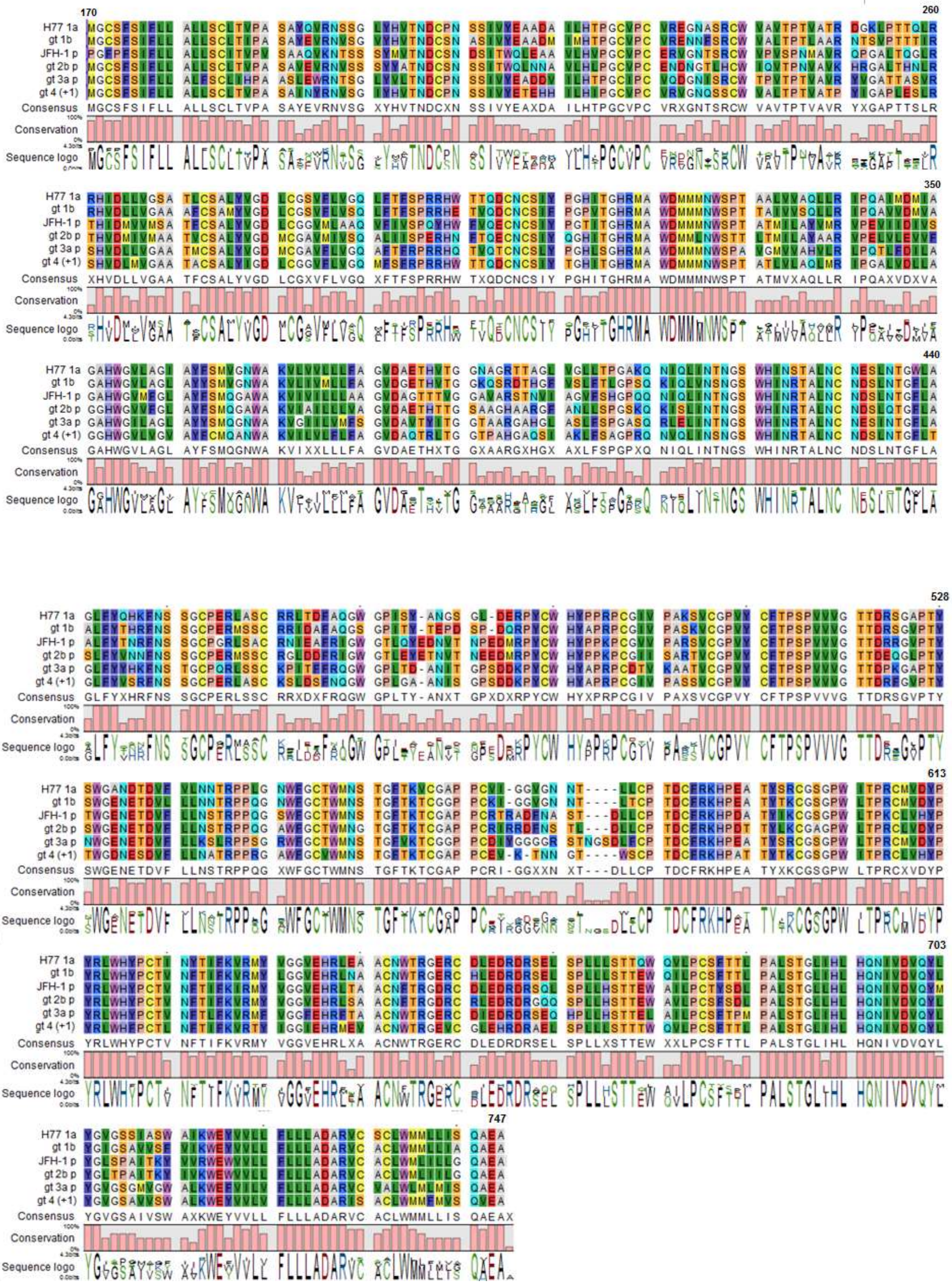


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Figure 3.11 Alignment of E1E2 protein sequences from the XG panel HCVpps

Alignments of protein sequences for the E1E2 glycoproteins expressed on the HCVpps used in the XG panel were generated in Genomics Workbench 7.0. Sequences are labelled by genotype except H77 (gt 1a) and JFH-1 (gt 2a). Percentage conservation at each aa is indicated below the consensus sequence. Amino acid numbering relates to the H77 sequence.

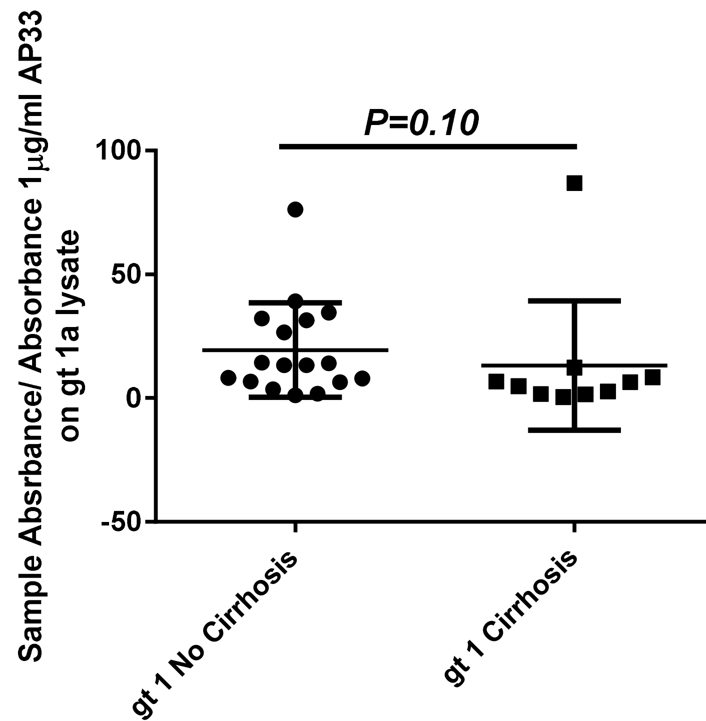

Figure 3.12 Absorbance of IgG from gt 1 infected CHCV patients and presence of cirrhosis.

Absorbance of purified IgG from individuals infected with gt 1 CHCV on gt 1a E1E2 was determined by ELISA as previously described. A standard curve of AP33 was used and the ratio of sample absorbance to absorbance of 1µg AP33 calculated. Individuals were categorised as having cirrhosis or not and the absorbance values between the groups compared using a Wilcoxon Rank Sum test. Mean and SD is shown.

3.10.3 HLA genotype and bNAb responses

While there are many possible explanations why individuals may mount a bNAb response, the SNP typing assays confirmed that SNP rs9275224 in the HLA-DQ gene is associated with the development of bNAbs in gt 1-infected patients (133). Genes in this region have already been identified in genome wide association studies as one gene which seems to influence the outcome of HCV infection (278). Given that such genes are involved in antigen presentation it is plausible this may reflect restriction in CD4 presentation of epitopes to B cells. In contrast, no association with this SNP was observed in the ability of IgG from gt 3-infected individuals to neutralise the standard gt 3a HCVpp (*P*=0.85, Fig. 3.13). This may be due to the limitations of small sample size and testing against one gt 3, alternatively it is possible that other HLA genes could be more important in humoral responses to infections of different genotypes. In support of this, recent research by the STOP HCV consortium, examining associations between amino acid polymorphisms and HLA type in gt 3 infected individuals, identified an association between 4 amino acid changes in E1E2 and HLA subtypes (aa 348I with B*13:02, 372I with C*08:02, 444Y with A*29:02 and 561L with C*15:02)(279). Similar research into viral characteristics and host HLA and neutralisation profiles would be useful in further determining the extent to which infecting viral strain and host genotype influence the development of bNAbs.

As gt 3 developed predominantly in the Asian subcontinent it is interesting to note that the unfavourable alleles explored in my study are less common in non-European, non-African populations (39% vs 47% in European populations(87)). Therefore gt 3 virus may have evolved to overcome the advantage that the

favourable alleles confer. Further studies will be required to distinguish between these possibilities.

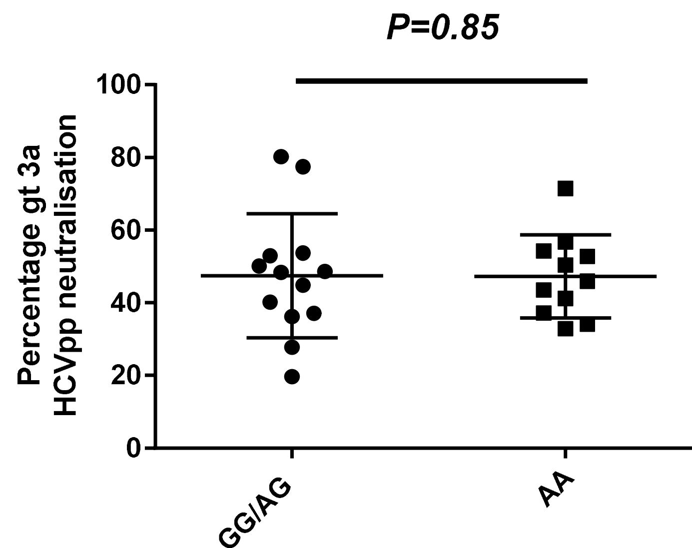

Figure 3.13 Percentage neutralisation of gt 3a HCVpps in individuals infected with gt 3 HCV analysed by rs9275224 genotype.

Neutralisation assays of gt 3a HCVpps were conducted as described previously as was rs9275224 genotyping. Percentage neutralisation of gt 3a HCVpp in individuals infected with gt 3 HCV was plotted against their rs9275224 genotype. The P value was calculated using a Wilcoxon Rank Sum test. Mean and SD is shown.

3.11 Chapter 3 Summary

- Breadth of antibody binding to the whole E1E2 protein is associated with lower HCV viral load

- Broad anti-HCV neutralising responses are associated with lower levels of liver fibrosis, raising the possibility for a protective role in chronic infection.

- Older and more obese patients are less likely to display bNAb responses, the reasons for this are unclear.

- Host HLA DQ genotype appears to play a factor in ability to produce bNAbs against gt1 HCV. Different host polymorphisms may be involved in producing antibodies targeted against different genotypes.

4 Investigation of neutralising antibody epitope targets and neutralisation-resistance mutations in chronically infected individuals

[Some of the work described in this chapter has been published in Swann *et al., J. Virol, 2016(251)* - the American Society of Microbiology copyright licence allows authors to reproduce their work as part of academic theses]

4.1 Introduction

Due to the error prone HCV NS5B RNA-dependent RNA polymerase, mutations in HCV sequences occur commonly with a large percentage of sequence variation between, and even within, genotypes(15). Selection of the variants produced within a host depends upon the fitness of the virus to infect and replicate and also on immune pressure from the host's innate and adaptive immune systems(280).

Only a small number of chronically infected individuals with bNAbs have been studied in detail with little information on the regions of the E1E2 glycoproteins that are preferentially targeted by the in vivo humoral response. A recent study demonstrated that some HCV infected individuals largely target one immunogenic domain whereas others produce antibody responses to multiple domains (118). The impact of antibodies binding different epitopes on neutralisation breadth has not been fully explored, although there are conflicting reports of simultaneous binding of antibodies targeting more than one region conferring additive or interfering effects on virus neutralisation (84, 281) . In addition, the underlying mechanisms by which some host and viral characteristics result in an effective neutralising immune response whereas others do not has not been fully defined.

Many examples of envelope amino acid substitutions which alter the entry characteristics of HCV and susceptibility to neutralisation by various monoclonal antibodies have been identified (see Table 4.1). Mutational escape from neutralising antibody responses, observed both *in vitro* and *in vivo*, are well-documented, with chronically infected individuals often possessing antibodies able to neutralise an earlier isolate of virus but not their current dominant strain (137). Viral diversification after acute infection has been associated with the likelihood of progression to chronic viral infection, perhaps because this gives the virus the best chance of escaping initial adaptive immune responses (282). Interestingly, several mutations that confer escape from broadly neutralising antibodies also appear to affect infectivity or use of certain entry proteins in functional testing (see Table 4.1), presumably because effective neutralising antibodies tend to target areas of the virus that are critical for cell entry. The mutations documented in Table 4.1 are in addition to information from alanine scanning data of antibody binding sites detailed in Table 1.2.

Table 4.1: Mutations detected in vivo or generated in vitro affecting cell entry or promoting escape from monoclonal antibodies

Glycoprotein interactions affected by mutation	Amino acid (aa) position change	Effect	Ref
SRBI/CD81	I347L	Rescues infectivity in Δ HVR-1 virus	(283)
SRBI	L399R	Key for interaction with SRBI	(284)
SRBI/CD81	Y507L,V514A,V515A	Modulate dependence on SRBI	(285)
SRBI	I262L, N415D	Restore infectivity in Δ HVR-1 virus	(286)
SRBI/CD81	G451R	Reduces dependency on SRBI/ enhances neutralisation sensitivity	(29)
E1/E2 interaction	M308I, T330A, L345M	Mutations restore infectivity and interact with aa 580-651 of E2 to enable Claudin-1 binding.	(41)
CD81	L438F	Reduces CD81 binding	(121)
Cell entry	F447 S458G, R478C	Enhanced infectivity	(287)
CD81	529-535	Core CD81 binding region - does not tolerate escape mutations	(121)
CBH-2	D431G, A439E	Escape from neutralisation	(121)
HC-11	N434D, T435A and L438F	Escape from neutralisation (NB reduction in infectivity)	(121)
AP33	L413A, N415A, G418A, W420A	Reduces binding of AP33 to E2	(146)
AP33, HC-1	N417S	Allows escape from neutralisation by AP33 and HC1 due to alteration in the glycan shield	(145)

AP33, CBH7, HC-1, HC-11	Y611A, R614, C652A	Reduces binding of AP33 and multiple conformational antibodies to E2. Probably changes structure	(146)
CBH-4B, CBH-4D, CBH-4G, CBH-5 and CBH-7	aa 611-631	Reduces binding of multiple conformational antibodies to E2. Probably changes structure	(288)
HC-84, HC-111, HC-33, AR3	R424S	Reduces neutralisation by polyclonal sera and monoclonal antibodies in an ancestral isolate “Bole” gt 1a but not in H77	(289)
HC33,	L403F, L408M	Reduces neutralisation sensitivity to HC33	(290)
AR4A	L403F, L438V,S686T, V720I	Reduces neutralisation sensitivity to AR4A	(290)
AR5A	L665W	Escape from neutralisation, requires compensatory mutations to maintain infectivity	(291)
HC84.22, CBH2, AR3A, CBH5, CBH2	D431E, F560Y, I538V, Q546L, T563V	Additive effect on neutralisation escape	(292)

In order to identify which epitopes would be the optimum targets for vaccine design and which may be susceptible to viral resistance, it is important to detail the regions of the envelope protein targeted by those with a bNAb response and identify naturally occurring escape mutations. I aimed to determine common E2 epitope targets for bNAb responses in gt 1 patients in the CHCV cohort and determine the impact of specific amino acid variations on virus functionality and susceptibility to antibody neutralisation. Several approaches were used to achieve these aims:

- Performing competition assays with selected antibodies to known epitopes to determine sites targeted by the polyclonal IgG response of individual patients.
- Analysing the sequence characteristics of HCVpps from the HCVpp panel less sensitive to neutralisation by individuals with bNAbs and by monoclonal bNAbs (251) (Chapter 3). By identifying common sequences in those resistant to neutralisation, I aimed to infer regions where escape from neutralising responses may occur.
- Studying the proportion of synonymous to non-synonymous nucleotide substitutions in viral sequences derived from the same patient in order to identify regions of the virus where mutations are advantageous to survival and regions where conservation of sequences is necessary. For those regions where mutation is advantageous, immune selection pressure can be inferred(292).
- Characterising the infectivity and susceptibility to neutralisation of HCVpp bearing closely related E1E2 sequences isolated from a single patient. By aligning sequences and grouping them by functional state I hoped to identify any single aa changes associated with change in functional status.

- In addition, I also aimed to determine if sensitivity to neutralisation was related to dependence on the cell entry protein SR-BI as previous groups have identified single aa mutations which can alter dependency on this molecule.

4.2 Common epitope targets of the broadly neutralising polyclonal IgG response

4.2.1 Competition ELISA for the identification of E2 epitopes targeted by patient IgG

In an effort to gain an understanding of the epitopes targeted by the IgGs from individuals with bNAb, I performed competition ELISAs using IgG from the 20 gt 1-infected patients that were tested for their ability to neutralise the IG1 panel (Chapter 3 Section 6, Table 3.7). These individuals had been categorized as broadly neutralising or narrow neutralising depending on the number of HCVpp strains in the IG1 panel they could neutralise.

Competition ELISA assays were designed to determine if patient derived IgG competed for binding to gt 1a sE2 with a panel of 6 well-characterized neutralising and non-neutralising anti-E2 monoclonal antibodies (HmAbs: CBH-4B, HC-1, HC-11, CBH-7, HC-84.1 and mAb AP33). Several of these antibodies bind to non-competing regions of the E2 protein and therefore have been described as targeting different immunodomains. Non-neutralising HmAb CBH-4B binds to a region designated immunodomain A, the neutralising HC-1and HC-11 bind to immunodomain B, which contains the CD81 receptor binding sites. CBH-7 and HC-84 also neutralise and bind to immunodomains C and D respectively (120). Lastly, the neutralising mAb AP33 binds to the linear epitope aa412-423 (also referred to as immunodomain E). Further details of the competition ELISA assay

are given in Materials and Methods and more information on binding sites of these antibodies is presented in Tables 1.2 and 2.5.

Six of the ten patient samples identified as broadly neutralising in the intra-genotypic analysis (see individuals neutralising >7/11 HCVpp strains in the IG1 panel, Table 3.7) competed with at least 3 of the HmAbs tested at the 50% level, and 9/10 reduced binding of 3 of the HmAbs by 45% or more. Conversely, in the narrow neutralising samples only one competed with more than 2 HmAbs at this level (sample 1010, Table 4.2). Furthermore, while 15/20 samples tested competed with HmAb HC-11 at the >50% level, the majority of those with broad neutralising activity inhibited HmAbs CBH-7 and HC-11 binding by >50% (6/10) compared to (1/10) of narrow neutralising samples. There was also a significant association of percentage inhibition of these antibodies with inter-genotypic neutralisation breadth (*P*=0.02) (Fig.4.1). Together these data suggest that those with a broadly neutralising phenotype tended to compete with antibodies at multiple sites on E2 within more than one immunodomain whereas those with a narrow neutralising profile target limited numbers of neutralising epitopes.

Table 4.2 Competition of purified patient IgG with mAbs to specific epitopes on E2

Patient IgG	Neutralisation Breadth in panel IG1	Immunodomain						No. domains bound
		aa412-423	A	C	B		B/D	
		AP33	CBH-4B	CBH-7	HC-1	HC11	HC-84	
1001/00	Narrow	18.3	19.9	22.9	51.9	58.7	11.2	1
1002/00	Narrow	7.2	1.0	1.1	0.0	4.7	-2.1	0
1010/00	Narrow	33.2	48.5	76.4	55.2	82.7	9.5	2
1022/00	Narrow	0.4	20.3	22.9	57.6	73.4	9.1	1
1030/00	Narrow	13.4	16.7	15.2	0.6	36.3	6.5	0
1034/00	Narrow	10.0	5.4	26.3	3.6	43.5	20.6	0
1036/00	Narrow	-0.1	-13.7	1.9	35.3	65.3	14.9	1
1037/00	Narrow	6.4	6.4	9.0	7.8	32.2	14.5	0
1045/00	Narrow	7.9	20.7	20.9	39.6	26.4	0.7	0
1047/00	Narrow	12.3	16.5	29.1	62.5	71.9	12.6	1
1072/00	Narrow	8.2	16.6	14.3	53.2	56.1	12.6	1
1003/00	Broad	5.7	26.1	71.7	80.4	77.9	37.5	2
1012/00	Broad	43.8	58.3	76.0	46.8	75.3	15.8	3
1013/00	Broad	33.9	9.9	6.1	14.1	57.0	13.1	1
1016/00	Broad	3.4	10.2	47.0	75.8	75.0	30.6	2
1023/00	Broad	19.5	37.3	82.8	68.9	77.5	-1.8	2
1031/00	Broad	10.9	49.4	50.2	51.5	83.4	13.9	2
1032/00	Broad	28.2	44.7	85.7	70.0	92.4	26.6	2
1035/00	Broad	8.6	18.0	53.9	63.2	77.4	20.1	2
1112/00	Broad	14.5	24.4	48.2	69.3	74.0	18.0	2

Competition ELISA of gt1 patient IgG with E2 monoclonal antibodies CBH-4B, HC-84, AP33, CBH-7, HC-1 and HC-11 was performed as described in Materials and Methods. The mean percentage of competition (i.e. reduction in binding of the monoclonal antibody) from 3 independent experiments is shown with higher percentages being highlighted in green whereas lower percentages are shown in red. Samples categorisation as broad or narrow neutralisation breadth is based on the number of strains of HCVpps neutralised in the IG1 panel as shown in Table 3.7.

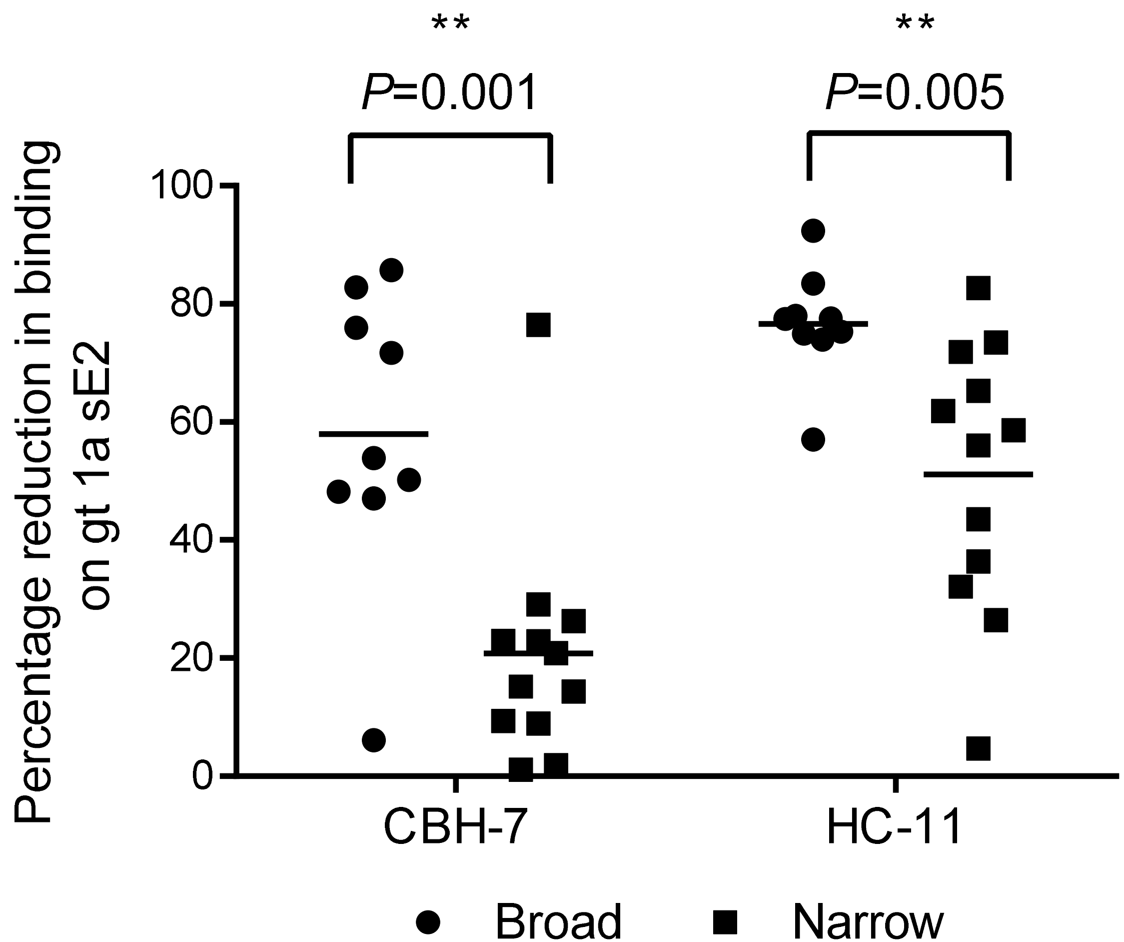

Figure 4.1 Association of competition of patient IgG against CBH-7 and HC-11 with breadth of neutralisation status

A competition ELISA of gt1 patient IgG with E2 monoclonal antibodies including CBH-7and HC-11 was performed as described in materials and methods. Briefly Immulon II plates (Nunc) were coated with sE2 and incubated with purified patient IgG at 200 µg/ml in PBSTM for 1h. Subsequently, monoclonal antibodies (mAbs) (listed in Table 2.5) to discrete E2 immunodomains (119, 120, 145) were added at a concentration close to their EC_{50}. The mean reduction in relative binding of each HmAb (calculated as percentage reduction in absorbance) on addition of patient IgG compared to PBSTM control was determined for each IgG sample. Competition for binding at these sites was compared between IgG samples classed as broad neutralisers and narrow neutralisers against the IG1 panel. *P* values were calculated using the Wilcoxon Rank Sum test.

4.2.2 Bioinformatic analysis of potential linear epitopes targeted by broadly neutralising polyclonal IgG responses

As sequence data were available for all of the 11 E1E2 proteins used in the intra-genotype 1 panel I aimed to determine if further bioinformatics analysis could identify linear antibody targets of the polyclonal responses of patient IgGs tested against this panel.

Dr Sreenu Vattipally from the Bioinformatics department at our Centre developed a program to cluster the HCVpp sequences in the panel using 10 and 20 amino acid (aa) sliding window. This program also compared the similarity of the clustering of HCVpp E1E2 sequences in each window to the pattern of clustering by the ability of each individual patient's IgGto neutralise the different HCVpp strains in the panel. As a control, the HCVpp neutralisation data for HmAbs HC-11 and HC-84.1, which have well characterised binding sites, was also included in the panel. For some of the windows an association was noted between HCVpp sequence differences at particular windows and the ability of patient derived IgGs to neutralise these HCVpps. These sequence windows were then flagged up as potential antibody binding sites for the patient derived IgG.

From this, predicted antibody binding sites were generated for some of the patient derived IgGs. Identified targets are listed in Table 4.3 below. For the HmAb HC-11, a region containing one of its previously identified binding sites (aa429-449) was located by the program. Some of our patient samples were also found to target similar regions to the shared HC-11/HC-1 binding site which complements the findings of patient IgG competition with HC-11 in the competition ELISA (see Table 4.2).

This analysis works on the basis that amino acid mutations in regions targeted by patient antibodies will reduce neutralisation overall. Of the five patients identified as targeting the shared HC-11 binding sites on bioinformatics analysis, two did not display significant cross-competition with any other broadly neutralising HmAb in the competition ELISA. This suggests that the majority of their neutralising antibody response may be directed against this region and that mutations here may confer resistance to their neutralising antibody response. For individuals with a polyclonal response targeting multiple epitopes, this program may have difficulty determining which regions are targeted as they may still be able to neutralise in the presence of mutations conferring resistance at one epitope. In addition, this bioinformatics analysis only looks for linear clustering of amino acids which limits its sensitivity as many bNAbs target discontinuous epitopes. This is supported by the fact that the majority of patients tested appeared to have antibodies competing with HC-1 and HC-11 but only 5 were detected by the program.

Other sites identified in some of the broadest neutralising individuals were not tested for in the competition ELISA, such as aa 632-642. It may be that mutations in this region confer a conformational change in other areas targeted by their antibody response. Using alanine scanning mutagenesis, Pierce *et al* (2016)(145), showed that mutation of single amino acids in the region 638-644 reduced binding of E2 to CD81 and multiple conformational antibodies (HC-1, HC-11,CBH-7 and HC-84). However, alanine scanning is not reliable in predicting the effect of substitutions with other amino acids more commonly seen *in vivo*. The effect of mutations in this region on bNAb efficacy requires further investigation.

Table 4.3 Predicted E2 antibody binding sites from bioinformatics analysis of neutralisation patterns.

Predicted E2-antibody binding sites based on subsequence clustering	Start Site 1 (aa)	End Site 1 (aa)	Start Site 2 (aa)	End Site 2 (aa)
C1001	613	623		
C1003	427	449		
C1010	427	449		
C1022	437	453		
C1023	563	573	632	642
C1031	401	411	428	449
C1032	415	425		
C1035	400	410		
C1036	437	453		
C1037	393	408		
C1072	400	411		
C1112	563	573	632	642
HC11	429	449		
HC84-1	437	453		

Sequence and neutralisation data were entered into a bespoke bioinformatics programme created by Dr Sreenu Vatiipally. This identified similarities short windows of HCVpp amino acid sequences with their sensitivity to neutralisation by patient derived IgGs or HmAbs. Only those samples where a probable site of antibody binding was identified are included in the table below. No probable sites of binding were found for patient samples 1012, 1013, 1016, 1022, 1030, 1034, 1045 and 1047.

4.3 Within patient amino acid variation and escape from autologous antibody responses

4.3.1 Amino acid selection within individual patients (dN/dS data)

In order to gain more information about the viral sequences targeted by individual's neutralising responses, I selected samples from a number of gt 1 virus-infected individuals from the CHCV cohort for clonal sequence analysis. This is described in more detail in Methods and Chapter 3 Section 3. Briefly, viral RNA was extracted from serum, cDNA libraries generated using random hexamers and E1E2 sequences were amplified using a gt 1-specific degenerate primer set (see Table 2.3, Methods). Resulting products of the expected size (1.8 kbp) were excised from agarose gel and extracted using a Qiagen Gel Extraction kit before inserting into the pENTR/DTOPO entry vector and transforming competent *E. coli* to enable clonal sequencing.

E1E2 amplification and subcloning of product in the pENTR D-TOPO vector proved challenging, therefore while sequencing was attempted for 15 patients, only 6 samples yielded more than 5 clones containing full length E1E2 (sample IDs 1012, 1013, 1034,1037,1047,1112). For one of these individuals (1047) 19/20 clones isolated were identical. For the other 5 patients, sequences from all clones generated were aligned and dN/dS plots were generated using MEGA 6 software. These plots compare the proportion of nucleotide substitutions at each codon which result in no amino acid changes (synonymous or dS) compared to those which result in an amino acid change (non-synonymous or dN). A negative value indicates amino acids which are relatively conserved with positive values indicating a tendency towards mutation (Fig. 4.2 a-f). The HVR-1 region (aa384-411) was the most mutated in all individuals with the exception of some conserved

amino acids (402 and 403) within this region as previously reported (39). When overall dN/dS scores were compiled for the 5 individuals studied, some commonly conserved and mutated regions were identified. These are listed along with any previously defined function in Table 4.4a and 4.4b and shown relative to key E2 regions in Fig. 4.3. It should be noted that while some of the individual dN/dS scores were positive or negative across patients tested, none of the values reached a *p* value of <0.05 at any of the amino acid positions, likely due to the small number of sequences included. Therefore, no firm conclusions can be drawn from this data.

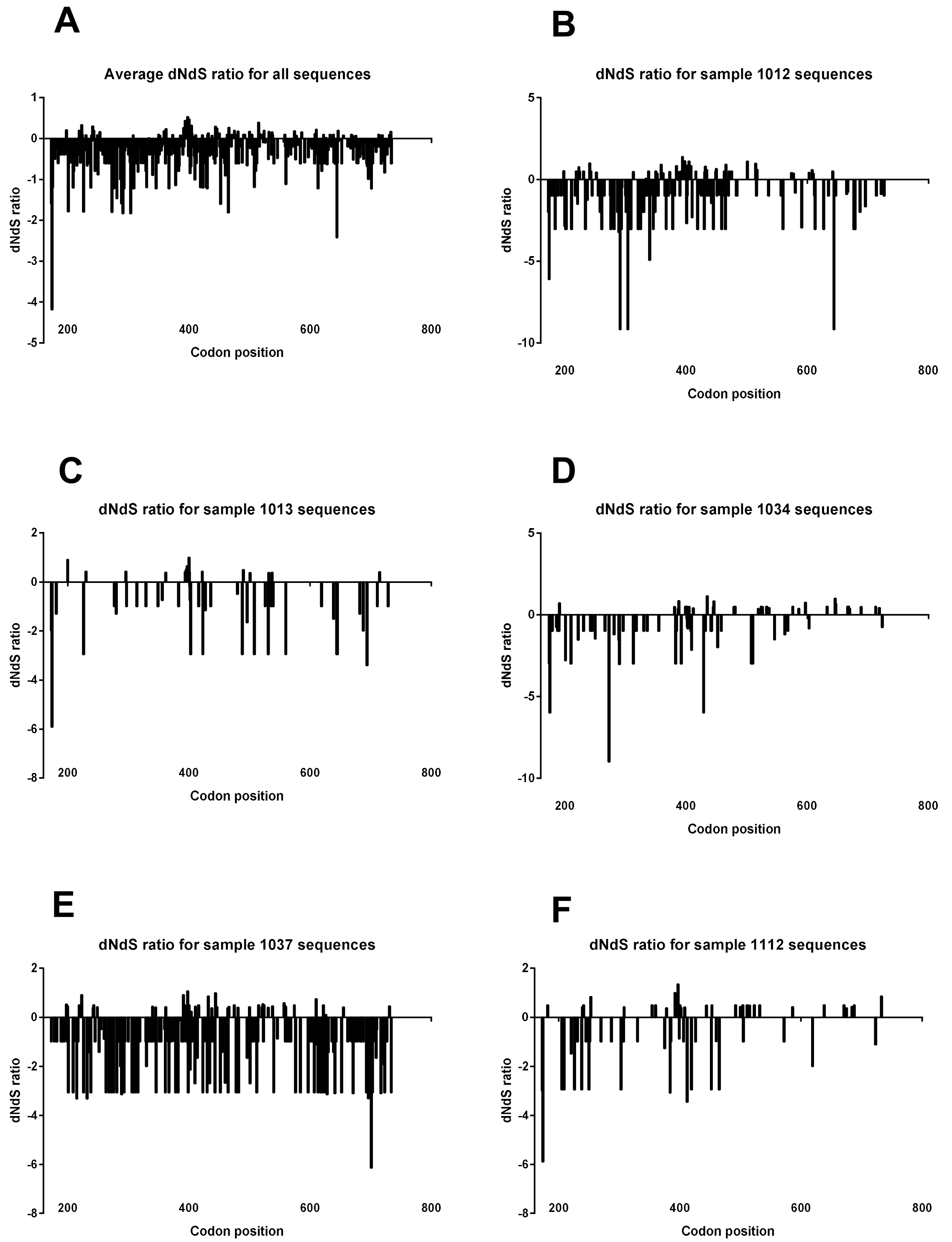

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Figure 4.2 dN/dS plots of sequences from individual patients.

The sequence for E1E2 was amplified as described in Materials and Methods and the subsequent PCR product cloned and sequenced using Sanger sequencing. Nucleotide sequences were aligned and analysed using the MEGA6 Hy-Phy programme to give a value for the ratio of nucleotide changes with alteration of an amino acid compared to the number of nucleotide changes associated with conservation of the amino acid. A positive ratio reflects relative positive selection of aa mutations whereas a negative ratio suggests relative conservation of the region. dN/dS ratio values are plotted for each amino acid with E1 starting at 192 and E2 terminating at aa746. None of the ratios reached statistical significance therefore *P* values have not been shown. An average dN/dS ratio for each position from all of the sequences was calculated and plotted in (A). The individual dN/dS plots are also shown from B-F from samples 1012 (B), 1013 (C), 1034(D), 1037 (E) and 1112 (F).

Table 4.4a Most conserved regions according to dN/dS ratio.

Conserved Regions	dN/dS (Sum)	Description	Ref
Amino Acid Position			
644	-12.12	Cysteine	(293) (44)
291	-9.16	Potential fusogenic region of E1	(294)
304	-9.16	Cysteine	(293)
273	-9.00	Just before fusogenic region	(294)
560	-6.01	Ectodomain - Mutation confers resistance to bNAbs - possible CD81 binding role or structural role.	(292)
419	-6.00	Conserved CD81 binding region targeted by AP33 antibody	(129)
465	-6.00	Unknown	
429	-6.00	End of HmAb HC-11 epitope	(121)
508	-5.96	Cysteine	(44)
226	-5.91	E1	
201	-5.85	HmAb H-111 epitope (antibody to this region blocks viral attachment to cells and infectivity)	(122)
452	-4.95	Cysteine	(44)
340	-4.94	E1 – unknown Function	(294)
289	-4.79	Fusogenic region of E1	(294)
250	-4.43	E1	
281	-4.26	Fusogenic region of E1	(294)
330	-4.00	E1 interaction with E2	(41)
383	-4.00	E1/E2 junction	
290	-3.89	Fusogenic region of E1	(294)
403	-3.42	A Conserved amino acid in HVR-1	(39)

Table 4.4b Most mutated regions according to dN/dS ratio.

Variable regions:	dNdS (Sum)	Description	Ref
Amino Acid Position			
397	2	HVR-1	
400	1.88	HVR-1	
394	1.82	HVR-1	
398	1.61	HVR-1	
405	1.6	HVR-1	
396	1.52	HVR-1	
501	1.5		
241	1.5	E1	
515	1.47	Region determining SRBI dependence	(285)
404	1.37	HVR-1	
388	1.33	HVR-1	
646	1		
395	0.96	HVR-1	
359	0.92	E1	
466	0.92	Non-neutralising linear antibody domain before HVR-2	(295)
399	0.92	HVR-1	
532	0.88	Near HmAb HC-11/HC-84 binding region	(124)
733	0.86	E2 transmembrane domain	
446	0.84	Target for multiple monoclonal antibodies	
253	0.84	E1	(124)
586	0.81	E1 interaction site	(41)

MEGA 6 software was used to calculate the dN/dS ratio for each codon position for the 5 patients studied. The sum of the ratio for all samples at a single codon position was calculated to determine if there were any commonly conserved or mutated positions. The literature was then examined to determine if these had previously been documented. The table shows aa position and function of the 20 most conserved and 20 most variable aa positions with references given where available.

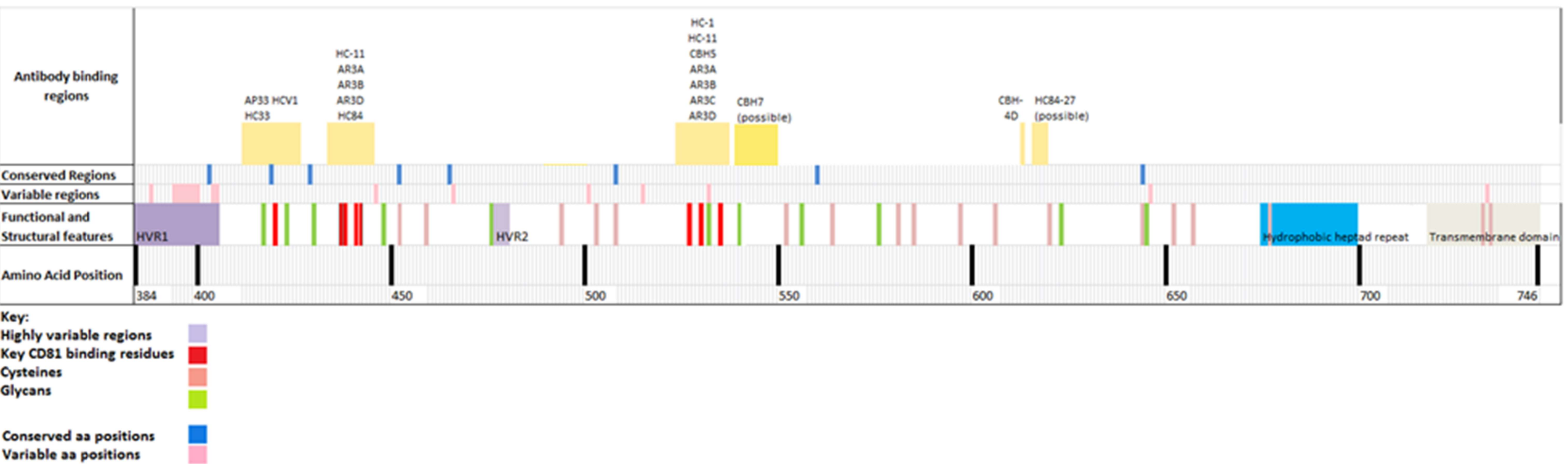


Figure 4.3 Most variable and conserved positions according to dN/dS data shown alongside key E2 residues.

dN/dS plots were generated as described above. The 20 most conserved and most mutated regions in E1E2 across all samples were determined (see Tables 4a and 4b). Those positions in E2 were plotted on the above figure showing key antibody binding and other structural residues.

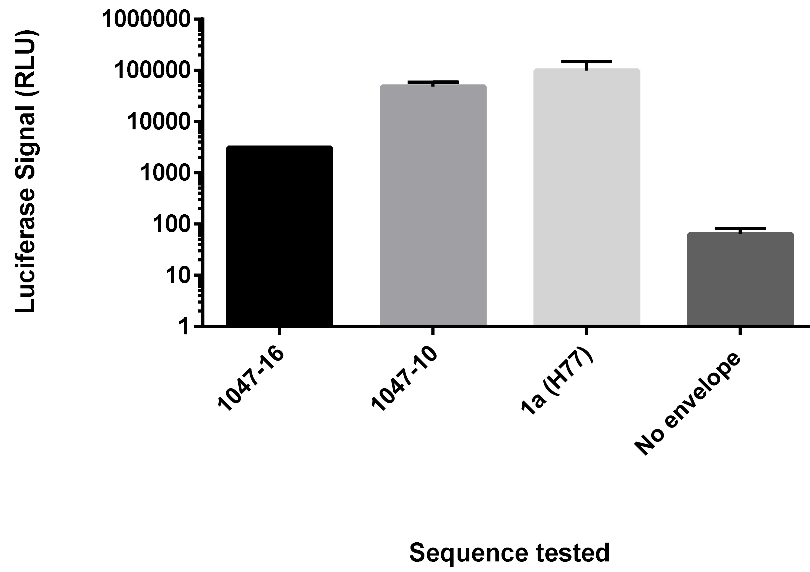
4.4 Interaction of autologous virus with host IgG in broad and narrow neutralising profile patients

In individuals with more than 5 E1E2 sequences obtained on clonal analysis, these sequences were inserted into a phCMV expression vector as described in Materials and Methods. The resulting expression vectors were tested for their ability to generate functional HCVpp. In 3 individuals, more than one E1E2 sequence produced functional HCVpps. Infectivity data for the sequences from patient 1012 is shown in Chapter 3, Fig. 3.3b. Infectivity data for patient 1047, 1013 and 1034 are shown below (Fig. 4.4a,b and c respectively). For patient 1047, sequence 1047-16 was approximately tenfold less infective than 1047-10. These were the only clones tested for patient 1047 as all of the other sequences obtained were identical to 1047-10. For the sequences tested from patient 1013, two out of 4 HCVpps showed infectivity at comparable levels. While other sequences were identified from this patient, several of these were not in frame in the directional cloning vector could not be transferred to the expression vector for analysis of functionality. From all the sequences tested in the HCVpp system for patient 1034, only one sequence (1034-11) showed significant infectivity to allow neutralisation assay testing although 1034-14 shows probable infectivity (Fig 4.4c). Only one sequence from patient 1037 was functional in the HCVpp system (1037-04).

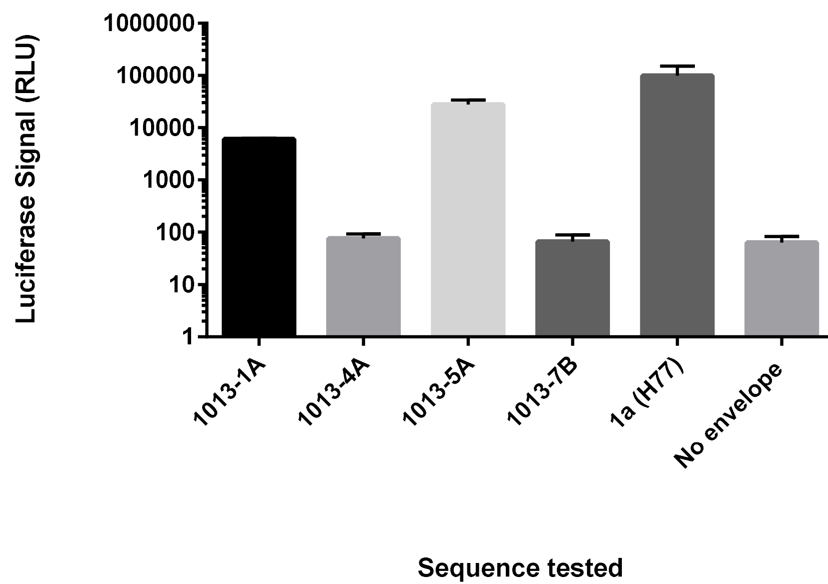
I subsequently tested the ability of serial dilutions of autologous IgGs from patients 1012, 1013, 1047 and 1037 to neutralise these sequences in vitro (Fig. 4.5) as described in Materials and Methods. Results of these assays were analysed alongside sequence data available for the clones from each patient and previous information about antibody targets and neutralisation ability of the patient derived IgG from the assays described in Chapter 3. The key findings from the

sequence analysis and dN/dS plots of sequences derived from individual patients in addition to autologous neutralisation assays (where it was possible to perform these) are detailed below.

A



B



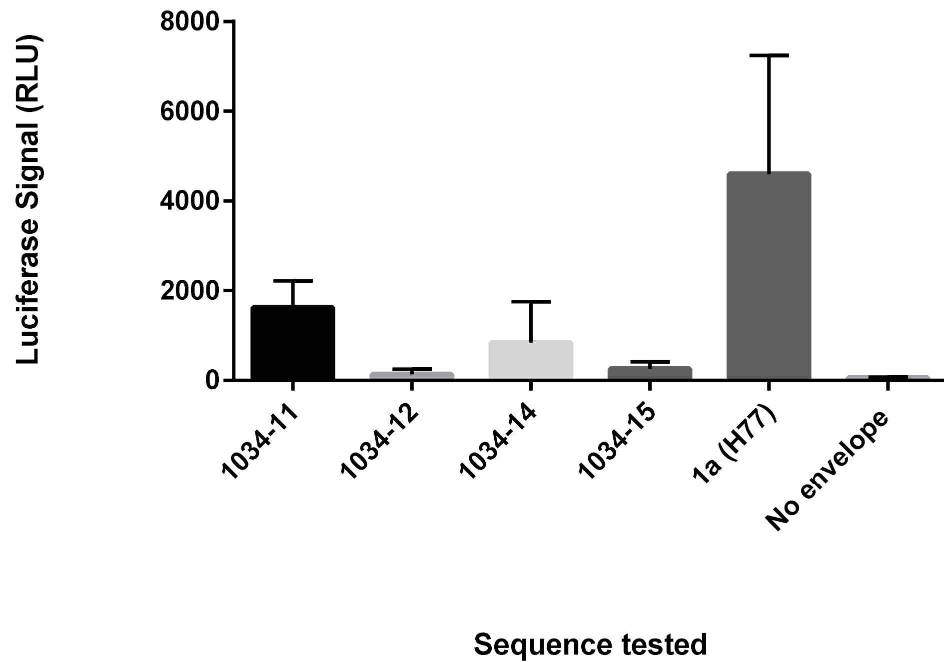
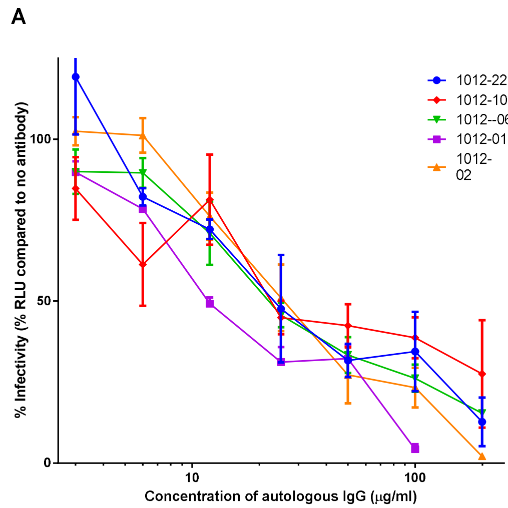


Figure 4.4 Infectivity of HCVpp bearing E1E2 derived from patients 1047, 1013 and 1034

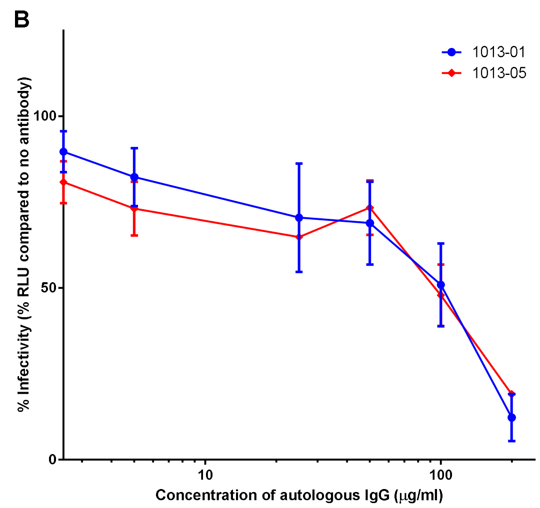
Huh7 cells were infected with HCVpp bearing different E1E2 sequences from patients (A) 1047, (B) 1013, and (C) 1034. At 72 h post-infection, cells were lysed and the level of infectivity determined by measuring the luciferase activity as relative light units (RLU). HCVpp carrying E1E2 from the gt 1a isolate H77c or no envelope were used as positive and negative control, respectively. Values shown are the mean result from three independent assays. Error bars show the SEM.

A



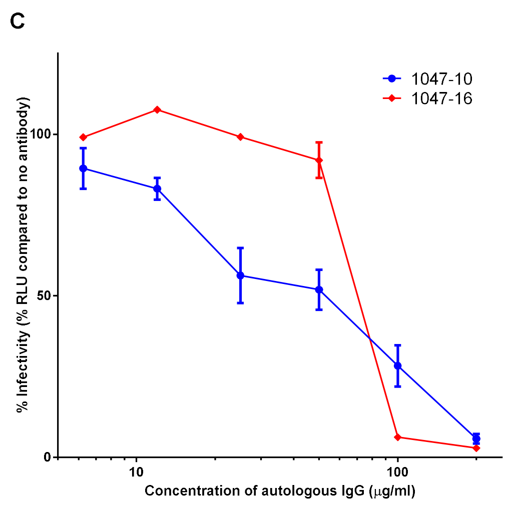
Patient Sequence	Autologous IgG IC 50 (µg/ml)
1012-01	12
1012-02	26.1
1012-06	22.6
1012-10	22.8
1012-22	23.2

B



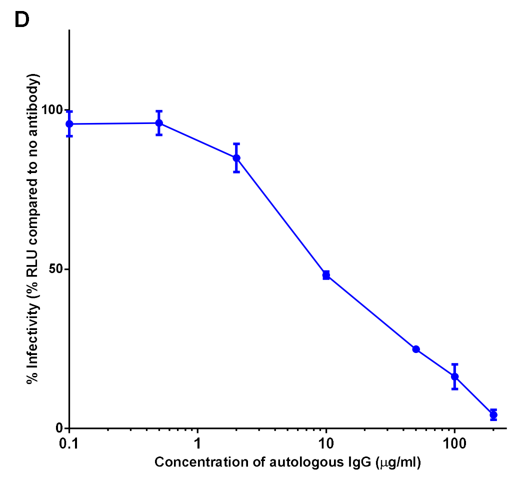
Patient Sequence	Autologous IgG IC50 (µg/ml)
1013-01	101
1013-05	97

C



Patient Sequence	Autologous IGg IC 50 (µg/ml)
1047-10 (478S)	25
1047-16 (478N)	75

D



Patient Sequence	Autologous IgG IC50 (µg/ml)
1037-04	9.8

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Figure 4.5 Relative neutralisation of patient-derived HCVpp by IgGs of autologous sera.

IgG was purified as described in Materials and Methods. HCVpp bearing E1E2 from different strains of envelope sequence derived from subject A) 1012, B) 1047, C) 1013 and D) 1037 were generated as described in Materials and Methods. 40µl of HCVpp containing media was incubated with decreasing dilutions of IgGs for 1 hour prior to adding to Huh7 cells. Percentage neutralisation of HCVpps was determined by calculating the relative percentage reduction in light units in wells with IgG compared to a PBS control. % Neutralisation is plotted against log concentration of IgG with error bars representing SEM. Relative IC50 values, as calculated using Graphpad Prism 6 software, are shown below each figure.

4.4.1 Analyses of the viral E1E2 sequences derived from patient 1012

For patient 1012, 20 diverse E1E2 sequences were identified by clonal sequencing. After constructing a phylogenetic tree (see Fig. 3.3a Chapter 3), 9 sequences representing the main clades had been selected for testing in the HCVpp system as described above. Of these 9, 5 were functional. From the neutralisation assay with autologous IgG, it became apparent that one of these, HCVpp 1012-01, was more sensitive to neutralisation by autologous IgG than the others (Fig. 4.5a). Analysis of a protein alignment in using MEGA 6 software was performed to determine any unique aa changes in this sequence (Fig. 4.6).

Sequence	Amino acid position																				
	198	200	219	223	237	241	242	243	252	313	334	359	362	373	384	386	387	388	391	394	395
1012-22	T	L	A	A	R	V	M	T	K	V	V	I	F	V	E	Q	V	T	S	Y	A
1012-02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
1012-06	.	.	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	T
1012-10	S	I	T	F	K	A	V	A	R	I	M	L	Y	I	D	H	T	I	T	R	T
1012-01	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Sequence	Amino acid position																			
	398	399	403	404	405	408	410	416	431	434	441	442	449	450	455	463	466	471	475	501
1012-22	G	F	F	K	L	R	N	S	E	T	L	L	S	T	R	T	A	P	T	N
1012-02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	S
1012-06	.	L	.	S	V	Q	K	.	A	.	.	F	.	S	.	.	.	.	A	S
1012-10	V	L	L	P	P	K	D	T	A	E	.	F	.	S	.	A	D	.	A	S
1012-01	.	.	.	.	.	.	.	.	.	.	F	.	F	.	.	.	.	L	.	S

Figure 4.6 Variable amino acid positions from alignment of E1E2 sequences derived from patient 1012.

Sequences from patient 1012 which are functional in the HCVpp system were translated into protein and aligned using MEGA 6 software. They were organized according to sensitivity to neutralisation by autologous IgG. Non-conserved aa between all sequences are show in the figure above. Amino acid changes which are unique to sequence 1012-01 are highlighted.

Analysis of the alignment revealed sequence 1012-01 had 4 unique aa differences compared to the other patient 1012 sequences tested in the HCVpp system: A223T L441F, S449F and P471L. The second of these, L441F, lies in a region identified as a key CD81 binding site and is targeted by a number of broadly neutralising monoclonal antibodies including HC-1 and HC-11and HC-84. IgG from this patient was previously shown to competitively inhibit binding of HC-1and HC-11 to E2 (Table 4.2) suggesting this individual has antibodies which bind in this region. S449F is adjacent to the 451 site identified by Grove et al as responsible for a SRBI entry-protein usage switch(29). The final aa change is in a region recognized by a linear antibody AP320 produced from mouse immunization studies within our lab (259) but is not known to be important in viral neutralisation.

The dN/dS plot for all twenty sequences isolated from pt 1012 (Fig. 4.2b) reveals areas of higher levels of non-synonymous mutations between 385 and 415 (HVR-1). Other areas of a high (>1) dN/dS ratio include aa 423-429 (CD81 binding region, target of HC-11 like antibodies), 443-446 (another CD81 binding region and target of HC-11 like antibodies), 460, 495, 509 and 511.

4.4.2 Analyses of the viral E1E2 sequences derived from patient 1047

While 20 clones were isolated from pt 1047, only clone 1047-16 differed from the other 19 by a single amino acid change, S478N, which lies within the HVR-2 region of E2. Therefore HCVpp 1047-16 (N478) was tested along with HCVpp 1047-10 bearing the majority sequence (S478) for neutralisation by IgGs derived from the autologous serum. S478 had an IC50 3 times lower than N478 (Fig. 4.5c). This serine to asparagine change in 1047-16 creates a potential N-linked glycosylation site in HVR-2. Further testing is required to confirm if this site is targeted directly by the individual’s Nabs or whether it induces some other conformational change allowing escape. Interestingly a study by Falowska et al. (2007) showed that adding a glycosylation site at aa 476 increased cell entry and decreased sensitivity to neutralisation, therefore it is plausible that the introduction of an additional glycosylation site adjacent to this region might amplify this effect(296).

IgGs from this individual compete with antibodies recognizing the E2 CD81 binding epitopes (HmAbs HC-1 and HC-11) (Table 4.2). It is possible that additional glycosylation of a region around aa 476 impairs binding at these regions. Escape from a neutralising response would usually result in positive selection, however, N478 is only a minority variant in our clonal analysis. As the other 19 sequences are the same and this mutation occurred distant to primer sites, it is unlikely to be

a result of PCR bias, however it is possible that this mutation was introduced as an error at the reverse transcription stage. Nevertheless, it is still interesting to study the effect of this amino acid change. Infectivity in the N478 HCVpp appears to be slightly lower than S478 (Fig. 4.4a) and the site 478 appears well conserved in gt 1a (>95% S, the next common variant being T) in an alignment of over 300 gt 1a E2 sequences from the Los Alamos database (data not shown) and conserved in 80% of all gt 1 E1E2 sequences held in the Los Alamos database (see Fig 1.12) therefore there may be some fitness cost to adding a glycosylation epitope at this site. McCaffrey *et al* showed that HVR-2 is essential for correct envelope formation and infectivity of virus suggesting that key mutations in this region may impair viral assembly or cell entry (286).

4.4.3 Analyses of the viral E1E2 sequences derived from patient 1013

From the 7 E1E2-encoding sequences generated from this patient, 2 HCVpp derived from them were functional on testing in the HCVpp system. While IgG from patient 1013 showed strong neutralising activity against other gt 1 HCVpp, e.g. being able to neutralise 5 of the HCVpp in the previously described intra-genotype 1 panel by >80% at 100 µg/ml (Table 3.7 Chapter 3), it has relatively weak neutralising ability against its own E1E2 sequences with an IC50 of approximately 100 µg/ml (Fig. 4.5c).

Sample 1013 is also intriguing as despite its IgG being a broad neutraliser of the IG1 panel, it has relatively poor ability to neutralise other genotypes in the XG panel. It also shows competition with HmAb HC-11-like antibodies and displays a degree of competition with mAb AP33 but not with HmAbs HC-1 or CBH-7 (Fig. 4.1).

Analysis of dN/dS ratio shows evidence of selection for most variability in the E2 HVR-1 and also some strongly selected sites in E1 (aa201), E2 aa 491, 502 and also the E2 heptad repeat region (Fig 4.2) (a structural motif found in viral fusion glycoproteins consisting of a repeating sequence of seven amino acids). This is interesting as this region is generally conserved (40). There is very little variability seen around the known CD81 binding regions targeted by many bNAbs, although some of these will be conserved to preserve infectivity. It is possible that the circulating variants sequenced have successfully escaped from antibodies directed at these regions and are no longer under immune pressure to mutate further, as reflected by low neutralising titre. Indeed the HCVpp derived from patient 1013 was poorly neutralised by both monoclonal bNAbs and IgG derived from chronically infected patients in our panel experiments (Chapter 3, Table 3.7) Negative selection of persistent variants in chronic infection is a well described phenomenon with resultant narrowing of viral diversity(297, 298). Alternatively, host antibodies may be targeting regions/residues outwith these epitopes.

4.4.4 Analyses of the viral E1E2 sequences derived from patient 1034

Clonal analysis for patient 1034 identified 20 diverse sequences. These were aligned and a phylogenetic tree constructed (Fig. 4.7). Four sequences from separate clades were selected for testing in the HCVpp system, unfortunately only one of the HCVpp tested was functional (1034-11, Fig. 4.4c). Although this HCVpp is relatively easily neutralised by the individuals with broadly neutralising antibodies, IgG from 1034 was unable to neutralise this HCVpp at a concentration of 100 ug/ml (data from IG1 panel, Chapter 3, Table 3.7), therefore a dose/neutralisation curve was not performed. This is consistent with the

classification of IgG from patient 1034 as poorly neutralising when tested against the IG1 panel (Chapter 3.6)

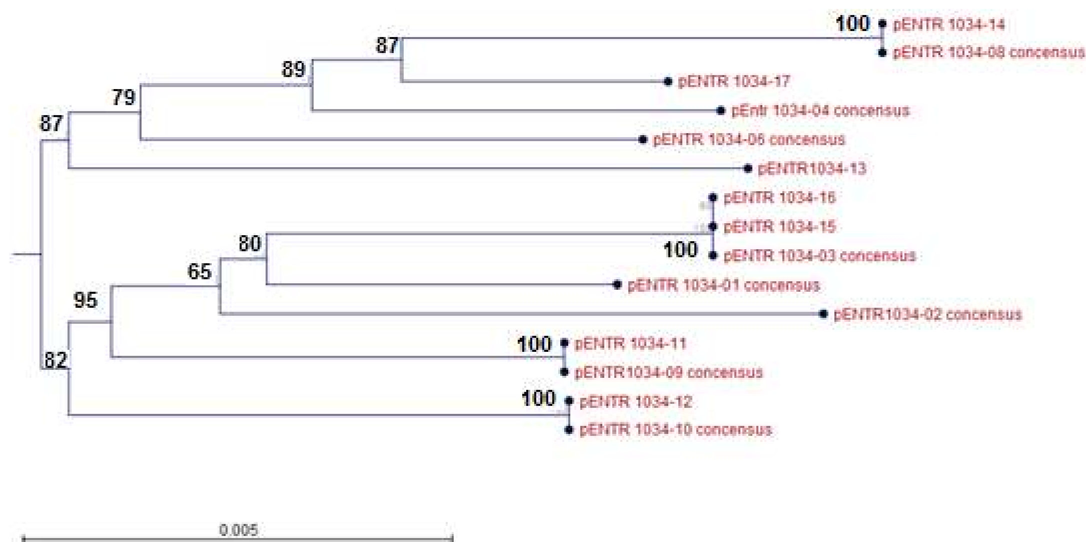


Figure 4.7 Phylogenetic tree of sequences isolated from patient 1034.

Five sequences were excluded from the alignment as they were incomplete. The evolutionary history for the remaining 15 sequences was inferred by using the Maximum Likelihood method based on the Tamura-Nei model(257). The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1726 positions in the final dataset. Evolutionary analyses were conducted in MEGA6(16).

dN/dS analysis of the regions of E1E2 which are relatively mutated or conserved in the variants available by clonal analysis reveals evidence of selection in the

regions aa 443, aa 445 and aa 530-538 which are regions targeted by the HmAb HC-11. This would fit with the finding that this individual has evidence of antibodies which compete with HC-11 but not with HC-1 (see Figure 4.1a). It is possible that mutations in this region to escape antibody pressure have resulted in an infectivity cost in the variants sequenced. dN/dS analysis also shows some evidence of selection at aa 647-648 and aa 668. The importance of these regions in cell entry and neutralisation has not yet been demonstrated.

4.4.5 Analyses of the viral E1E2 sequences derived from patient 1037

Six viral sequences were obtained for pt 1037. Three of these were tested for infectivity, however only one of these (1037-04) was found to be sufficiently infective in the HCVpp system for use in neutralisation assays. This sequence was neutralised by autologous purified IgG with an IC50 of 9.8 μg/ml (Fig.4.5d).

The IgG derived from patient 1037 was found to have a narrow neutralising capability when tested against the Gt1a panel (Chapter 3 Table 3.7). Bioinformatics analysis (Section 4.2.2) identified HVR-1 as a potential target for antibodies from this patient. Indeed, dN/dS plots revealed the most variable aa positions were within the HVR-1 region or E1 (Fig 4.2e). In addition, the amino acid sequence was variable in the SRBI dependence region (aa 515) identified by Lavie et al.(285). Competition assays with HmAbs to known epitopes revealed no evidence of significant binding at these sites apart from weak inhibition of HC-11. It is therefore likely that this individual's humoral response preferentially targeted the HVR-1 region with the virus adapting to remove dependence on HVR-1/SRBI interaction for cell entry. Immune responses preferentially targeting the HVR-1 region are well documented in acute HCV and associated with progression to chronic infection (282).

4.4.6 Analyses of the viral E1E2 sequences derived from patient 1112:

While 10 sequences were identified on clonal analysis, unfortunately no functional HCVpps could be generated for these sequences. This individual did show evidence of broadly neutralising activity against 11 HCVpp strains in the IG1 panel however (Chapter 3, Figure 3.7), and so I examined sequence data for any evidence of selection pressure in the clones isolated.

dN/dS ratio analysis showed peak areas of variability in E1 aa232-254, E2 HVR1, and separate E2 amino acids between 493 and 533 (Fig. 4.2f). This section contains a CD81 binding region targeted by the neutralising HmAbs HC-1 and HC-11and the HmAb e137 which recognisesE2 residues W529, G530, and D535 that are crucial for E2-CD81 interaction(299).

4.5 Single amino acid changes associated with infectivity and resistance to neutralisation

4.5.1 Non-infective E2 producing sequences

As described above, some HCVpp incorporating E1E2-encoding sequences derived from patients were non-infectious. To test whether this phenotype was due to lack of viral glycoprotein expression or inability of specific E2 glycoprotein sequences to form infective HCVpp, the lysates of HEK-293T cells transfected with 8 constructs from patient 1012 were analysed for the presence of E2.

For the purposes of this experiment, non-functional or non-infective sequences were designated as those which gave a RLU signal of <5 times the background (i.e. lysates of cells not transfected with E1E2-expressing construct) on testing in the HCVpp system. Functional sequences were determined as those with a RLU

signal >10 times the background. One HCVpp (12-08) gave an intermediate signal therefore was included separately in the analysis. The lysates of the HEK cells used to produce these HCVpps were tested using an ELISA for binding to mAb AP33 and HmAb CBH-4B as described in Materials and Methods (Section 2.4.8). As a negative control, lysate from HEK cells transfected with a non E1E2 containing plasmid was used. Presence of E2 was confirmed with an absorbance reading of greater than 2 times that of the negative control (see Fig. 4.8).

For those non-infective clones where E1E2 production was confirmed, MEGA 6 software was used to align these sequences with infective ones derived from the same individual and identify amino acid differences only present in non-infective isdolates. Comparison of amino acid changes unique to non-functional sequences highlighted several regions of importance (Table 4.5).

Sequence 1012-11 has a single unique amino acid change (M/L608P) compared with infective sequences from the same patient. Mutations in this region have already been identified as having a dramatic effect on CD81 and conformational antibody binding(145, 288) and it is likely that rather than being a CD81 binding epitope *per se*, this region is critical for the structure of the envelope proteins facilitating correct binding. It is interesting to note, that while this sequence has clear evidence of AP33 binding, it gave one of the lowest positive values for conformational antibody binding, again suggesting disordered structure.

The data also highlight the critical role of E1 which has been relatively neglected compared to E2. Two non-functional sequences showed single aa changes in the region 294-296 this is adjacent to Perez-Berna's proposed fusogenic region of E1(294). Therefore, it is possible that these changes modulate the function of this region. Finally, the sequence from patient 1012-20 had 2 mutations compared to

infective strains, both at uncharacterized regions of E1 (R213H and G350D). Interestingly a recent paper described attenuated infectivity in a virus with an alanine inserted at aa 213. This strain also used a different class of Claudin to the un-mutated strain (Claudin-6 compared to Claudin-1 in un-mutated virus), and displayed reduced binding to CD81 which may have had an impact on infectivity in Huh7 cells (300). Further exploration of the effect of mutating these regions of E1 may yield new insights into the E1 structure and role in HCV cell entry.

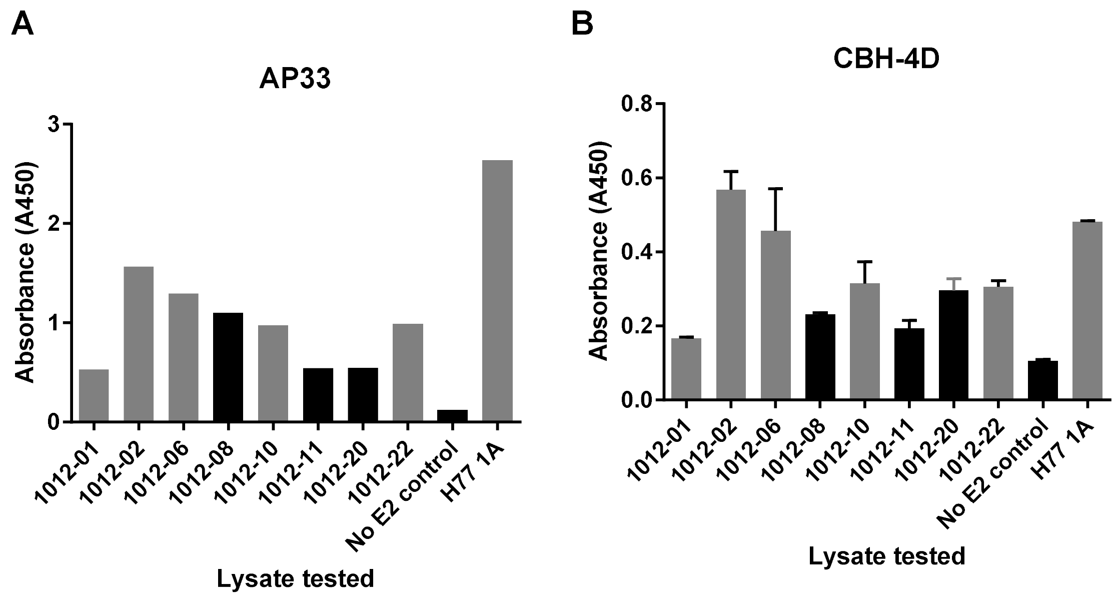


Figure 4.8 ELISA of HEK cell lysate transfected with plasmids containing E1E2 sequences from patient 1012 to determine E2 expression.

HEK cells were co-transfected with plasmids containing E1E2 sequences derived from patients 1012 together with Gag-Pol and luciferase-expressing plasmids in order to create HCVpps (as described in Materials and Methods). Supernatant media containing HCVpps was filtered and tested for infectivity of Huh7 cells. At 72 h post-infection, the infectivity was determined measuring luciferase activity as RLU reading 10 times greater than negative control (see figure 3.3b for infectivity data). ELISA of lysates from HEK cells transfected with pHCMV-E1E2 plasmids producing non-infective HCVpps was performed to determine synthesis of E2. Lysates were tested using A) a linear antibody (AP33) and B) conformation sensitive antibody (CBH-4B). Anti-mouse antibody A4416 and streptavidin-HRP respectively were used as a secondary substrate. Lysates from HEK cells transfected with a pHCMV plasmid not containing a E1E2 sequence were used as a negative control. Absorbance readings of two times control were used as a cut off to determine presence of E2. The results shown are from a single experiment containing 3 replicates. Error bars represent SEM.

Table 4.5 Mutations in non-functional E2 producing sequences compared to sequences from the same patient producing functional (infective) HCVpps.

Isolate	Amino acid changes*	Comments
1012-08	S294P, S401N, V515M	HVR-1 and SR-BI dependence switching regions(aa 515) (285) NB intermediate signal for infectivity, see Fig 3.3b
1012-11	M/L608P	Possible role in structural integrity
1012-20	R213H, G350D	Possible effect on CD-81 binding and other entry protein usage

*Amino acid substitutions only found in non-functional sequences were noted and their function documented (where known).

4.5.2 Amino acid mutations associated with resistance to neutralisation by monoclonal antibodies and polyclonal patient-derived IgGs

The experiments described in Chapter 3 using the IG1 panel of 11 gt 1a HCVpp characterized the ability of polyclonal IgG derived from single patients and HmAbs to prevent Huh7 infection by HCVpps bearing diverse envelope sequences. However, examining the patterns of HCVpp neutralisation across the panel, some HCVpp appeared relatively resistant to neutralisation by individual HmAbs and patient IgG samples. In particular those bearing UKN1A20.8 (1Av220), 1012-02, 1013-01 and UKN1B_5.23 glycoproteins were only neutralised by a minority of patient IgG and HmAbs. (Chapter 3, Table 3.7 and Table 4.6).

Table 4.6: Number of patient derived IgG samples and HmAbs able to neutralise each E1E2 sequence in the HCVpp G1 panel.

Sequence	No. Individual patient IgG samples neutralising to >50% (n=21)	No. Neutralising Monoclonal Panel antibodies neutralising to >50% (n=4)
1Av220	0	1
UKN1B_5.23	3	3
1013-01	9	0
1012-02	11	0
1Av93	12	2
UKN96	13	2
1034-11	17	2
1037-04	18	3
ET10	18	3
H77	19	4
UKN181	20	3

As sequence information was available for all of the HCVpps in the IG1panel, I aimed to analyse the sequences of the HCVpps resistant to neutralisation by the antibodies tested to determine if any particular amino acid changes in the envelope protein conferred this resistance. Figure 4.9 shows the percentage conservation of each amino acid in an alignment of the gt 1 E2 sequences used in the IG1 panel (A) in comparison with an alignment of all the E2 sequences stored in the Los Alamos database (B). This gives a picture of the most variable regions within the protein and demonstrates that the amino acid variability within the IG1 panel covers the majority of variable sites in genotype 1 sequences.

Detailed methods for the testing of neutralisation against the IG1 panel are described in Materials and Methods. To identify aa changes which were predominantly found in neutralisation-resistant envelope sequences (and therefore could possibly be responsible for escape from neutralisation) I used a similar technique to that described by Bailey *et al*(292). Briefly, for each HmAb or polyclonal sera, HCVpp E1E2 aa sequences were aligned using MEGA6 software and arranged in order of resistance to neutralisation by that antibody. Amino acid differences from the sequence most sensitive to neutralisation were highlighted. Amino acid changes only present in HCVpp relatively resistant to neutralisation by a particular HmAb or polyclonal sera (i.e. infectivity over 50% in the presence of the patient IgG or monoclonal antibodies being studied) were identified manually from these alignments and recorded in Fig. 4.10a and 4.10b. Mutations present in all neutralisation-resistant HCVpp sequences but not sensitive sequences were colour coded in red as these are most likely to have an impact on function. These sites have also been shown on a linear diagram of E2 (Fig 4.11) alongside previously identified naturally occurring escape mutants(292) and other relevant mutations from the literature as detailed in Table 4.2.

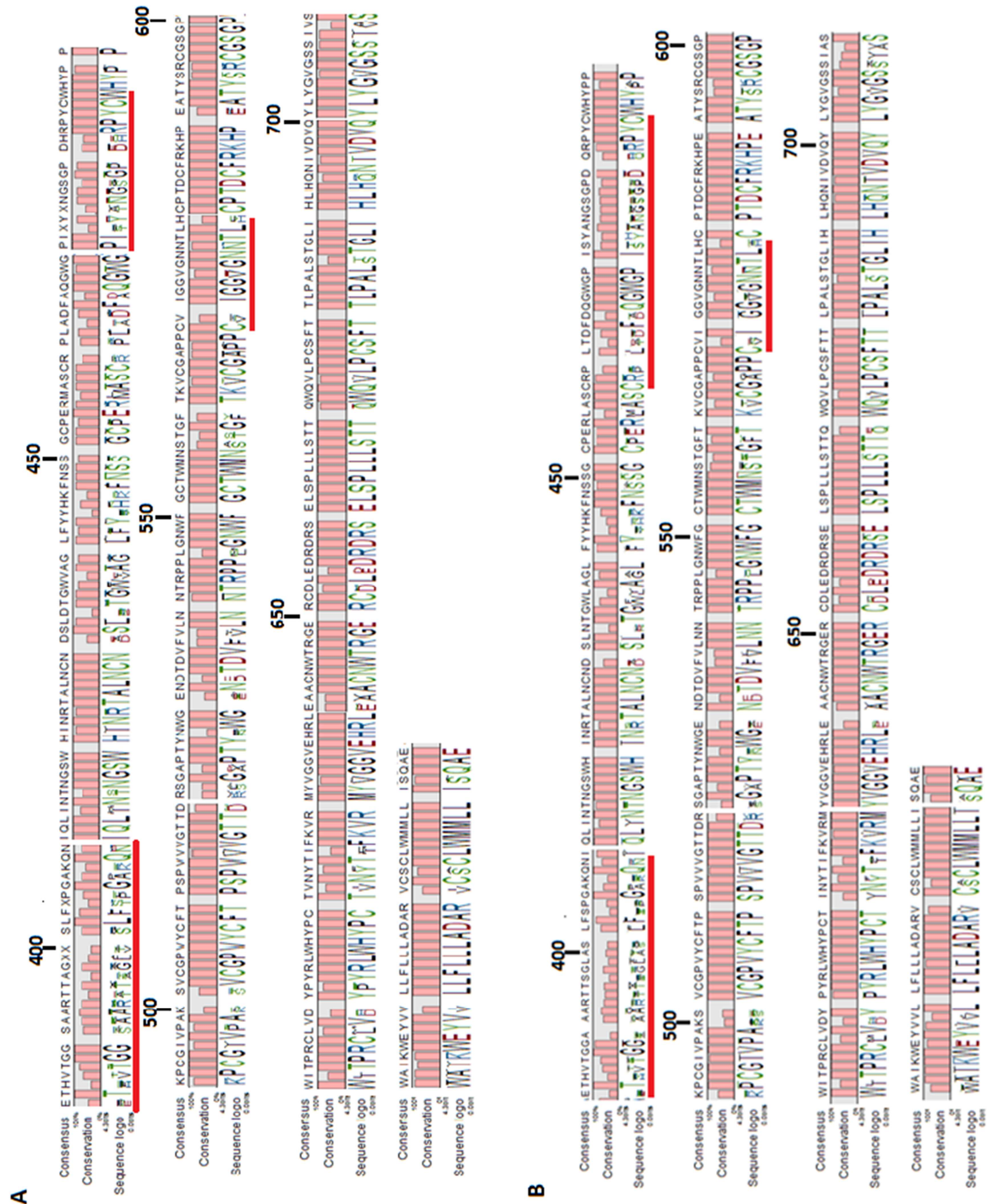


Figure 4.9 Percentage conservation of each amino acid position and consensus amino acid sequence in E2 for alignments of A) Panel of 11 gt 1 HCVpp E1E2 sequences B) All complete envelope sequences from Los Alamos HCV database.

Alignments of all envelope sequences for A) the intra gt 1 HCVpp panel and B) complete E1E2 sequences uploaded to the Los Alamos HCV database (https://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html accessed 23/02/2016) were created using MEGA 6 software. These were imported into the CLC Genomics Workbench 7 program which calculated percentage conservation at each amino acid position.

While no universal amino acid motifs were noted to confer resistance, both HCVpp 1012-02 and 1Av220 contained the L442 mutation which has previously been associated with escape from neutralising monoclonal antibodies. Sequence 1012-02 also bears the previously identified escape mutation E431 (292).

Some of the other amino acid differences associated with mAb resistance fell in well-characterized binding sites for the broadly neutralising monoclonal antibodies used (e.g. 434, 438, 442, 444), however, others were distant to previously identified regions of escape (e.g. 500, 528, 570) (Fig. 4.11). Some of these sites have been identified as having functional importance in previous work (Table 4.2). In particular, Wasilewski *et al* identified aa570 as one which had mutated relatively early on in the evolutionary history of HCV suggesting that this region may play a key part in escape from the host response (289). However, their study did not identify any advantage against neutralisation by polyclonal sera.

It is likely that some of the amino acid substitutions identified are coincidental or compensatory rather than being primarily responsible for resistance to neutralisation, while others may confer structural changes. Nevertheless, further characterization of these regions may yield helpful insights into the interaction between the immune response and HCV.

A

E1 E2 Position where mutation is associated with resistance to HmAb	H 222	T 223	A 235	V 240	V 242	T 243	T 255	T 256	Q 257	S 268	I 313	I 340	E 384	H 386	V 387	N 391	R 394	A 395	T 396	A 397	T 400	P 405	T 416	I 422	E 431	N 434
Monoclonal Antibody (hMAb)																										
CBH-7	M L						A		E T	G A	V					A K		Q D				S L	S			
Ap33						A						V		Q Y	T		Y Q			S T				V	A	D T
HC-11		S	V N			A						V	G N	Q R Y	T					S					A	D E T
HC-84			V		M			A						Q R Y			Y Q H		A V	S	A S					T

E1 E2 Position where mutation is associated with resistance to HmAb	L 438	F 442	Q 444	A 446	S 473	D 481	Q 482	K 500	S 501	R 521	S 522	A 524	S 528	E 531	S 557	T 558	F 560	V 570	I 606	D 641	A 642	E 655	S 686	I 714	V 720	V 731
Monoclonal Antibody (hMAb)																										
CBH-7		L			G R			L S		K		V			A		Y	N K I						V		
Ap33	V		Y H																		V	D				
HC-11	V		Y H	D		E G			N T		A L		G R			S			L M						I	I
HC-84		L	Y		G R			L Q		K			R N		A		Y	N I		E	V			V		

B

E1 E2 Position	A235	V240	V242	T243	T255	T256	Q257	S268	I313	I340	E384	H386	V387	N391	R394	A395	T396	A397	T400	P405
Polyclonal IgG																				
1112		A						A G				R							S	S
1012	V		M L				E T	A G				Q R Y				Q D				S L
1013	V F						E T	A G								Q D				S L
1034	V N		A							V	G N	Q R Y	T					S	A S	
1037	V F						E	A G								Q D				S L

E1 E2 Position	E431	N434	L438	F442	Q444	A466	D481	Q482	K500	S522	E531	T558	F560	V570	D641	E655	A642	S686	V720	V731
Polyclonal IgG																				
1112													D							
1012									L S					N K I	N					
1013		D							L S					N K I	N					
1034	A	D E T	V		Y	D	E G	D H		A L	S D	S				D	V	T	I	I
1037		T		L					L S					N K I	D N					

Figure 4.10 Amino acid (aa) position changes in HCVpps in sequences resistant to neutralisation by A) monoclonal antibodies and B) polyclonal IgG derived from patients.

The ability of antibodies (either HmAb or patient IgG) to neutralise the 11 E1E2 HCVpps in the gt1a panel was determined as described in Materials and Methods. Subsequently the sensitivity of the panel to neutralisation by each individual HmAb / patient IgG was considered in turn. For the antibody being tested, the 11 E1E2 sequences from the IG1 HCVpp panel was categorized as resistant (<50% neutralisation), intermediate (<60% neutralisation) or sensitive (>60% neutralisation). An alignment of these 11 sequences was then analysed to determine if there were any aa changes only present in sequences resistant to neutralisation by the antibody being tested. Amino acid substitutions present in a majority of resistant +/- intermediate sequences but not in any sensitive sequences were identified. These were classified according to number of resistant sequences containing the mutation as follows: Weak (shown in green) present in 50% of resistant sequences and at least one intermediate sequence, Moderate (shown in orange): present in >50% of resistant variants +/- present in intermediate variants. Strong (shown in red): Present in all resistant variants or a majority of resistant variants and a majority of intermediate variants. Aa positions are labelled. Those aa positions highlighted in red type were previously identified by Bailey et. al (2015)'s study of a intergenotypic panel tested against a number of monoclonal antibodies, including HmAb HC-11(292).

Antibody binding regions

AP33 HCV1 HC33

HC-11 AR3A AR3B AR3D HC84

HC-1 HC-11 CBH5 AR3A AR3B AR3C AR3D

CBH7 (possible)

CBH-4D

HC84-27 (possible)

Previously Identified Escape Mutations

Possible Resistance Mutations

Conserved aa

Variable aa

Functional and Structural features

HVR1

HVR2

Hydrophobic heptad repeat

Transmembrane domain

Amino Acid Position

384 400 450 500 550 600 650 700 746

Key:

Neutralisation

Alanine Scanning

Entry characteristics

HVRs

Conserved aa

Variable aa

Key CD81 binding site

Cysteines

Glycans

Figure 4.11 Possible aa positions of resistance mutations to monoclonal bNAb and polyclonal purified patient IgG marked on linear E2 protein.

This figure shows a linear representation of E2 with aa positions of interest marked as per the key, Aa position mutations which were present on E2 sequences relatively resistant to neutralisation by monoclonal bNAb or polyclonal patient derived IgG characterised as having bNAb were identified as described above. In addition, previously published aa mutations which have resulted in escape from antibody binding and neutralisation or a change in cell entry characteristics are marked on the figure. The details and references for these are given in table 4.1 and table 1.2. Other structural features of the polyprotein and conserved and mutated regions in the sequences obtained from our cohort from dN/dS analysis (see Figure 4.1) are marked as indicated.

4.6 Differential use of the cell entry protein SRBI

4.6.1 Use of the SRBI receptor and resistance to neutralisation

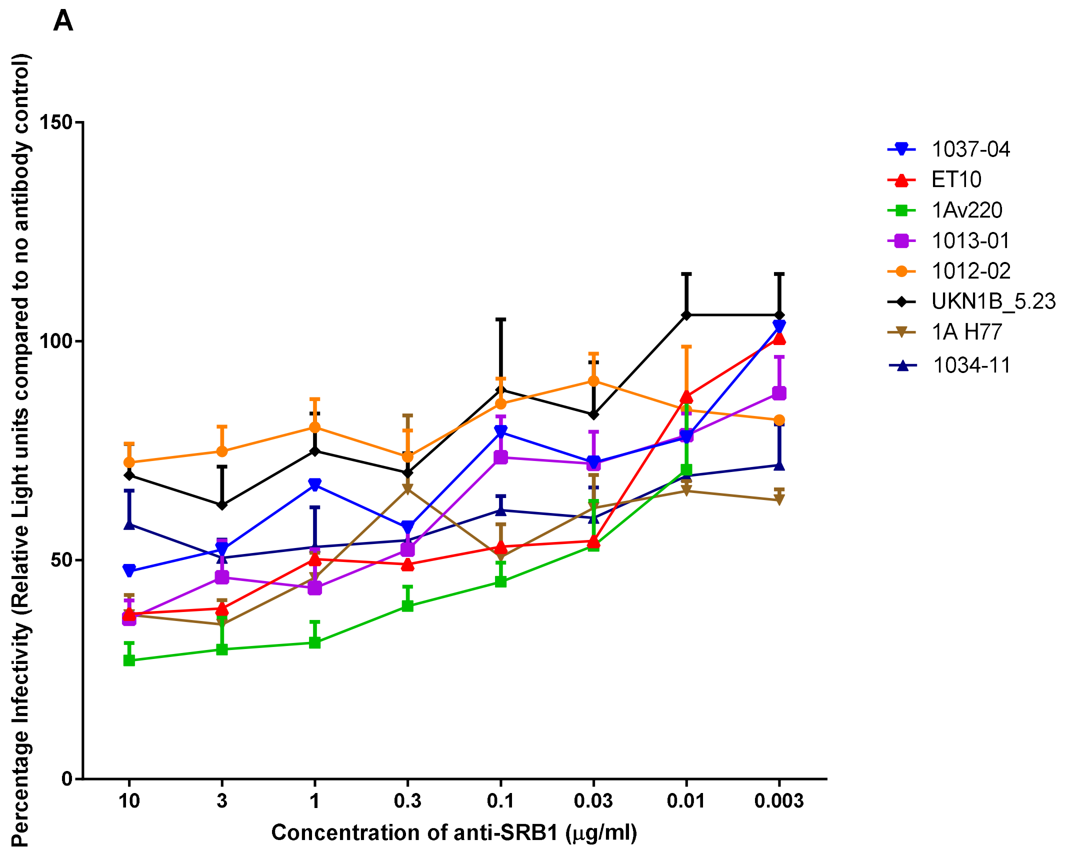
The SRBI receptor is proposed to play an important role in HCV cell entry by binding to the E2 HVR-1 and inducing a conformational change in the envelope polyprotein which exposes the CD81 binding regions of E2(283). *In vivo* SRBI primarily functions as a high affinity receptor for HDL incorporating Apolipoprotein A-I(114). HCV lipoviral particles also incorporate this apolipoprotein which may enhance the use of this receptor to gain cell entry(301). In addition, HCV envelope protein mutations have been discovered that increase or decrease usage of SRBI and in some cases, alter sensitivity to neutralisation (285, 290). SRBI dependence, unlike CD81 binding, can vary without necessarily compromising infectivity, as HCVpp are able to infect hepatocytes in the absence of HVR-1(286). In the dN/dS analysis of sequences from the CHCV cohort, HVR-1 was identified as the most variable region (Fig. 4.2).

As discussed above, some HCVpps from the IG1 panel were less sensitive to neutralisation by patient IgG compared to others when expressed in the pseudoparticle system, namely 1012-02,1013-01, UKN1B_5.23 and 1Av220 (see Chapter 3 and Table 4.6). In addition, within patient 1012 sequences, increased sensitivity to autologous neutralisation was noted in sequence 1012-01 (Fig. 4.5a).

Variable SRBI usage could explain some differences in neutralisation sensitivity observed in the patient-derived sequences. Therefore the diverse IG1 panel of 11 HCVpp and four functional HCVpp derived from patient 1012 were tested for their

infectivity in the presence of serial dilutions of anti-SRBI antibody (further details in Materials and Methods).

Among the IG1 panel of HCVpps, 2 of the sequences most resistant to neutralisation by monoclonal antibodies and polyclonal patient derived IgG also had reduced sensitivity to SRBI blockade (1012-02 and UKN1B_5.23) compared to the rest of the panel (Fig. 4.12a). However, sequence 1Av220, which was the most resistant to neutralisation in the IG1 panel, was sensitive to SRBI blockade compared to the other HCVpp tested. In addition, while sequence 1012-01 is more sensitive to neutralisation by autologous IgG than the other patient 1012 derived sequences, there was no appreciable difference in SRBI sensitivity (Fig. 4.12b: note no isolate had infectivity reduced by 50% even at a concentration of 10µg /ml of anti-SRB1 antibody).This suggests that even if SRBI dependence is responsible for modulating sensitivity to neutralisation in some isolates, this is not a universal feature.



Sequence	IC50
1037-04	3.11
ET10	1
1Av220	0.03
1013-01	1.12
1012-02	>10
UKN1B_3.23	>10
1A H77	2.55
1034-11	3.11

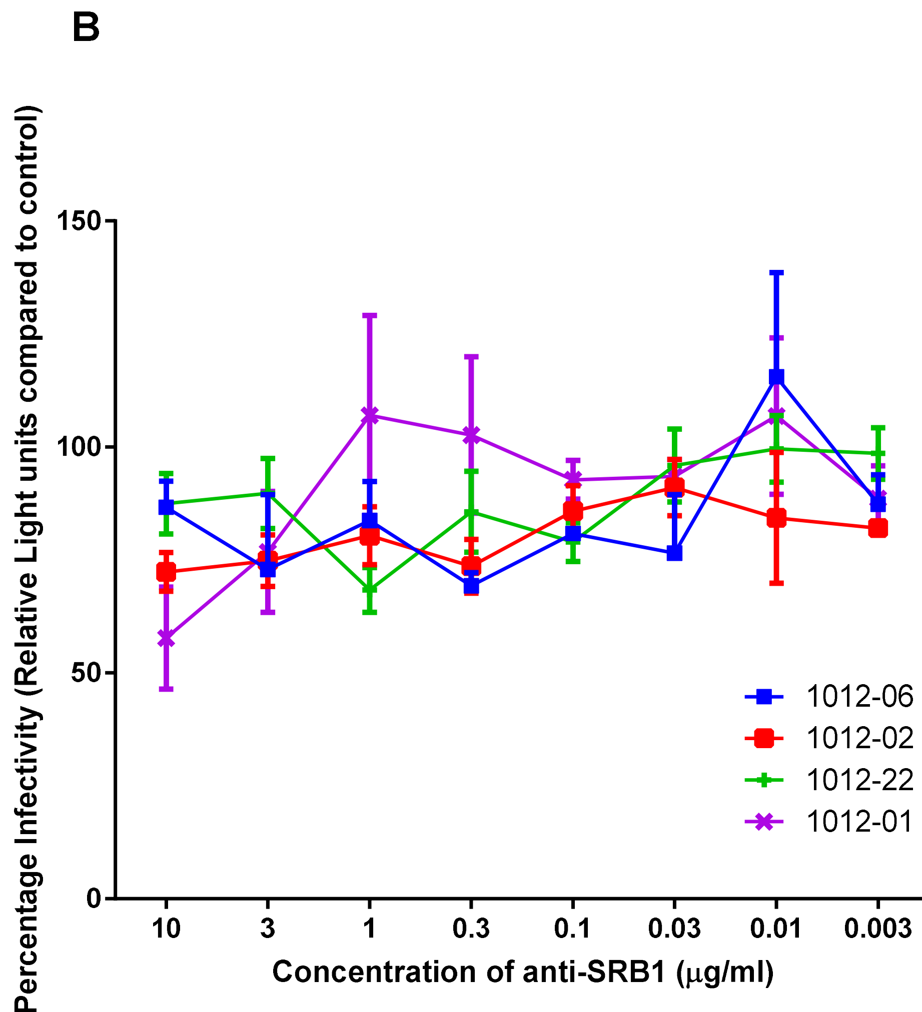


Figure 4.12: Comparison of sensitivity of HCVpp to SR-BI blockade.

A selection of HCV pseudoparticles derived from patient sequences were tested alongside the standard laboratory sequences H77 1A for ability to infect Huh7 pre-incubated with increasing concentrations of anti-SRBI antibody as described in Materials and Methods. A) Infectivity of HCVpp bearing sequences derived from different patients are shown against concentration of anti-SRBI antibody. IC50s were calculated in GraphPad Prism 6 and displayed in a table below the graph. B) Infectivity of HCVpp bearing sequences derived from a single patient (1012) are shown against concentration of anti- SR-BI. As no concentration tested inhibited infectivity by 50% IC50 calculations were unreliable and are not included in this graph. The results presented represent three independent experiments with each sample tested in duplicate, due to a shortage of HCVpps Av220 was not tested at the lowest concentration of anti-SRB1. Error bars are the SEM.

4.7 Chapter 4 Discussion

4.7.1 Epitope targets of polyclonal bNAbs

Our data suggests that those gt1-infected individuals who mount a broadly neutralising response direct antibodies at more than one neutralising domain on E2 i.e. domain B (targeted by HmAb HC-11) and domain C targeted by HmAb CBH-7. In contrast, those with a narrower neutralising response appear to target only one region, usually that recognized by the HmAb HC-11. Previous studies have shown that different regions of E2 interact to prevent neutralisation, with some requiring a conformation change to expose CD81 binding epitopes, therefore it is likely that an antibody response interfering with multiple regions of E2 may be more effective than a IgG targeting a single epitope (286). This is in keeping with recent data from Carlsen et al which demonstrated synergy in neutralisation when using a combination of two antibodies against differing viral glycoprotein domains(126) although a study examining the effect of AP33-like antibodies found interfering effects at lower concentrations(135).

Bioinformatics analysis of E2 sequence and susceptibility to neutralisation by polyclonal IgG has confirmed the presence of antibodies to previously characterized targets such as the HC-11 binding sites. In addition, it also identified a linear epitope, aa 632-642, as having importance in antibody escape. This region has not been previously documented as having importance for bNAbs but has been shown to impact on CD81 binding in alanine scanning models(145). This finding requires further confirmatory testing *in vitro*.

Identification of epitopes targeted by bNAbs was restricted to the range of sequences contained in the HCVpp panel and by the monoclonal antibodies used. It would be possible to use alternative methods such as peptide and phage display capture, which divide E2 into small linear epitope portions to capture antibodies directed at these portions, to explore additional regions which may be important in bNAb binding. Studies of small cohorts of patients have characterised different immunophenotypes of anti-HCV antibody responses using such methods. However, these have limited ability to detect antibodies directed at discontinuous epitopes (118) therefore our data are a valuable complement to information that might be gained from such studies.

4.7.2 Information gained from dN/dS ratio analyses

While this analysis of individual amino acid mutations has focused on a small number of selected individuals, studying their viral sequences and antibody responses in depth yields some interesting findings. Examining dN/dS ratios highlights different domains which are likely to be under immune pressure from the host. Some of the variable regions identified corroborate information already gained from competition ELISAs indicating mutation to escape from immune pressure in known CD81 and HmAb binding regions such as aa446 and 532. Despite this, a few individuals had evidence of persistence of envelope sequences which were more sensitive to neutralisation by autologous IgG. While one might expect these variants to be eradicated in favour of more resistant variants, a recent paper has suggested that persistence of more antibody reactive variants may be beneficial to the persistence of resistant variants by maintaining an ineffective antibody response (termed antigenic co-operation)(302).

While non-conserved regions (i.e. positive dN/dS) were less consistent between individuals, some common areas of positive selection were highlighted. As expected, the highest ratios were found in E2 HVR-1 however some sites in E1 and less well studied regions of E2 were revealed (e.g. aa 241,253,359,501 and 646) , particularly in individuals with broadly neutralising antibodies. While it has not been possible to study these sites during the course of my research due to time constraints, future work involving identification and isolation of antibodies binding to these regions may help determine if they are contributing to the broadly neutralising response.

4.7.3 Amino acid changes associated with resistance to bNAb and mAb neutralisation

Despite being a marker of immune pressure, the dN/dS ratio data did not highlight all of the amino acid mutations associated with antibody resistance in the HCVpp panel. This may be because mutations which confer an advantage may already have been heavily selected for in the population. Therefore, evidence of this selection may be lost by sampling at a single time point. In addition, there are many limitations of clonal sequence analysis in analyzing viral population diversity and the number of clones we obtained was relatively small. However, studying within patient evidence of selection is a powerful tool which can give a more accurate picture of the effect of intra-host pressures than a simple comparison of all sequences stored in online databases. Next generation sequence analysis of the whole envelope proteins and bioinformatics analysis of neutralisation against larger panels of envelope sequences will provide more robust tools for identifying areas of selection pressure from neutralising antibodies and escape.

The analysis of aa mutations in resistant IG1 HCVpp sequences yielded a number of possible resistance mutations. Some of these have already been reported as

conferring resistance to bNAbs, in particular aa 438 for domain B antibodies(292). Despite this, polyclonal IgG from the broadest neutralising individuals tested were able to prevent infection by sequences which appeared resistant to neutralisation by the 'broadly neutralising' monoclonal antibodies in our panel. For example, patient 1012, from whom the sequence 1012-02, was derived, was able to neutralise this pseudoparticle with an IC-50 of 26.1 μg/ml. This suggests some of the antibodies contained within the isolate were capable of preventing cell entry despite mutations conferring resistance to anti-HCV IgG responses from other hosts. This may be due to production of a more potent bNAb, or may be explained by a synergistic effect of multiple bNAbs present in the polyclonal IgG response.

In addition to the previously known aa substitutions conferring resistance to HmAbs, several new mutations which may be associated with escape from neutralisation were identified. In particular for the less well characterized HmAb CBH-7 aa 570 was identified as a possible site for escape mutations. Interestingly this site was one of the few not tested for its effect in Pierce et al's study using alanine scanning to identify key antibody binding sites(145, 303).

4.7.4 SR-B1 dependance and sensitivity to neutralisation

It is intriguing to note that two of the most poorly neutralised E1E2 sequences in the panel tested (1012-02 and UKN1B_5.23) are relatively insensitive to SRBI blockade. This may be due to escape from the effect of anti-HVR-1 antibodies, produced in the majority of patients who progress to chronic infection. However, this would not explain its insensitivity to neutralisation by monoclonal antibodies targeting the CD81 binding regions. E2 HVR-1 has been proposed to have an important effect in shielding CD81 binding sites from the immune response, therefore structural changes which alter dependence on SRBI may also impact on

the ‘shielding’ effect of the hypervariable regions(286). However, a recent paper by Zuiani et al identified 9 mutations in the Gt 2a JFH-1 envelope sequence which increased sensitivity to bNAbs HC84.28 and H77.39 (which binds a similar region to AP33) and reduced dependence on SRBI for cell entry (304). This suggests that from the relationship between bNAbs sensitivity and SRB1 is complex.

Our data has identified several regions on E2 which may be responsible for escape from bNAbs, some of which have not previously been described. The impact of single aa mutations on neutralisation sensitivity is difficult to define, as mutations conferring resistance in one viral strain may not have the same effect in another, presumably due to compensatory changes elsewhere in the envelope sequence(292). Further investigation of patients with bNAbs and *in vivo* escape of envelope proteins from recognition by these antibodies will help inform vaccine design. In addition, further work could include directional mutation of sensitive sequences to introduce amino acid changes at these sites to evaluate their impact on infectivity and resistance to neutralisation.

4.7.5 Limitations of the findings

While much previous work on neutralisation has focused on experiments using the HCVpp system (123, 276) as I have done in this set of experiments, this system does have some limitations. As mentioned previously, the structure of HCVpps is not identical to the envelope of live virus, especially as it does not incorporate lipid which can mask important epitopes(161). In addition, the assumption through studying neutralisation in vitro is that antibodies eliciting a strong neutralising response would likely be effective in preventing infection *in vivo*. However, in humans, many sub-viral particles are produced which do not contain viral

RNA(141). As these particles have no infectious potential but bind neutralising antibodies, these may reduce the impact of the antibody response *in vivo*.

4.8 Chapter 4 Summary

- Hepatitis C virus entry characteristics, the viral epitopes targeted by the host IgG response and the neutralising efficacy of this polyclonal response vary widely between individuals and viral quasispecies.
- Individuals with broadly neutralising polyclonal IgG tend to compete with the non-overlapping monoclonal antibodies HC-11 and CBH-7 suggesting they target multiple CD81 binding epitopes.
- Bioinformatic study of the ability of patient derived envelope sequences to form functional HCVpp, their sensitivity to neutralisation and cell entry protein usage can yield useful information on single aa sites which may play a crucial role in these processes.
- While the CBH-7 epitope has not been fully mapped, amino acid substitutions at E2 positions 416 and 570 may impair its ability to neutralise.
- Mutations in the region surrounding aa 608 are likely to have a role in maintaining the structure of the E1E2 heterodimer to allow it to retain function, and mutations around the putative fusogenic region of E1 (aa 274 to 291) may also have a significant effect on cell entry.
- Insertion of an extra glycosylation site at position 476 in the HVR2 region of E2 may have an effect on sensitivity to neutralisation.
- Dependence on SRBI for cell entry varies widely between gt 1 sequences. Lower levels of dependence on SRBI may protect against neutralisation

from antibodies raised *in vivo*, however this is not a universal feature of 'hard to neutralise' gt 1 E1E2 sequences.

5 Association of anti-HCV envelope antibody reactivity and neutralisation with long-term liver outcomes in a historically recruited cohort

5.1 Introduction

In Chapter 3, I described an association between bNAb antibody responses and lower levels of liver fibrosis in chronically infected individuals. The association between anti-HCV antibodies and liver outcomes has also been noted by Hamed *et al* (277), albeit using total IgG binding to E1E2 by ELISA as a predictor. Anecdotal reports have also reported HCV-infected individuals with inherited hypogammaglobulinaemia and those treated with the anti-B cell monoclonal antibody Rituximab have a more aggressive disease course suggesting antibodies have a protective effect (270, 271).

This contrasts with the understanding that hepatic damage induced by HCV is largely immunopathic; that is, when the immune response fails to clear viral infection, inflammation, necrosis and subsequent fibrosis result in the continuing presence of virus in hepatocytes. While much of this damage occurs through direct activation of the innate inflammasome by HCV, the adaptive immune component may contribute by local release of pro-inflammatory mediators and through cytotoxic effects (305). Therefore, induction of immune responses without clearing virus may have a deleterious effect.

Determining any causative effect that antibody responses have on disease outcomes is challenging in HCV infections as pathology develops over many years and in the present era, guidelines recommend treating all of those infected. Many infected individuals also have other risk factors for progression of liver disease(218). Here, I aimed to explore the hypothesis that a broad anti-envelope

antibody response early in the liver disease process is associated with rate of progression of liver disease, using samples and clinical data from a cohort of historically recruited individuals with HCV infection.

5.2 Selecting the Progressor and Non-Progressor cohorts

For this study, ethical approval was granted to use serum samples from a tissue bank of HCV infected samples from 198 individuals taken between1999 and 2005. These individuals had been recruited by Professor Peter Mills (Gartnavel Hospital, Glasgow) as part of a liver biopsy study, and each subject had clinical data recorded, liver disease staging with liver biopsies and serum samples taken at the point of recruitment. Clinical data was maintained in a database with follow up outcome data being available for 98 patients within the database. For simplicity, I will refer to this group of individuals as the 'Historical cohort'.

I used anonymised clinical and biopsy data to identify two groups of patients of interest from this cohort:

'Progressor' group: defined as those with a Metavir fibrosis score of 0, 1 or 2 on their initial liver biopsy who went on to develop clinical cirrhosis as evidenced by further biopsy, Fibroscan >12.5 kpa, clinical decompensation, development of varices on endoscopy or confident radiological evidence of cirrhosis.

'Non-Progressor' group: defined as those with Metavir fibrosis score 0, 1 or 2 at the time of sampling who did not show progression to cirrhosis as evidenced by subsequent biopsy or Fibroscan. Those achieving an SVR within 5 years of sampling were excluded, as were those with other liver disease on biopsy.

As age and genotype can have an influence on liver disease outcomes, for each of the 'Progressor' patients, 2 Non-Progressors infected with the same genotype and of a similar age were included. For 2 of the Progressor individuals, only 1 suitable Non-Progressor match could be found. Final demographics of the 25 selected patients are shown in Table 5.1.

Table 5.1 Demographics of Progressors and Non-Progressors from Historical Cohort

	Progressors (n=9)	Non-Progressors (n=16)	*P*-value
Age at sampling (years) (range)	35 (r 24 - 44)	37.5 (31-47)	0.71
Median year of sampling (range)	1999 (1999-2002)	2001 (1999-2004)	0.22
Gender – Male (%)	6 (67%)	7 (44%)	0.41
IVDU (%)	8 (89%)	8 (50%)	0.09
Duration of infection at cirrhosis or censor (years) (range)	23 (8-31)	18 (8-33)	0.07
Alcohol excess (%)	4 (44%)	6 (38%)	1.0
Ever treated (%)	0 (0%)	2 (13%)	0.52
Genotype 1 (%)	5 (55%)	7 (44%)	0.69

P-values calculated in SPSS v.22 using the Chi squared test for categorical data and t-test for continuous data. Alcohol excess was recorded in the clinical database if hospital or GP records included a diagnosis of alcoholic liver disease, alcoholism, alcohol withdrawal or regular consumption of >50 units of alcohol per week.

5.3 Cross genotypic-HCVpp-neutralising IgG shows a trend to association with non-progression

For each of the selected individuals, serum IgG was purified according to the protocol described in Materials and Methods. This IgG was then tested for its ability to prevent Huh7 infection by HCVpp bearing envelope sequences from the cross-genotypic (XG) panel (gt 1a,1b, 2a, 2b, 3a and 4) as described in Chapter 3 and Materials and Methods. Briefly, subject IgG was incubated with HCVpp at a final concentration of 100µg/ml and the mixture added to Huh7 cells. The Luciferase signal from HCVpp incubated with IgG was compared with control wells where only PBS had been added. Healthy control IgG was also used as a negative control. Individuals reducing infectivity by a mean of >50 % were recorded as having neutralising activity.

The number of genotypes neutralised by the Progressor and Non-Progressor groups are compared in Fig. 5.1, where no association was seen between number of genotypes neutralised and progression status. Interestingly, the number of genotypes neutralised by the historical cohort as a whole was fewer compared to the CHCV cohort (*P*=0.006, Fig. 5.2). Only 5 individuals in the historical cohort neutralised >4/6 subgenotypes. Therefore to allow sufficient numbers to compare samples better at neutralising with those poor at neutralising, all subjects able to neutralise 3 or more subgenotypes were categorised as “broadly neutralising” and the remainder as “narrowly neutralising”. A Kaplan Meier plot was generated to determine time to progression or censor in the broadly and narrowly neutralising groups, this showed separation of the survival curves with narrow neutralisers tending to have a shorter time to cirrhosis, however this difference was not significant (Fig. 5.3 P=0.18). Finally the demographics of those with broadly neutralising and narrowly neutralising phenotypes were compared. No significant

differences were observed but those with a broader binding profile tended to be older at time of sampling (Table 5.1).

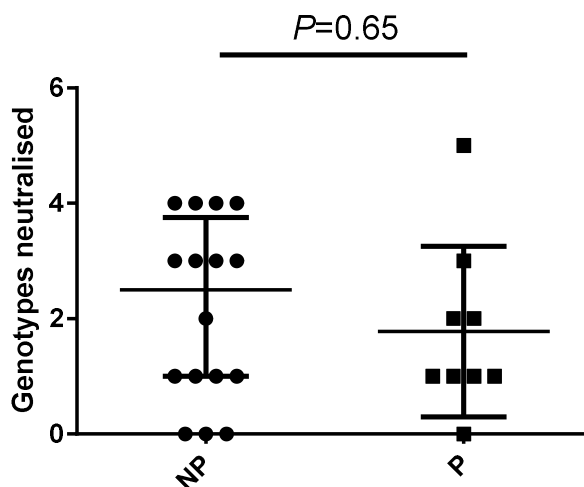


Figure 5.1 Number of XG HCVpp neutralised by purified IgG from Progressor and Non-Progressor individuals.

Purified IgG from each sample was tested for its ability to neutralise HCVpp bearing E1E2 from 6 different subgenotypes in the XG panel as described previously. The number of HCVpp subgenotypes neutralised by >50% is shown for individuals in the Progressor (P) and Non Progressor (NP) groups. The *P* -value was calculated using the Wilcoxon Rank Sum test. Median and interquartile range are shown.

Figure 5.2 Number of XG HCVpp neutralised by purified IgG from Historical Cohort and CHCV cohort

Purified IgG from each sample was tested for its ability to neutralise HCVpp bearing E1E2 from 6 different subgenotypes in the XG panel as described previously. The number of HCVpp subgenotypes neutralised by >50% is shown for individuals in the Historical cohort described in this chapter and the CHCV cohort described in Chapter 3. The *P*-value was calculated using the Wilcoxon Rank Sum test. Median and interquartile range are shown.

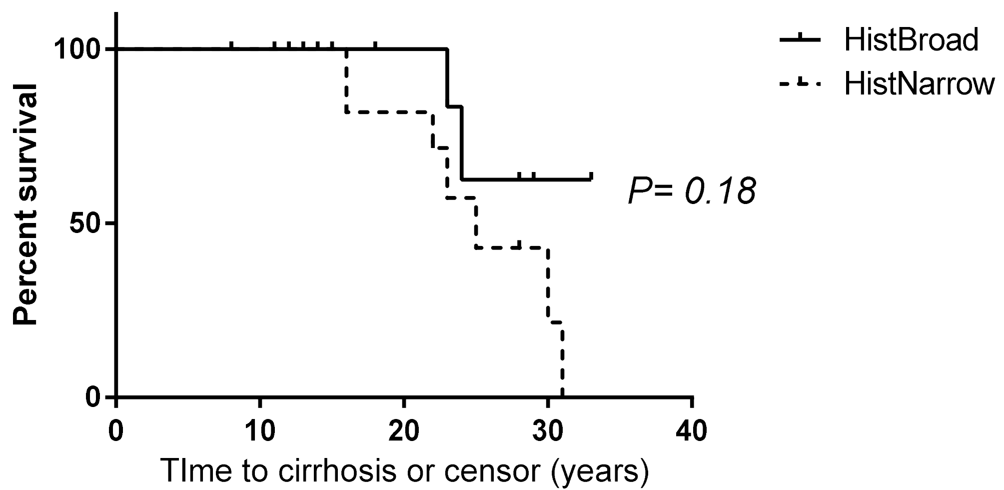


Figure 5.3 Kaplan-Meier plot of cirrhosis free survival in individuals from the Historical cohort stratified by breadth of neutralisation in the XG HCVpp panel

Individuals from the historical cohort (Progressors and Non Progressors) were divided into two groups - those who neutralised 3 or more of the HCVpp in the XG panel (HistBroad) and those who neutralised fewer than 3 (HistNarrow). The time from initial sample to development of cirrhosis (or censor where cirrhosis had not developed) was plotted. Censor was taken as date of death or last clinic appointment. Graph and statistical analysis was carried out using GraphPad Prism v.6. Censored data points are indicated by a small bar in the survival plot.

Table 5.2 Characteristics of broadly and narrowly neutralising historical cohort patients.

	Broadly Neutralising (n=10)	**Narrowly Neutralising** (n=15)	**p**
Age at sampling (years) **(range)**	38 (31-47)	34 (24-46)	0.16
Year of sampling (median)	2001 (1999-2002)	2001 (1999-2002)	0.71
Gender – Male (%)	4 (40)	9 (60)	0.42
IVDU (%)	7 (70)	9 (60)	0.69
Date of presumed infection (median) (range)	1988 (1978-2001)	1988 (1980-2000)	0.82
Time from infection until cirrhosis or censor (median years) (range)	23 (8-33)	22 (8-31)	0.75
Alcohol excess (%)	3 (30)	5 (33)	1
Treated before initial sample (%)	1 (10)	1 (7)	1
SVR post sample (%)	2 (20)	4 (27)	1
Genotype 1 (%)	3 (30)	9 (60)	0.22
Progressor (%)	2 (20)	7 (47)	0.23

P values calculated using SPSS v.22 using Chi squared test for categorical data, Wilcoxon rank sum for ordinal data and t-test for continuous data.

5.4 IgG binding to diverse E1E2 glycoproteins shows a trend to association with progression

IgG from individuals in the cohort was tested for strength of binding to E1E2 proteins of the subgenotypes from the XG panel expressed in HEK-293T cells as detailed in Chapter 3 and Materials and Methods. Briefly, lysate from HEK-293T cells transfected with plasmids containing E1E2 sequences from the selected subgenotypes was used in a GNA capture ELISA. A standard curve of humanised mAb AP33 (hAP33) (249) was used as a positive control.

As detailed in Chapter 4 Section 4, there is no accepted cut –off for a 'positive' value in this assay. Moreover, I was interested in comparing those with stronger and weaker signals in the cohort rather than ascertaining the presence of anti-envelope antibodies, which would be expected to be present in a majority of those with chronic infection. Therefore, to allow comparisons to be made within the cohort, individuals were ranked according to their relative absorbance to each genotype (with rank 1 being highest level of binding). The sum of ranks for the individual for each genotype was obtained and this final number used to rank the cohort for breadth of cross-genotype binding.

Time to progression was compared between the highest ranked (1-13) and lowest ranked (14-25) individuals using a Kaplan Meier survival plot. This showed a shorter time to cirrhosis in those with a broader glycoprotein binding profile (i.e. higher ELISA rank Fig. 5.4 P=0.049). A comparison between the capacity of IgG-mediated virus neutralisation and the IgG HCV E1E2 binding rank revealed a lack of correlation between two parameters (Fig. 5.5, Spearman's correlation coefficient r=0.05). This diagram also highlights the association of glycoprotein binding and

progressor status with progressors tending to cluster in the higher ELISA ranks, while non-progressors cluster in the lower ones.

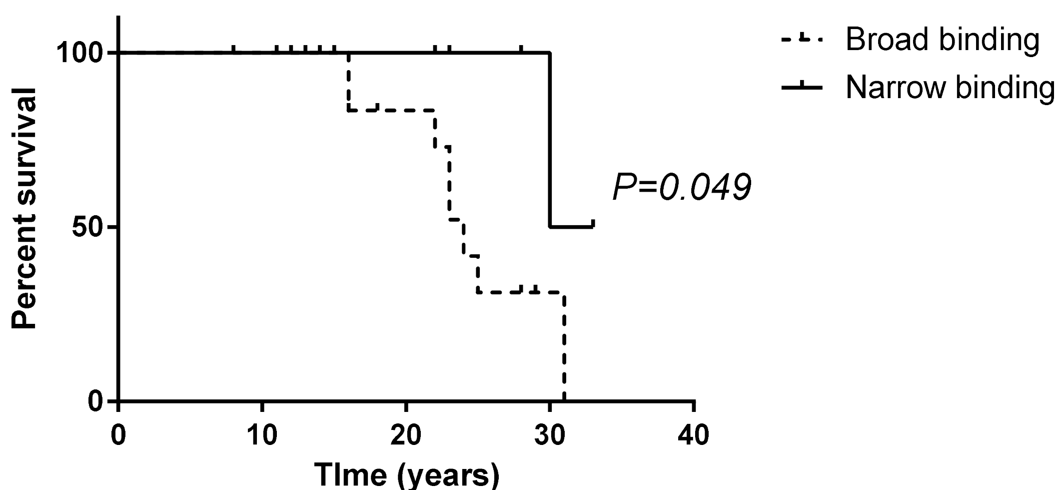


Figure 5.4 Kaplan-Meier plot of cirrhosis free survival in individuals from the Historical cohort stratified by breadth of binding to E1E2 from the cross genotype HCVpp panel

Purified IgG from individuals from the historical cohort was tested by ELISA for ability to bind E1E2 containing lysate from 6 different subgenotypes of HCV as described in Materials and Methods. Each individual sample was ranked for ability to bind each genotype with1 assigned to the highest absorbance reading and 25 the lowest. Each individual's ranks across the genotypes were summed and the individual given an overall rank. The cohort was then divided in half into the broadest binders (numerical ranking closest to 1, termed 'Broad binding') and the weakest binders ('Narrow binding"). The time from initial sample to development of cirrhosis (or censor where cirrhosis had not developed) was plotted for each group. Censor was taken as date of death or last clinic appointment. Graph and statistical analysis was carried out using GraphPad Prism v.6, censored data points are indicated by a small bar in the survival plot.

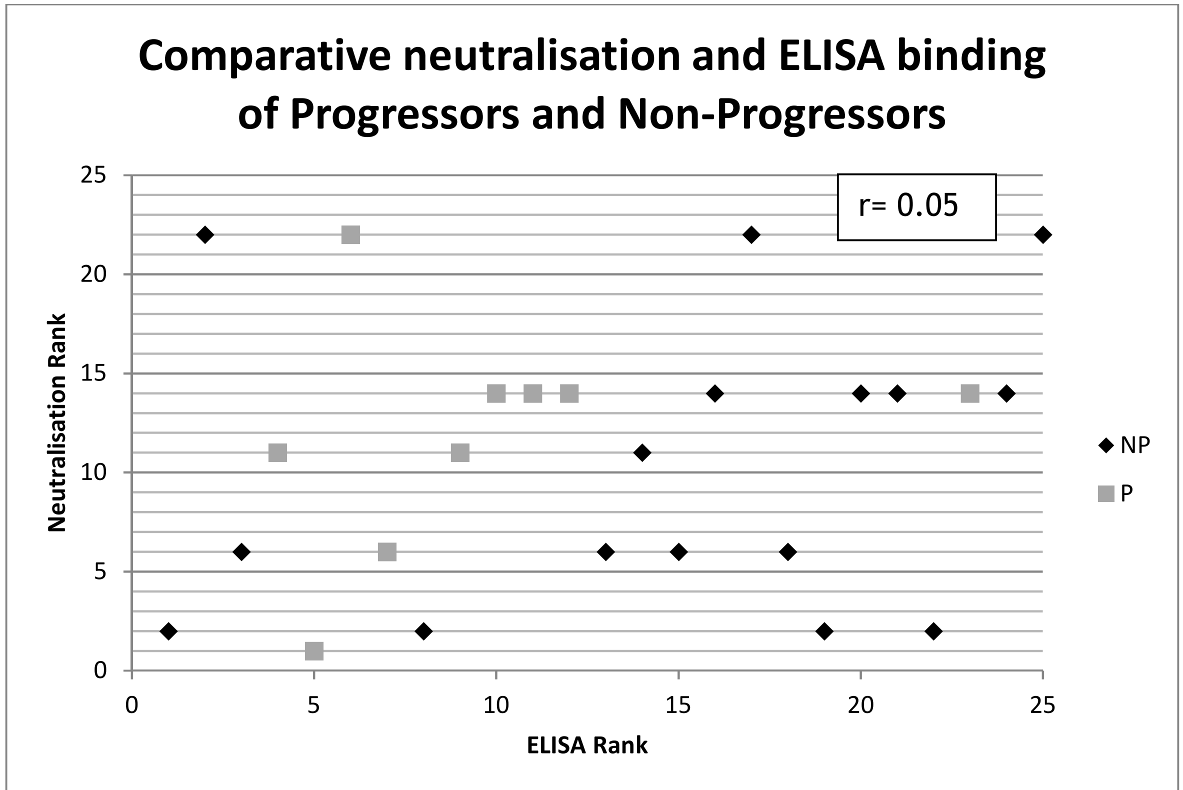


Figure 5.5 Scatter plot of historical cohort samples according to rank for breadth of neutralisation and ELISA binding.

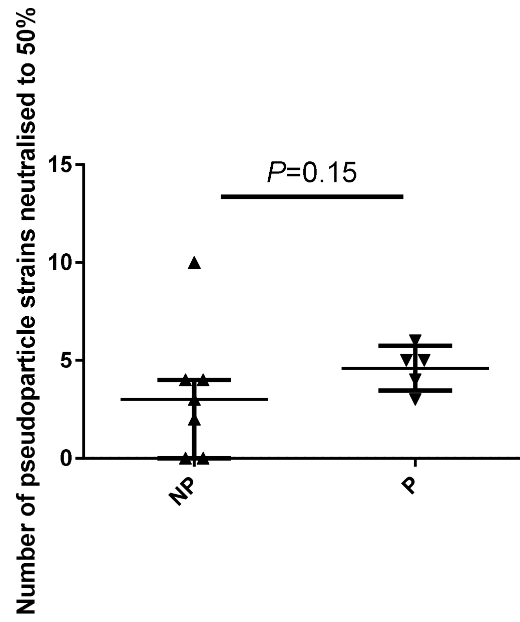
Purified IgG from individuals from the historical cohort was tested for breadth of neutralisation of HCVpp and ELISA binding to a cross-genotype panel of 6 subgenotypes as described in Materials and Methods. Each individual sample was ranked for ability to bind each with1 assigned to the highest absorbance reading and 25 the weakest. Each individuals ranks across the genotypes were summed and the individual given an overall rank. The individual samples were also ranked based on the number of HCVpps they could neutralise (1 being able to neutralise the most HCVpp types). The Spearman's correlation coefficient (r) was calculated and is shown on the graph.

5.5 Intra-genotypic neutralisation of gt 1 HCVpp by gt 1-infected historical cohort IgG shows no association with progression

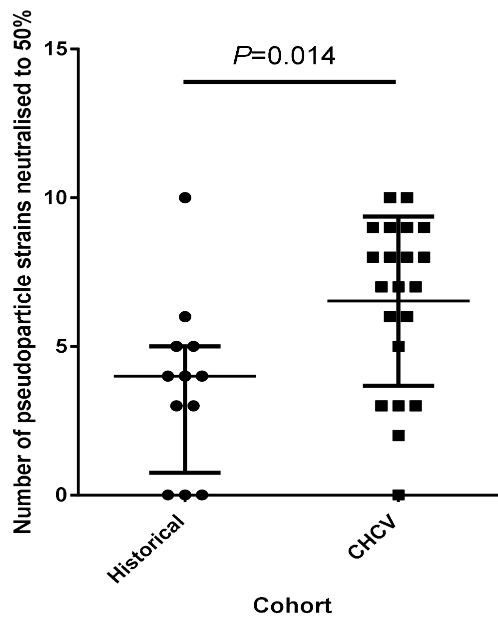
To determine if breadth of neutralisation within genotype 1 was associated with progression, IgG from the 12 gt 1-infected individuals in the historical cohort were tested for their ability to prevent the IG1 panel of HCVpps infecting Huh7 cells. This panel was developed as described in Materials and Methods and represents a wide variation of sequences at key CD81 binding regions within the E2 glycoprotein of gt 1 HCV. It should be noted that only 3/9 of the broadly cross-genotype neutralising samples were from gt 1-infected individuals.

There was no significant difference in number of HCVpp neutralised between Progressor and Non-Progressor individuals (Fig. 5.6a) although there was a possible, non-significant trend to greater numbers of HCVpp neutralised in the progressor group *(P*=0.16). Again, the overall number of HCVpp neutralised by the individuals in the gt 1-infected historical cohort was significantly lower than in the more recently recruited CHCV cohort (Fig. 5.6b).

A



B



Legend for Figure 5.6 on following page

Figure 5.6 Number of HCVpp strains in the IG1 panel neutralised by IgG from the Historical cohort.

A) IgG from gt 1-infected patients in the historical cohort were tested for neutralisation ability against the panel of 11 gt 1 pseudoparticles as previously described. A) The historical cohort results were split according to progression status (Progressors (P) and Non Progressors (NP)) and the median number of HCVpp neutralised was compared between the two groups using the Mann Whitney U test. B) Comparison of the number of IG1 panel HCVpps neutralised by the gt 1 infected subjects in the Historical cohort with the gt 1-infected subjects in the CHCV cohort. Number of pseudoparticle strains neutralised by both groups were plotted on the graph and a statistical comparison made using the Mann Whitney U test. Median and interquartile range are shown.

5.6 Breadth of intra-genotypic neutralisation of gt 1 HCVpp by gt 1-infected historical cohort IgG and association with SNP *rs9275224*

The SNP *rs9275224* in the HLA-DQB gene has previously been associated with breadth of neutralisation within genotype 1 and in Chapter 3 an association with gt 1 neutralisation breadth was demonstrated in the CHCV cohort. Here, I aimed to determine if there was any association between presence of the favourable alleles AG/GG and neutralisation breadth in the historical gt 1-infected individuals.

As no whole blood was available, DNA was extracted from serum of individuals in the historical cohort using the Qiagen Viral RNA kit without a DNAse step. 1µl of the eluted DNA solution was used as described in Materials and Methods with a custom Applied Biosystems qPCR probe for SNP typing. I was able to type 11 of the cohort using this method. Four of the cohort had the unfavourable SNP (AA), none of which neutralised more than 4/11 genotypes. However there were too few samples to demonstrate a clear relationship between SNP and neutralisation

breadth (Fig. 5.7a *P*=0.37). There was no relationship between cross-genotypic neutralisation and SNP type (*P*=0.78 Fig. 5.7b). In addition only one of the SNP AA individuals was in the Progressor group suggesting no strong relationship between the SNP and progression. Again, the numbers are too small to draw any firm conclusions.

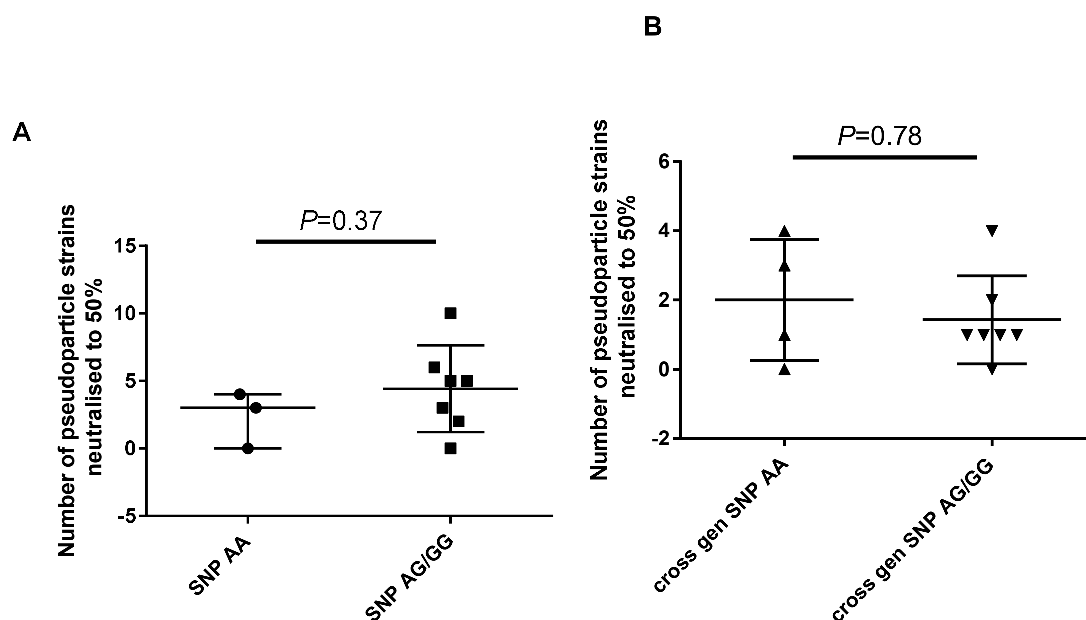


Figure 5.7 Association of rs9275224 SNP genotype with breadth of intra-genotype 1 and cross-genotype neutralisation

A) 10/12 historical cohort individuals tested in the intra-genotype 1 panel were typed for the HLA DQ-B1 SNP rs9275224 as described in Materials and Methods. The number of HCVpp strains neutralised in those bearing the AA SNP compared to the more favourable AG/GG SNPs were compared. B) The number of HCVpp genotypes neutralised in the cross-genotypic panel was compared by SNP rs9275224 genotype for the 11 individuals in the historical cohort who had been typed. Statistical comparisons were made using the Wilcoxon Rank Sum test. Median with interquartile range is indicated.

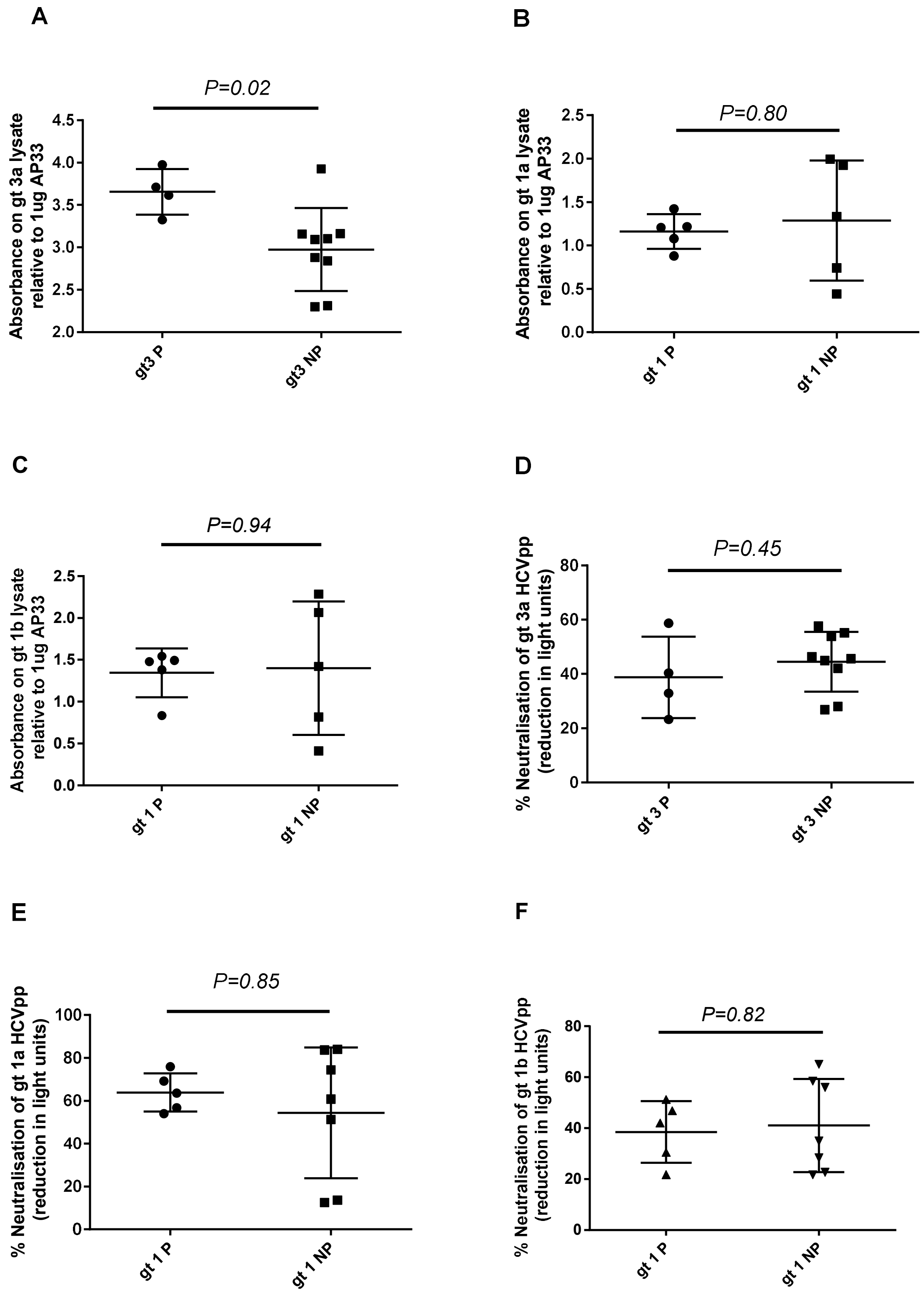
5.7 Genotype specific responses and progression status

As half of the progressor cohort were infected with genotype 3, I also analysed the ELISA and neutralisation data to explore whether genotype-specific responses were associated with progression status in this cohort.

When the strength of ELISA binding to gt 3 E1E2 in those infected with gt 3 virus was examined, those in the Progressor group displayed significantly stronger binding compared to the Non-Progressors (Fig. 5.8a P=0.02). However, no significant difference in glycoprotein binding to gt1a or gt1b was observed between P and NP groups in those who were infected with gt 1 (Fig 5.8b and c).

In those infected with gt 3, there was no association between ability to neutralise the laboratory prototype gt 3 HCVpp and progression status (Fig. 5.8d). Similarly, there was no difference between gt 1-infected individuals in the Progressor and Non-Progressor groups in their ability to neutralise HCVpps bearing gt 1a or gt 1b glycoproteins (Figs. 5.8e and f).

I further analysed the absorbance readings on ELISA against different genotypes, separating the samples by infecting genotype. Interestingly while those infected with gt 1 showed no difference in absorbance to gt 3 lysate between Progressor and Non-Progressor groups (Fig. 5.9a), those infected with gt 3 in the Progressor group had significantly higher binding to gt 1a (P=0.03) and 1b (P=0.02) by ELISA compared with gt 3-infected Non-Pogressor patients (Fig. 5.9b).



Legend for Figure 5.8 on following page

Figure 5.8 Analysis of the association of progressor status with strength of patient IgG binding to HCV glycoproteins (A,B,C) and neutralisation of HCVpp bearing HCV glycoproteins (D,E,F) derived from the same genotype as the subject's HCV infection.

Data from ELISA (A,B,C) and HCVpp neutralisation assays (D,E,F) conducted as described in Materials and Methods and as detailed in Sections 5.3 and 5.4 were further analysed to examine whether there was any association between progression status and individuals' ability to bind or neutralise envelope glycoproteins from the same HCV genotype that they were originally infected with. Bars shown are mean and standard deviation. Statistical comparisons were made using the Wilcoxon Rank Sum test.

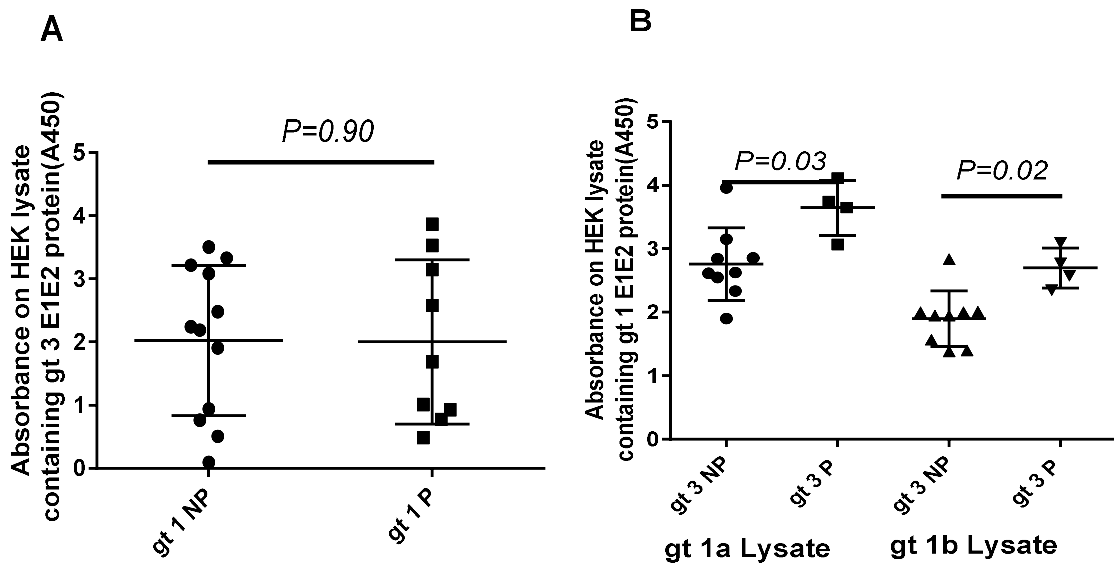


Figure 5.9 Analysis of progressor group and binding strength of patient IgG to HCV glycoprotein by ELISA from A) gt 3 in gt 1 infected individuals and B) gt 1 in gt 3 infected individuals

Data from ELISA assays conducted as previously described were analysed to examine whether there was any association between progression status and individuals ability to bind HCVpp bearing an envelope glycoprotein of a different genotype to that they were infected with. For gt 1-infected individuals, their ability to bind to gt 3 E1E2 lysate was plotted by progression status (A). For gt 3-infected individuals, their ability to bind to gt 1a and gt 1b E1E2 lysate was plotted by progression status (B). Bars shown are mean and standard deviation. Statistical comparisons were made using the Wilcoxon Rank Sum test. Mean and SD are indicated.

5.8 Chapter 5 Discussion

The findings from this historical cohort are limited by a number of factors including small sample size, variability of the cohort with multiple confounders and scarcity of samples. Nonetheless, the historical sample bank represents a valuable resource as it provides a rare snapshot of antibody status early in the disease course for those who are known to go on and develop significant liver disease. Such cohorts are challenging to obtain for many liver diseases, as typically liver pathology develops over decades with many individuals presenting to medical attention at a late stage in their disease process. It is unlikely that we could expand this HCV-infected cohort with prospective recruitment in the UK at the present time as advances in therapy should ensure that the majority of individuals presenting without significant liver fibrosis are treated before they progress to cirrhosis.

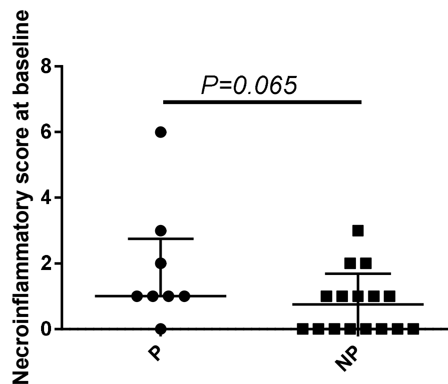
I attempted to determine if neutralising antibody status at baseline could predict development of significant liver disease. While no statistically significant association was observed, there was a possible trend towards faster disease progression in those without broadly cross-genotype neutralising antibodies. De Jong *et al.* have shown that neutralising antibodies have the potential to clear established HCV infection *in vivo* (144). A broadly neutralising response should be better able to cope with envelope sequence variation making viral escape more difficult. Reducing the number of hepatocytes infected with virus would be a plausible mechanism by which such antibodies could delay disease progression. However, it is possible that viral characteristics, such as slower evolution of virus, may be linked to both slower progression and a narrower antibody response.

The small sample size of this historically collected cohort makes it difficult to draw any firm conclusions as any effect would have to be very large to be detected in the presence of other potentially confounding factors. .

While broader neutralising antibodies may have a protective effect, there was a significant association between broader binding to viral glycoproteins by ELISA assay and progression status. This was particularly evident in the 4 individuals infected with gt 3 HCV who had progression of their liver disease. With such a small cohort, it is possible that there are confounding factors resulting in both a strong ELISA response and progression of their liver disease. However, from the clinical data available for these individuals, none had a history of alcohol excess and their other demographics (Table 5.1) were comparable to the cohort as a whole.

It is possible that high titres of non-neutralising anti-envelope antibodies may have a cytopathic effect. The degree of histological inflammation at baseline has been linked to progression in HCV (306). On reviewing the necroinflammatory scores on biopsies from the cohort there was a non-significant trend towards higher levels of inflammation in the progressor group (P=0.065 Figure 5.10a), however there was no association between levels of inflammation and glycoprotein binding as measured by ELISA (P=0.43 Fig. 5.10b)

A



B

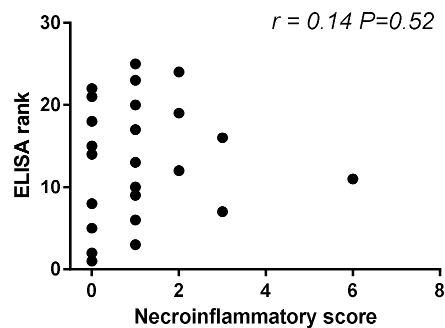


Figure 5.10 Liver biopsy necroinflammatory score at baseline with A) progression status and B) breadth of IgG binding to envelope glycoproteins of different genotypes by ELISA.

Histological Activity Index necroinflammatory scores for the historical cohort liver biopsies were taken from clinical pathology reports issued. Where none had been given but the description of inflammation levels was sufficient to allocate a score, one was assigned to that sample. There was insufficient information to assign an inflammation score to one of the biopsy samples which was excluded in this analysis. Scores were then plotted against A) Progressor status as defined previously (with median and interquartile range shown,) and B) ELISA binding rank determined as described previously from ELISAs using purified patient IgG binding to E1E2 from 6 sub-genotypes of HCV. In this case a rank of 1 indicates the strongest binding response across all sub-genotypes. The Wilcoxon Rank Sum test was used to calculate the P value in A. In B the value given as *r* is the Spearman's correlation co-efficient, a non-parametric measure of correlation with its corresponding *P* value.

Work from one group has suggested that a significant interfering effect from non-neutralising antibodies may exist (307). In those of the Historical cohort with the broadest ELISA binding, 6 were broad neutralisers and 7 narrow neutralisers. Within the broad ELISA/broad neutraliser group 2/6 progressed to significant liver disease whereas in the broad ELISA/narrow neutraliser group 6/7 progressed (P=0.10 Fishers exact test). While there are many possible confounders, this could indicate that those who have a broad binding but not neutralising response may have a tendency to more aggressive liver disease. The identification of individuals who had broad ELISA binding but narrow neutralisation also reinforces the findings in Chapter 3 that a neutralization response is not simply a function of overall levels of anti-'whole E1E2' antibodies.

Another interesting observation from this cohort was the difference in neutralisation breadth between this cohort and a more recently recruited cohort. There are several possible explanations for this:

1) These individuals were sampled earlier in their disease course than the CHCV cohort. The Historical cohort were sampled a median of 13 years following probable exposure to HCV wheras the CHCV cohort were sampled at a median of 23 years following probable infection. Neutralising antibodies usually take up to a year to first appear in chronic HCV infection (308). In addition, HCV envelope proteins tend to evolve over time to escape neutralising antibodies, with the humoral response lagging behind (309). It is likely that, in chronic infection, a polyclonal antibody response with neutralising properties develops in reaction to viral evolution and exposure to a variety of viral epitopes over time.

2) Degradation or denaturation of antibody samples over time. Serum from the historical cohort was stored at -70 for 10-15 years before use whereas the CHCV cohort antibodies were used within 1-2 years of sampling. While it appeared that none of the serum vials had been used prior to this study, it is possible that they were exposed to previous freeze-thaw cycles during their long period of storage. However, the anti-gt 1a and -gt 3 glycoprotein absorbance readings obtained by ELISA for these antibodies were comparable to those obtained for the CHCV cohort suggesting that similar quantities of antibodies with functional E1E2 binding ability remained in these samples although quality of these antibodies is harder to measure.

3) The sequences tested against are genetically distant to the virus that the Historical cohort was exposed to. While both the CHCV cohort and the Historical cohort were recruited from the same geographical region (Glasgow), HCV envelope sequences can evolve rapidly even within hosts. Therefore, it is possible that the circulating gt 1 and gt 3 viruses in Glasgow at the time these individuals were infected differ in important ways from the strains from which the gt 1 panel were derived (in samples recruited from 2006-2013). This alone would not explain the reduced level of neutralisation against the cross genotype panel. However, the viral population over time is shaped by adaptation to host responses, which balance viral fitness to infect and replicate against the need to escape adaptive and innate immunity (310, 311). If those infected with more 'modern' HCV sequences raise antibodies to a virus containing common 'escape' mutations their antibody response may target more conserved regions of the virus and be effective against a wider range of strains.

In an effort to explore this, we attempted to extract viral RNA from the stored serum and amplify E1E2 sequences using methods previously described. However no PCR product was generated for any of the samples suggesting RNA may have degraded. Further attempts to compare viral envelope sequences from these time points, perhaps using next generation sequencing methods, would help explore this theory.

5.9 Chapter 5 Summary

- Historically collected biological samples from individuals at an early stage of a chronic liver disease are a valuable resource to identify features which predict progression, albeit the small number in this cohort limits the conclusions which can be drawn from this data.

- A cross-genotypic bNAb response shows a non-significant trend to association with slower progression of liver fibrosis, however the numbers studied in this cohort are too small to draw any firm conclusions on influence on progression or host predictors of bNAb responses.

- Broad ELISA binding appears to predict more rapid progression of disease, particularly in gt 3-infected individuals and those who do not have a corresponding bNAb response. The mechanism which links these two features is not clear but interfering antibodies or immunopathic effects may play a role.

- The IgG samples in these historical samples have significantly narrower neutralising profiles than those collected from a contemporaneous cohort of chronically infected individuals. This may be due to i) duration of infection ii)

differences in infecting viral sequences or iii) deterioration of sample during storage.

6 Spontaneous clearance of HCV following viral relapse in the presence of potent, broadly neutralising anti-envelope antibodies

6.1 Introduction

Studying those who are able to control and clear HCV infection can yield valuable insights for vaccine design. While broad T cell responses were originally thought to be the most crucial for HCV clearance in the acute phase, a growing body of evidence has shown that early production of broadly neutralising antibodies (bNAbs) are also important (113, 131, 133, 137).

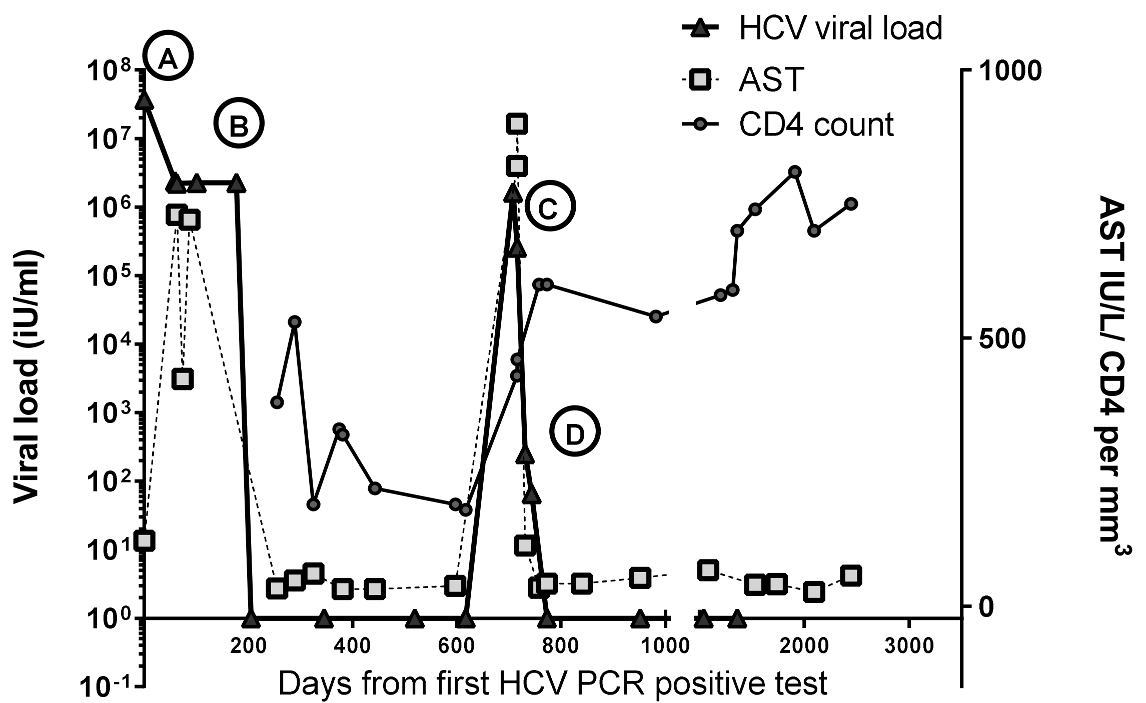
Previous work by Dr Emma Thomson had identified a cohort of HIV-positive individuals acutely infected with HCV (the St Mary's cohort – see Materials and Methods for further details). From this cohort of 99 individuals, six had undetectable viral levels on IFN-based therapy but relapsed following cessation of treatment. Next generation sequencing conducted by Dr Tamer Abdelrahman confirmed the relapsing strain was present prior to therapy indicating that these were cases of true relapse rather than reinfection (312). On clinical follow up of individuals with viral relapse, two were found to have spontaneously cleared their virus without further therapy. This is a newly described phenomenon which I have termed "secondary spontaneous clearance".As it follows a pattern of initial antigen exposure with subsequent re-exposure to the same virus followed by viral clearance similar to the principles of protection by vaccination, it may have particular significance for vaccine development.

Therefore, with permission from and in collaboration with Dr Emma Thomson, I planned to study the humoral anti-HCV response for one of these individuals infected with gt 1a HCV. I aimed to characterise this individual's antibody

response in detail during initial infection, relapse and subsequent HCV clearance to determine if neutralising antibodies could have played a significant part in this "secondary spontaneous clearance".

6.2 Clinical characteristics of the individual studied (patient ET)

For ease of reference I have termed the individual whose samples were used in this study ET. This individual was receiving antiretroviral therapy (HAART) for HIV infection at the time of developing acute HCV. Diagnostic laboratory testing of stored samples confirmed a HCV gt 1a infection with a duration of less than 3 months. As there was no drop in viral load after 12 weeks of presumed infection, 24 weeks of pegylated interferon and ribavirin therapy was commenced as was accepted standard therapy at the time (2009). Despite apparent viral clearance during therapy, 12 weeks later the individual was found to have detectable HCV RNA (Fig. 6.1) suggesting relapse. Illumina sequencing of the HCV E2 HVR-1 region at this point confirmed re-emergence of a minority strain (conducted by Tamer Abdelrahman, Abdelrahman et al, 2015, Hepatology) (312). However, on subsequent samples up to 5 years following relapse, the virus was not detected. Serum samples were available for viral sequencing and IgG purification from Timepoint A (at time of initial infection, TPA), Timepoint C (at time of relapse following treatment, TPC) and Timepoint D (taken less than 1 month following spontaneous clearance of infection, TPD). The sample from Timepoint B (mid therapy, TPB) was at a time during interferon exposure therefore while clinical laboratory results were available from this sample, serum from this TP was not suitable for testing for neutralisation ability and not used further in this study.



Clinical Timepoints:

A - HCV Infection detected **B** - Dual therapy commenced

C - Relapse detected **D** - "Secondary spontaneous clearance"

Figure 6.1 Clinical events and diagnostic laboratory tests during primary infection, treatment, relapse and secondary spontaneous clearance in patient ET.

Graph showing clinical events and laboratory parameters at these times (Liver enzyme ALT, HCV viral load and CD4 count reflecting the control of HIV infection). These samples were taken as part of the St Mary's cohort study and laboratory parameters measured using standard methods by the diagnostic laboratory at St Mary's hospital. Times of sampling for TPA, TPB, TPC and TPD are indicated on the graph as A, B, C and D.

6.3 Sequence analysis of whole E1E2 from TPA (pre-treatment) and TPC (relapse)

Viral RNA was extracted from serum from TPA and TPC using the QIAamp Viral RNA Kit (Qiagen) as per protocol. The E1E2-encoding sequence was amplified using degenerate primers for gt 1a with a directional tag to enable subcloning using the pENTR/D-TOPO directional cloning kit (ThermoFisher Scientific) (see Materials and Methods for protocol and Chapter 3 for further detail on PCR optimisation). Initial amplification was performed using this protocol by Ms Eva McGregor, all subsequent steps were performed by me).

The amplification products were gel-purified and subcloned using the pENTR/D-TOPO vector kit. For the sample from TPA, few clones were obtained using the pENTR kit. Therefore further subcloning was conducted using the TA cloning vector CloneJet PCR cloning kit (ThermoFisher Scientific) system. 20 colonies were selected for each TP, grown in selective media, plasmid DNA extracted using the Qiagen Miniprep kit and purified DNA sequenced by the Sanger method (GATC Biotech).

Only 6 complete unique sequences were returned for both time points. These sequences were aligned and consensus sequences generated using CLC Genomics Workbench 7 software. The translated consensus sequences for each time point were compared using a MEGA6 software package. This clonal analysis of the HCV envelope protein sequences demonstrated the majority variant of virus isolated before therapy (Timepoint A) and at relapse (Timepoint C) differed by 6 amino acids, mainly in the HVR1 region (T223A, V386F, T396A, G401S, K408R) and N574D (Figs. 6.2 and 6.3). The evolution of the HVR-1 region suggests this is likely to have been the target of an early, ineffective, adaptive humoral

response, similar to previously shown sequence evolution within hosts progressing from acute to chronic infection (140).

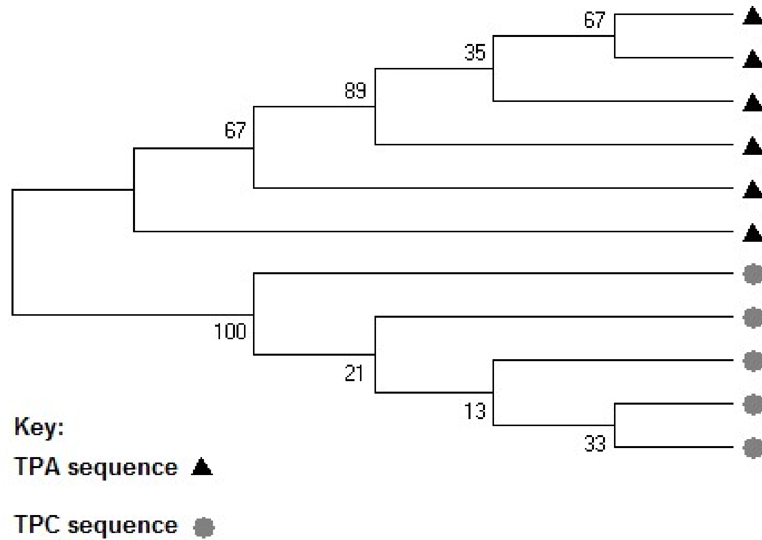


Figure 6.2 Molecular Phylogenetic analysis of sequences from TPA and TPC by Maximum Likelihood method

Sequences from both time points obtained by clonal analysis were aligned. From this alignment, the evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (257). The tree with the highest log likelihood (-2542.6456) is shown. The bootstrap value (i.e. the percentage of trees from 1000 generated using this data in which the associated taxa clustered together) is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Alignment and evolutionary analyses were conducted in MEGA6 (16).

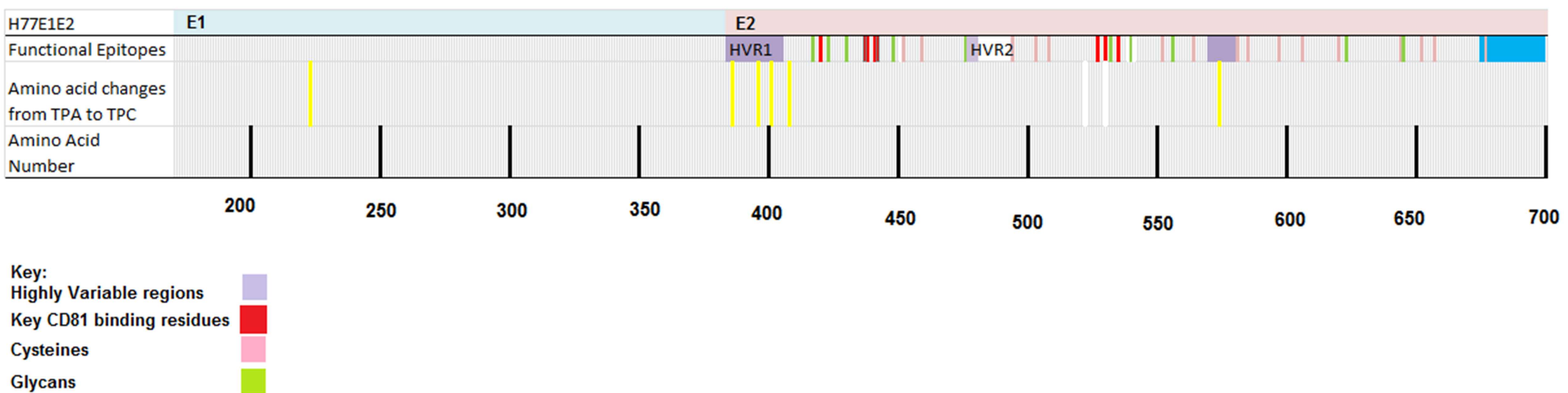


Figure 6.3 Position of variable amino acids between consensus sequence of TPA and TPC.

Clonal analysis of viral envelope sequences at TPA and TPC was performed as described above and as in Materials and Methods. After aligning sequences in Genomics CLC Workbench 7 to generate a consensus sequence for each TP. These consensus sequences were compared using MEGA 6.0 software and the sites of variability indicated in yellow above. Other structural features of the envelope protein are shown as detailed in the key.

6.4 [1]Infectivity and receptor usage of HCVpp bearing sequences from TPA and TPC is equivalent

The consensus E1E2 sequences from TPA and TPC were transferred into a phCMV gateway expression vector and used to manufacture HCV pseudoparticles (HCVpp) as described in previously in Materials and Methods. Media containing these HCVpp was harvested and their ability to infect HUH7 cells confirmed. HCVpps bearing TPA and TPC sequences were comparably infective on all assays (less than 0.5log difference in luciferase signal in relative light units, Fig. 6.4).

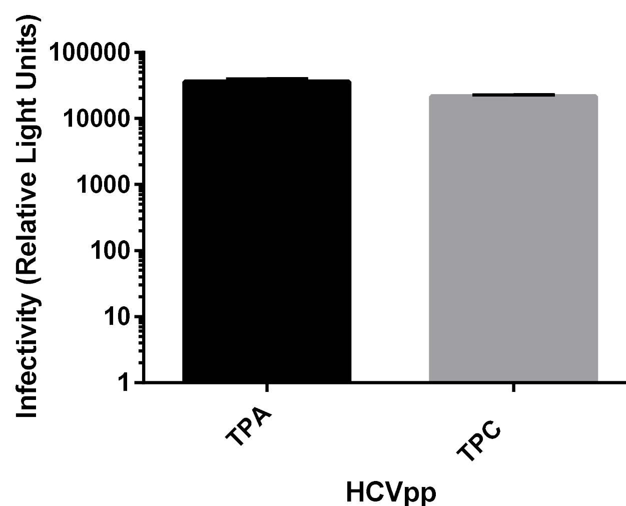


Figure 6.4. Relative infectivity of HCVpp bearing consensus envelope sequences isolated from TPA and TPC.

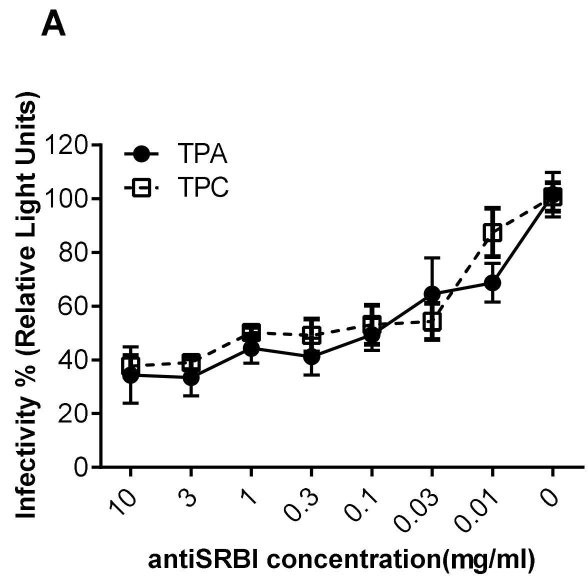
Sequences were isolated and HCVpp generated as described above and in Materials and Methods. 40 µl of HCVpp were used to infect Huh7 cells and relative infectivity determined using the BrightGlo Luciferase Assay (Promega). Results shown are from three independent experiments. Error bars show the SEM.

[1] NB the E1E2 sequence from TPC has been used in the IG1 panel under the name ET10

As HVR-1 mutations have been associated with adaptations in entry receptor usage, I aimed to determine if altered use of entry proteins was a factor in the differential ability of the host to control the primary and relapsed infection. Therefore I set out to assess the sensitivity of pseudoparticles bearing sequences from TPA and TPC to anti-SRBI and anti-CD81 antibody blockade.

Briefly, HCVpp were generated as described previously. Prior to adding these to Huh7 cells, the cells were incubated with 40 µl modified DMEM containing anti-CD81 antibody (clone JS-81, BD Biosciences) at decreasing concentrations from 3 µg/ml to 0.04 µg/ml or anti-SRBI mAb151-NP1 (252, 260) at decreasing concentrations from 10 µg/ml to 0.03 µg/ml. After 1 hour, the DMEM was removed and 40 µl of test pseudoparticles were added or DMEM added to control wells. These were incubated for 4 hours and then medium changed. Infectivity of pseudoparticles in the presence of cell receptor blockade was determined using a BrightGlo Luciferase Assay (Promega), as described previously, at 72 hours post-infection.

The above assays revealed both HCVpp were equally sensitive to anti-SRBI and -CD81 blockade. There was no appreciable difference between the receptor dependence of the two sequences (Fig. 6.5).



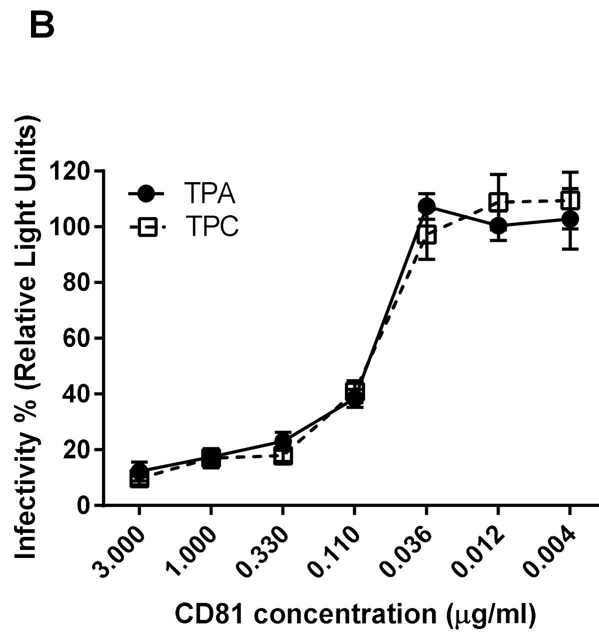


Figure 6.5 Sensitivity of pseudoparticles bearing E1E2 glycoprotein sequences derived from TPA and TPC samples to blockade of entry proteins A) SRBI and B) CD81.

As described in Materials and Methods, pseudoparticles were added to Huh7 cells which had been pre-incubated with serial dilutions of either A) anti-SR-BI antibody or B) anti-CD81 antibody in DMEM. Relative infectivity was determined by measuring luciferase levels as described in Materials and Methods. Results shown are from three independent experiments. Error bars represent the SEM.

6.5 Secondary spontaneous clearance is associated with neutralizing antibodies targeting E1E2 proteins

IgG from TPA, C and D were tested in GNA ELISA assays and HCVpp neutralisation assays for their ability to bind to and neutralise both previously generated HCV E1E2 sequences from different genotypes (the XG panel described in Materials and Methods and Chapter 3) and to neutralise HCVpp bearing autologous E1E2 glycoprotein sequences from TPA and TPC.

Briefly, ELISA analysis to detect antibodies against E1 and E2 glycoproteins was performed as described previously (313). ELISA plates coated with GNA were used to capture E1E2 glycoproteins from HEK-293T cell lysates for gt 1a, gt 1b and gt 3a. These are the same HCVpp from these genotypes used in the XG panel described in Materials and Methods. IgG purified from each time point was subsequently added at a concentration of 200 µg/ml and bound human IgGs were detected using HRP-anti-human IgG antibody (Sigma A0170) and TMB (3,3', 5, 5'-tetramethylbenzidine, Sigma) substrate. IgG from healthy controls (recruited at the same time as the CHCV cohort – see Chapter 3) were used as a negative control. A dilution curve of the humanised anti-HCV E2 mouse monoclonal antibody AP33 (129) was included as a control. Absorbance values were measured at 450 nm and normalised according to the AP33 curve to enable comparison of results obtained from separate ELISA plates. Purified IgG was also tested for its ability to prevent infection in vitro by HCVpp from the 6 subgenotypes of the XG panel (gt 1a, 1b, 2a, 2b, 3a and 4). These assays were conducted as described in Materials and Methods at a concentration of 100 µg/ml. Neutralisation assays were also conducted against the autologous HCVpp generated from TPA and TPC sequences. Due to the small volume of sample available for TPA, there was insufficient purified IgG available for use in this assay. Therefore serum from TPA,

TPC and TPD was heat-treated to deactivate virus and their ability to prevent infection from the HCVpp bearing autologous E1E2 sequences tested at serial dilutions of serum from 1:50 to 1:40000 in PBS.

While there was evidence of a weak anti-envelope antibody response from IgG purified from TPA (Fig. 6.6), this had no significant ability to prevent infection of Huh7 against the XG panel of HCVpp from differing genotypes (Fig. 6.7a). Serum from this timepoint was also unable to prevent infection by HCVpps bearing autologous viral sequences from both TPA and TPC, even at a 1:50 dilution (Fig 6.7b and c). In contrast, IgG and serum from TPC andTPD showed robust binding to and neutralising activity against a number of HCV genotypes (Fig. 6.6 and 6.7a) with potent protection against autologous viral sequences (Fig. 6.7b and c). The IC50 for serum from TPC was 1:3300 against the TPA HCVpp and 1:10000 against the TPC HCVpp with overlapping confidence intervals suggesting no significant difference in neutralisation sensitivity between the sequences. Serum following resolution of infection (TPD) displayed persistent neutralising activity with an IC50 in the region of 1:500 for autologous HCVpp from both time points (Figs. 6.7b and c).

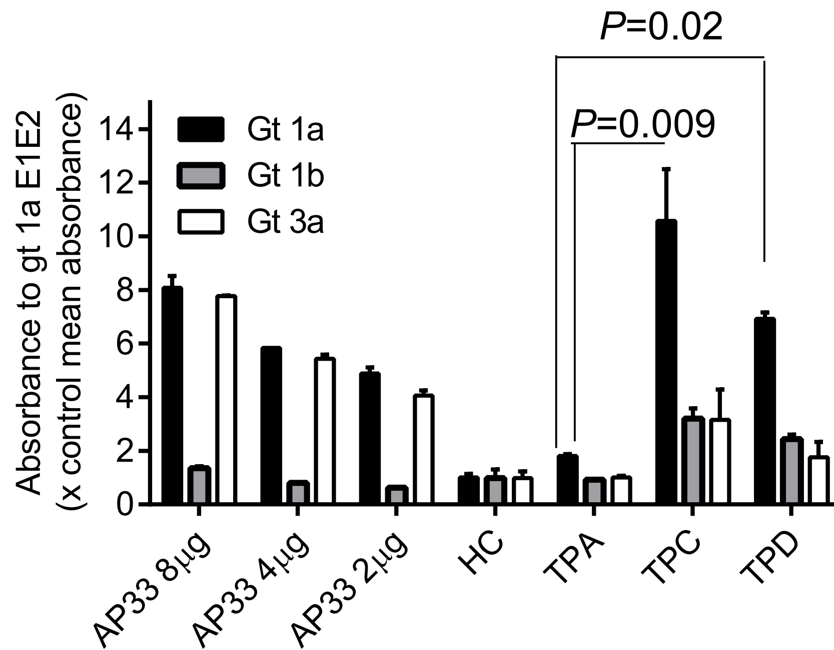
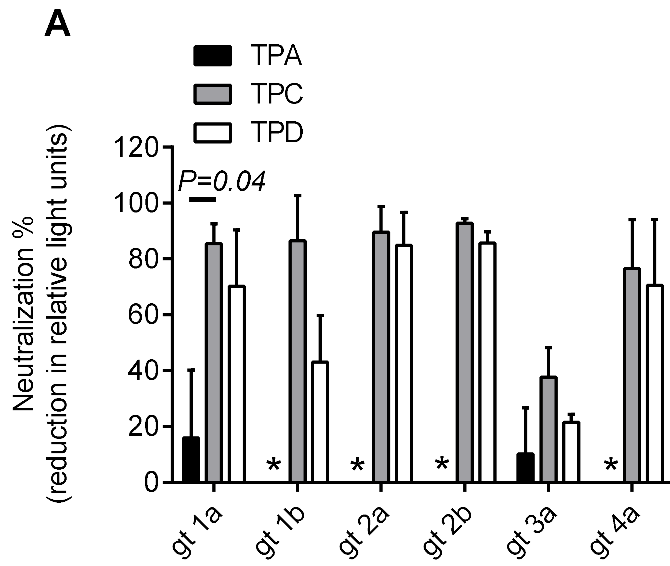
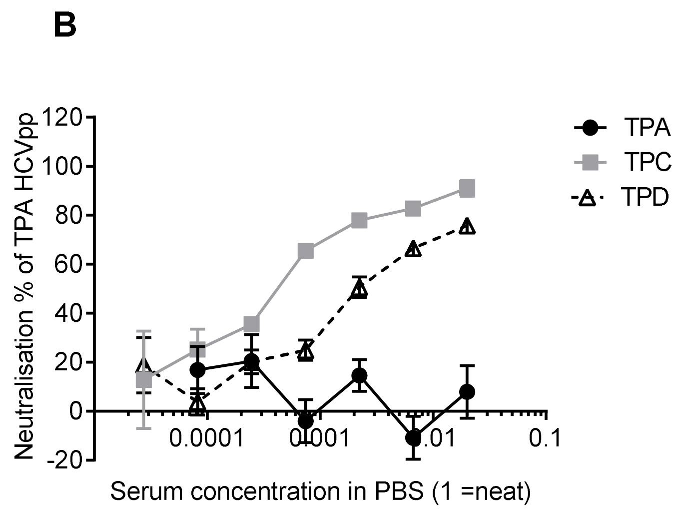


Figure 6.6 Relative binding of purified IgG to E1E2 lysate from gt 1a, gt 1b and gt 3a.

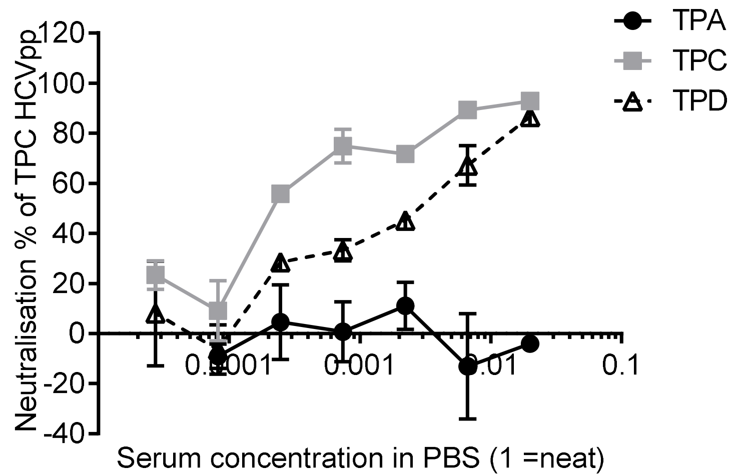
Graph show absorbance of purified IgG tested at 200 µg/ml to HEK-293T cell lysate containing E1E2 proteins from differing standard genotypes as described in Materials and Methods. AP33, a broadly reactive antibody recognising a conserved linear epitope in HCV envelope glycoprotein E2 was used as a positive control at the concentrations shown in the graph. HC samples were used as a negative control and absorbances plotted as a ratio of mean sample absorbance to mean HC sample absorbance. Statistical differences in absorbance between TPA and TPC and D were determined using the Wilcoxon rank sum test. Results shown are from a single experiment due to paucity of serum, each sample was tested in duplicate. Error bars represent the SEM.





Timepoint	IC-50
A	N/A
C	1/3300
D	1/500

C



Timepoint	IC 50
A	N/A
C	1/10 000
D	1/500

Figure 6.7 Neutralisation of HCVpp by IgG and serum from TPA, C and D.

A) Pseudoparticles bearing E1E2 proteins from the XG panel were generated as described in Materials and Methods. Purified IgG from TPA, C and D was tested for their ability to prevent infection from these pseudoparticles at a concentration of 100 µg/ml. * indicates that there was insufficient IgG from TPA to test for neutralisation against gt 1b, 2a and 2b and 4 pseudoparticles. The criterion for positive neutralisation is 50% reduction in relative light units. B+C) Neutralisation of HCVpp bearing envelope proteins of autologous viral sequences from B) TPA and C) TPC by serum diluted in PBS . The results shown are mean % reduction in HCVpp infection compared to no serum control i.e. percentage reduction in relative light unit signal on luciferase assay. Serum from TPA, C and D (post clearance) were heat-treated to inactivate any live virus, then tested in serial dilutions in PBS for the ability to neutralise the HCVpp as described in the IgG neutralisation assay in Materials and Methods. Results shown are from three independent experiments. Error bars represent the SEM.

6.6 Competition of neutralising antibodies with mAbs to known neutralising epitopes on E2

In an effort to gain an understanding of the epitopes targeted by the neutralising IgGs from TPC and TPD, a competition ELISA assay was performed to determine if they competed for binding to E2 with some well-characterized neutralising and non-neutralising antibodies to discrete immunodomains. The antibody panel and competition ELISA are described in Materials and Methods and Chapter 4 Section 2. It included non-neutralising HmAb CBH-4B, neutralising HmAbs HC-1, HC-11, CBH-7 and HC-84.1 and mAb AP33 (120) (119) (124) (129).

To determine whether purified IgG contained antibodies targeting similar domains, we incubated test IgG on purified sE2 coated plates at a concentration of 200 µg/ml in PBSTM in addition to control wells containing PBSTM only. Subsequently biotinylated antibodies from the above panel were added to these wells at a concentration close to their EC50 (119, 121, 255, 314) (Fig. 2.4). HP conjugated secondary substrates were added and binding measured as previously described (see Materials and Methods and Chapter 4). The reduction in relative binding of each biotinylated antibody (calculated as percentage reduction in absorbance) in the presence of IgG compared to control was determined for TPC and D.

This revealed the IgG antibody response at Timepoints C and D competed for multiple binding sites on E2, in particular they demonstrated competition with monoclonal antibodies known to target CD81 binding sites with broad neutralising activity (Fig. 6.8).

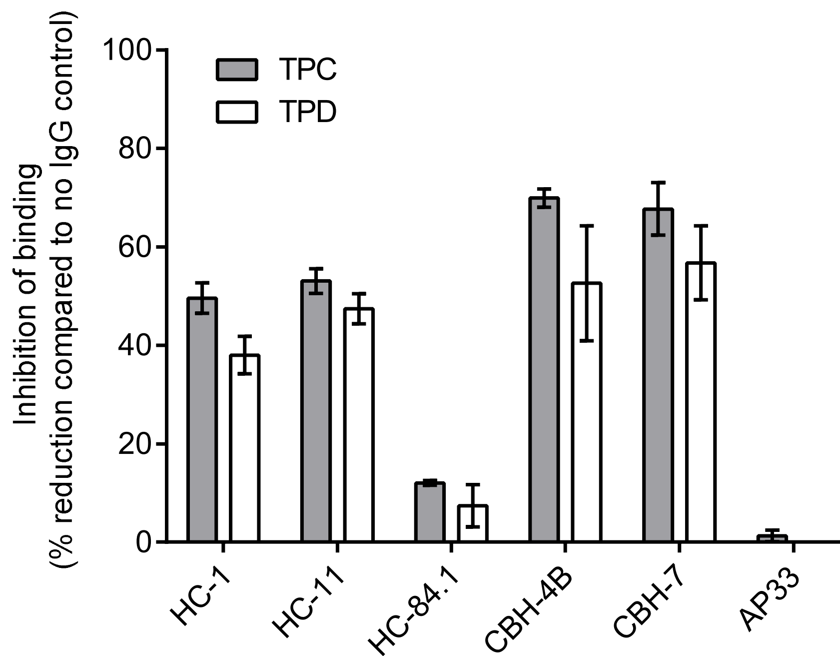


Figure 6.8 Ability of neutralising IgG from Timepoints C and D to compete with antibodies to known epitopes on E2.

The ability of purified IgG at 200 µg/ml to competitively inhibit binding by biotinylated monoclonal antibodies with known epitopes to E2 was determined as described in Materials and Methods. Percentage reduction in binding signal from the test monoclonal antibody in wells where IgG had been added compared to those where only PBSTM had been added is plotted on the graph above. The results shown are from three independent experiments. Error bars represent SEM.

6.7 Chapter 6 Discussion

Viral infection and resolution resulting in protection on re-exposure to a similar virus forms the basic principle of immunisation, being used in several primate models of HCV immunity (148, 315). Deliberate human re-exposure to HCV is unethical but this case provides a rare example of a natural version of such an experiment.

This report is the first detailed molecular study of viral populations and humoral immune responses in secondary spontaneous hepatitis C viral clearance after

post-therapy relapse. As another patient with a similar clinical pattern was also identified in the St Mary's cohort, this suggests that secondary spontaneous clearance may be a more common phenomenon than previously understood. Many clinics do not conduct routine viral load testing following confirmed relapse after therapy and such individuals may not be tested again until being considered for further treatment. Following presentation of this data, I have been informed of several anecdotal reports of individuals who relapsed after interferon based therapy but were found to be HCV PCR-negative on assessment for further therapy [personal communications from Barclay S, Thomson E and data from the Scottish HCV clinical database (265)]. The individual described in these experiments was HIV-positive as was the other patient in this cohort with a similar clinical picture. It may be that this phenomenon is more common in those with HIV where fluctuations in CD4 count may affect the efficacy of the adaptive immune response.

Cases of re-infection following therapy with spontaneous clearance of the reinfection have been previously reported (316, 317) and population data suggests that treated individuals who remain at high risk for re-infection have lower incident infection rates than naïve individuals (318). These results suggest that adaptive immunity may play a protective role following successful anti-viral treatment with IFN. I cannot exclude the possibility that the patient here was re-infected with the same strain of virus during follow-up although he did not report high risk activity during this time.

These findings provide new evidence of the importance of the anti-envelope antibody response in clearance of HCV infection after treatment. Those who are able to resolve HCV infection acutely tend to have a favourable immunogenetic profile which will also influence their likelihood of clearing further infections. A

meta-analysis of acutely-clearing individuals who spontaneously cleared on re-infection showed association with the IFNL4 CC genotype(77) while the HLA-DQB1 locus has been linked to the ability to raise an early, broad neutralising antibody response leading to resolution of acute infection (113). In the majority of HCV-infected individuals production of neutralizing humoral responses is delayed, which may explain the initial lack of functional antibodies in our patient (276, 280) . Patients co-infected with HIV have a particularly delayed response, often resulting in later diagnosis of infection (117). Therefore, it is encouraging to demonstrate that, even in an individual who did not have a favourable acute response (and who was co-infected with HIV), it is possible to generate sterilising antibody-mediated immunity.

The majority of HCV-infected individuals with relapse post-therapy develop chronic viraemia. Viral escape from both the T cell and antibody response has been described in detail in chronic infection (137, 319, 320). One key feature of our subject's antibody response at TPC was targeting of multiple CD81 binding epitopes. A multispecific T cell response is associated with HCV clearance and in Chapter 3, I described how individuals possessing antibodies targeting multiple CD81 binding epitopes of E2 (competing with monoclonal antibodies HC11 and CBH7) have the ability to neutralise the majority of a panel of gt 1a E1E2 variants (251). Other researchers have also identified a synergistic effect of monoclonal antibodies targeting separate epitopes when used together (126). This may have been an important factor in preventing viral escape from neutralising antibody response, leading to eventual clearance.

A further factor which may have favoured resolution of infection here was the short duration of the initial infection. In chronic HCV and HIV infections with persistent antigen stimulation, T cells may display an exhausted phenotype (106, 107). While

no direct effect on B cells has been demonstrated, they are known to display the activation and exhaustion marker molecule PD-1 on chronic exposure to antigen (109). In addition, given the co-stimulatory role of CD4+ T cells and complex interplay of B and T cell immune responses in viral clearance, it would follow that a persistent defect in the T-cell repertoire could have significant implications for B cell function. It has already been shown that such exhaustion is reversible after short periods of infection, however once chronic infection is established, an exhausted phenotype persists (108). It may be that development of neutralising antibodies generated in chronically infected individuals may fail to prevent re-infection but, in combination with cellular responses, such antibodies may be more likely to be effective if generated by brief exposure to antigen as in acute resolution of infection or vaccination. Timing of exposure and reappearance of virus may have been particularly important in this HIV-infected patient, as the substantial reduction in CD4 count on IFN therapy may have resulted in a diminished B cell functionality followed by a recovered response after completion of therapy.

6.8 Chapter 6 Summary

- This is the first sequencing confirmed case of spontaneous clearance of viral relapse after interferon-based therapy where the presence of broadly neutralising antibodies has been demonstrated.

- Despite differences in the E2 HVR-1 sequence between initial infection and relapse, there was no effect on infectivity or dependence on SR-BI for Huh7 entry when tested in the HCVpp system.

- Broadly neutralising antibodies were identified from samples during and 1 month following secondary spontaneous clearance which were able to neutralise a range of envelope sequences both autologous, and of different genotypes.

- These antibodies appeared to compete with mAbs targeting multiple neutralising epitopes on E2.

- These findings support the importance of neutralising antibody responses in clearance of HCV infection and highlight the potential for NAb to contribute to viral clearance on second exposure.

7 Anti-E1E2 antibody responses in a cohort of uninfected individuals at high risk of repeated HCV exposure

[The work described in this chapter has been published in Swann and Mandalou[2] *et al., J. Viral Hepatitis, 2016 (321)* with permission for reproduction of this work under the Creative Commons Attribution License]

7.1 Introduction

In the search for a prophylactic vaccine, it is especially relevant to study individuals who appear to have natural resistance to HCV infection. Individuals who have repeated probable exposure to HCV through regularly injecting drugs and sharing injecting paraphernalia are at very high risk of HCV exposure. Up to 90% of long term users with these risk factors show serological evidence of previous or current HCV infection (322) . However, a proportion of individuals in this high risk group appear resistant to HCV infection, and this is an increasingly well documented phenomenon (323). Conventionally HCV infection status is determined using a PCR assay to detect viraemia and an Enzyme Immunoassay (EIA) to detect antibodies to core and non-structural HCV proteins. Individuals at high risk of exposure who show no serological evidence of past or current infection have been termed exposed but uninfected (EU) and a number of such cohorts have been defined in previous studies (92-94, 324).

[2] P Mandalou recruited the cohort and provided their samples and clinical data. I designed and conducted the experiments described in this chapter with some assistance from P Mandalou.

EU cohorts have been shown to be immunologically distinct from both healthy controls and those who spontaneously clear HCV infection (see Table 1.1 in Introduction) (91-93) with raised levels of natural killer cell activity, IL6/IL8 and TNF-α activity compared to IDUs infected with HCV (94, 95). There are also substantial differences in the immunogenetics of individuals in EU cohorts, those who spontaneously resolve acute HCV infection and those with chronic HCV(96). HLA-C1 and KIR2DL3 genes are over represented in both EU and spontaneous resolver groups, whilst prevalence of the IL28B (IFNL3) polymorphism is more prevalent in spontaneous resolvers but similar in EU and chronically infected cohorts (93, 95, 97, 98). In terms of adaptive immunity, EU cohorts have been shown to display HCV-specific T cell responses in up to 60% of individuals (92).

By definition, EU cohorts do not show detectable antibody responses to core and non-structural proteins in a diagnostic assay, suggesting cellular infection and replication has not taken place. However, the role played by antibodies directed at the envelope protein, which may prevent cellular infection, has not yet been explored. As described previously, bNAbs are thought to play a significant role in acute clearance of HCV (133). Neutralising antibodies (NAbs, capable of preventing infection by one or more strains of HCV,) are also upregulated in those who acutely clear HCV infection on multiple exposures. Therefore, it is possible that such antibodies may provide a degree of protection from infection in the IDU EU population (113). Primate studies using HCV gt 1a envelope proteins as a vaccine substrate have shown that NAb were raised in the majority of animals immunized on 2 occasions. These were able to prevent *de novo* infection and had some activity against strains of virus from different genotypes (315). If one accepts that previous evidence suggests these individuals have indeed been exposed to HCV virions or at least some of the proteins contained therein, it would not be

surprising to find an adaptive anti-envelope response which could possibly confer a protective effect. Therefore, I aimed to determine the presence of functional anti-HCV envelope antibody responses in an IDU EU cohort of with a high risk of repeated exposure to HCV.

7.2 Defining the cohort

The EU cohort described in this study is detailed in Materials in Methods. Briefly IDU were recruited between 2003 and 2014 from a variety of locations in Plymouth, UK as previously described (92, 93) (clinical subjects recruited and clinical data collected by Professor M. Cramp and Dr P. Mandalou, South West Liver Unit Plymouth). Individuals were screened for evidence of current or past HCV infection and they completed a confidential structured questionnaire to collect demographic data and a detailed injecting history. For this study only those with no evidence of previous HCV infection who were judged to be at substantial risk of HCV exposure based on a >1 year history of injecting drug use and regular sharing of injecting equipment were included. Serum samples were taken from all individuals and where possible, follow up clinical information and clinical samples were obtained.

As a control group samples from chronically infected individuals in the CHCV cohort and their associated healthy controls (HC) were used (see Materials and Methods and Chapter 3 for further details) (325).

A total of 42 EU subjects were included, 5 of which had samples from multiple time points. Eight healthy controls and eight CHCV subjects were also included. Demographics of these cohorts are shown below (Table 7.1).

Table 7.1 Demographics of Exposed Uninfected and control cohorts

	Exposed Uninfected (EU) n=42	Chronic HCV (CHCV) n=8	Healthy Controls (HC) n=8
Mean age at sampling (years ± SD)	34±8.3	52 ±6.3	47±18.2
Ethnicity (% Caucasian)	42 (100)	8 (100)	8 (100)
Sex (% Male)	37 (89)	5 (63)	4 (50)
Median estimated year of infection	NA	1981	NA
Current IDU (%)	42 (100)	ND	0 (0)
Mean age at commencing IDU (year) ± (SD)	21.8 ± 5.2	18.5±6.5	NA
Mean lifetime injecting episodes (range)	4128(52-21900)	ND	NA
Duration of IDU (years ± SD)	7.6 ± 4.3	ND	NA
Sharing needles/syringes (%)	30 (72)	ND	NA
Sharing any injection equipment (%)	42 (100)	ND	NA
Sharing with IDU known to suffer from HCV infection (%)	12 (28.5)	ND	NA

EU data supplied by M Cramp and P Mandalou. ND indicates no data collected on this category in this group and NA indicates not applicable to this group.

7.3 Detection of anti-E1E2 IgG responses in exposed uninfected subjects

In order to detect an IgG response to all regions of the envelope protein, a GNA capture ELISA was performed as described in Materials and Methods. Purified IgG

from serum samples tested against plates coated with lysate from 293T-HEK cells transfected with phCMV plasmids containing E1E2 sequences from gt 1a and gt 3a (see Table 2.2). As there was no way of determining which genotype of HCV these individuals may have been exposed to, it was initially planned to test against other genotypes including gt 1b, however scarcity of sample from the EU cohort combined with a relatively weak binding from CHCV individuals on the gt 1b plate used for optimisation rendered this impracticable. For each test subject, 50 µl of IgG in PBSTM at a concentration of 200 µg/ml was added to wells in duplicate. The ELISA assay was performed as described in Materials and Methods, with HRP conjugated mouse-anti human IgG Fc (Sigma A0170) used as the secondary antibody at 1:5000 concentration (2µg/ml).

7.3.1 Reactivity of EU IgG to HCV E1E2 protein lysate

Median absorbances for the GNA capture ELISAs between the different cohorts were compared using a Wilcoxon Rank Sum test. The EU cohort median absorbance on gt 1a E1E2 lysate was significantly higher in than the HC group (P=0.038) (Fig. 7.1a). While there was a trend towards overall higher absorbance readings against gt 3a E1E2 in the EU group than in controls, this did not reach significance (P=0.067, Fig. 7.1b). However, three samples showed levels of binding to gt 3a lysate comparable to chronically infected controls.

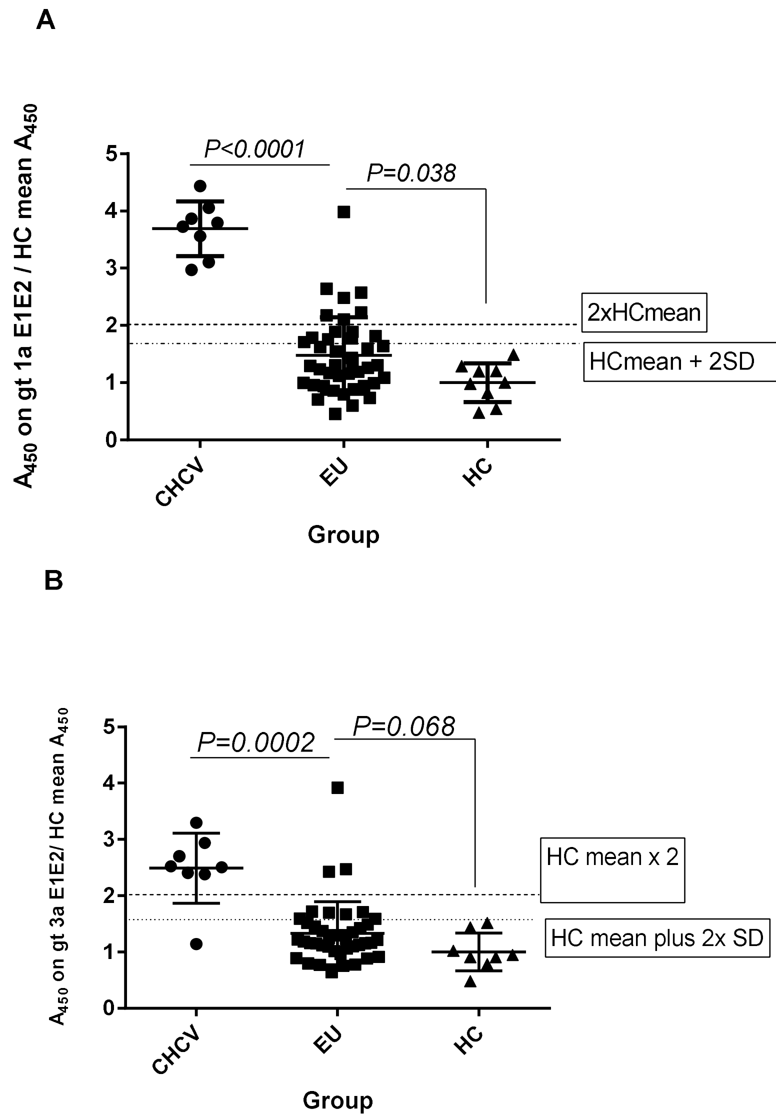


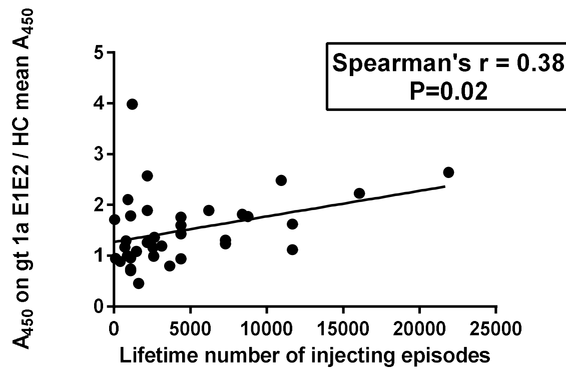
Figure 7.1 EU individuals show elevated IgG reactivity to HCV envelope proteins.

Purified IgGs were tested for their ability to bind HEK-293T-expressed E1E2 in a GNA-capture (A) gt 1a and (B) gt 3a ELISAs as described above. To allow comparison between plates, the healthy control (HC) panel was tested on each ELISA plate and absorbance values adjusted according to the HC mean. For those individuals where serum from multiple time points was available, each time point was tested individually but only one value for each individual is plotted. Statistical differences between the groups were calculated using the Wilcoxon rank sum test. Bars are included to show values corresponding to HC mean plus 2 standard deviations and 2 times the HC mean, both accepted arbitrary cut offs for developing a diagnostic assay.

7.3.2 HCV exposure risk and E1E2 reactivity

Logically, individuals more frequently exposed to HCV envelope proteins should show stronger antibody reactivity to these. EU IgG absorbance levels to gt 1a and gt 3a E1E2 were analysed for association with detailed reported injection frequencies for those 37 subjects where data was available. Non parametric analysis showed a significant correlation between IgG reactivity against gt 1a E1E2 and a higher number of lifetime injecting episodes (Spearman r 0.38, P=0.02, Fig. 7.2a), although this association was not seen with gt 3a reactivity (Fig. 7.2b). While self-reported behaviours are not always recalled accurately it would appear that those who are more frequent risky injectors have higher levels of anti-gt-1a HCV envelope IgG.

A



B

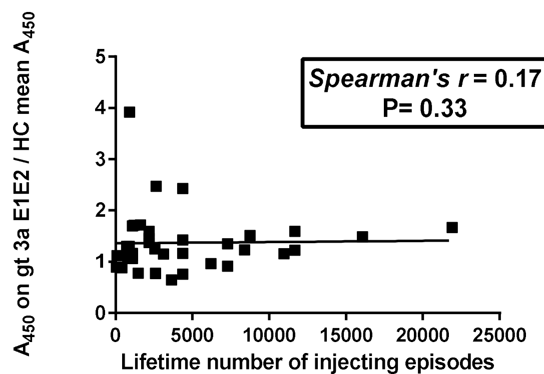


Figure 7.2 Correlation between IgG reactivity to either gt 1a (A) or gt 3a (B) E1E2 and total number of lifetime injecting episodes.

Absorbance values for the binding of EU cohort IgGs to E1E2 proteins from gt 1a- and gt 3a-transfected HEK-293T cell lysate were determined as described previously. Duration of reported injection drug use was multiplied by frequency of reported injection drug use to obtain an estimate of total number of lifetime injecting episodes. This was plotted for 37 EU (injection data not available for the remaining 5).

7.3.3 Reactivity of IgG to purified soluble E2 (sE2) by ELISA

As it is possible that those with frequent risky injection practices may also develop antibodies to human tissues, I aimed to eliminate possible cross-reactivity against HEK-293T cell antigens and medium components. To do this, serum samples from all individuals were diluted 1:50 in PBSTM and tested for IgG reactivity to Immulon® plates (Thermo Fisher Scientific) directly coated with 1 μg/ml gt 1a sE2 (produced by Ania Owsianka from the H77 E2 sequence as detailed in Materials and Methods). There was significantly higher absorbance of IgG from EU to sE2 than HC (P<0.01, Fig. 7.3).

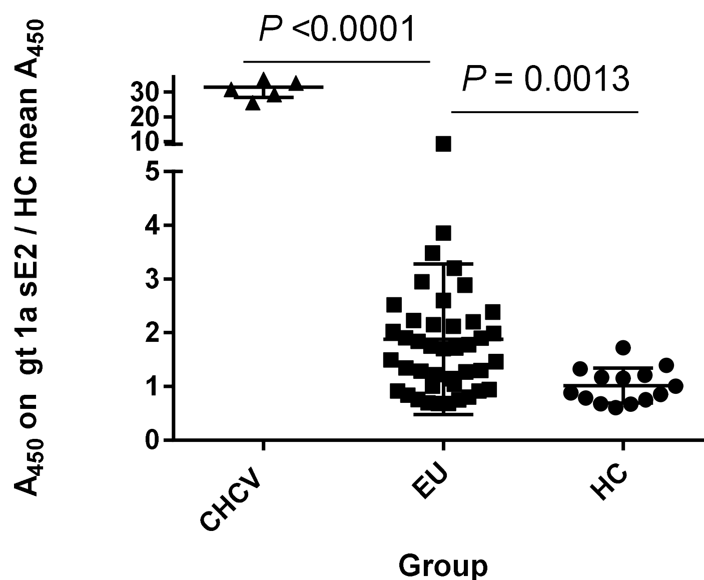


Figure 7.3 IgG reactivity to gt 1a sE2 is evident in EU individuals.

Diluted serum (1:50 in PBTSM) was added to plates coated with purified gt1a sE2 protein and absorbance determined for IgGs from EU, HC and CHCV individuals using a modified ELISA protocol described in Materials and Methods. Statistical differences between the groups were calculated using the Wilcoxon rank sum test. Mean and standard deviations are shown.

7.4 Detection of anti-E1E2 IgM responses in exposed uninfected subjects

In order to establish whether there was evidence of recent exposure to HCV envelope proteins, I developed an assay to test for evidence of IgM response to E2. While anti-HCV IgM persists in chronic infection, it wanes following viral clearance, with levels declining 8 weeks following exposure (326), (327). Therefore, elevated levels in the EU cohort would tend to suggest recent contact with HCV proteins. IgM is challenging to work with in that it tends to bind non-specifically and with lower affinity than IgG. In addition, IgMs can be difficult to purify as they are soluble under a narrower range of conditions than IgG and are more susceptible to becoming denatured (328) . Therefore, I took several steps to optimise an ELISA assay for detecting anti-envelope IgM, using the HC and CHCV groups prior to testing IgGs from the EU subjects. As I planned to use dilute serum (diluted 1:50 in PBSTM) rather than purified IgM in this assay, I also included purified IgG as a negative control to ensure there was minimal secondary antibody cross-reactivity.

Firstly, I tested only against purified sE2 (further details in Materials and Methods) to reduce the possibility of binding to other cellular proteins. I introduced three adsorption steps prior to adding samples to the ELISA plate. This involved incubating samples on a GST-coated plate in an attempt to remove non-specific binding antibodies. Despite this optimisation, high levels of absorbance from HC samples were still observed compared to CHCV samples (Assay 1 see Fig. 7.4). By increasing the concentration of Tween-20 in PBST used in dilution and wash steps to 0.5% (high Tween PBST) and adjusting the concentration of secondary antibody (anti-IgM- HRP conjugate, Abcam) to 1:500 (2µg/ml) from 1:1000 (1µg/ml), HC absorbance readings were reduced to a level where CHCV and HC

IgG binding levels could be easily distinguished (Assay 2 see Fig. 7.4). I then used this optimised assay to determine if anti-E2 IgM could be detected in the EU subjects.

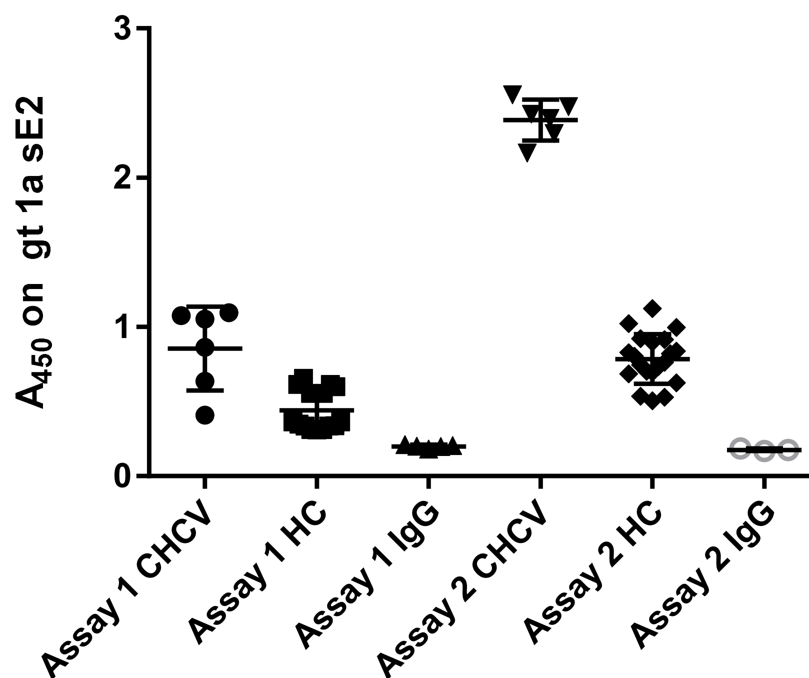


Figure 7.4 Optimisation of IgM ELISA

Serum samples from the CHCV and HC cohorts were used to optimise an ELISA to detect IgM binding to plates coated with gt 1a sE2. CHCV and HC serum samples were diluted to 1:50 in PBSTM. Purified IgG samples (at 200 µg/ml) were also used to ensure that there was no cross-binding with the secondary antibody. In Assay 1 PBSTM with a standard (0.1%) concentration of Tween-20 was used and secondary antibody (anti-human IgM HRP conjugate, Abcam) was used at 1µg/ml. In the second assay (Assay 2) the same samples were used with high concentration Tween-20 (0.5%) PBST in all dilution and wash steps and secondary antibody was used at 2µg/ml. Mean and standard deviations are shown.

Using this assay to test serum from the EU cohort, EU samples showed significantly higher median IgM binding to sE2 than the HC group (*P*=0.001 Wilcoxon Rank Sum, Fig. 7.5) with several individuals displaying levels comparable to the CHCV group. Overall 30 (71%) of the EU individuals showed levels of IgM binding >HC mean+2xSDs, 23 (55%) had absorbances >2x HC mean and 22 (42%) displayed absorbance levels at HC mean + 3x SDs.

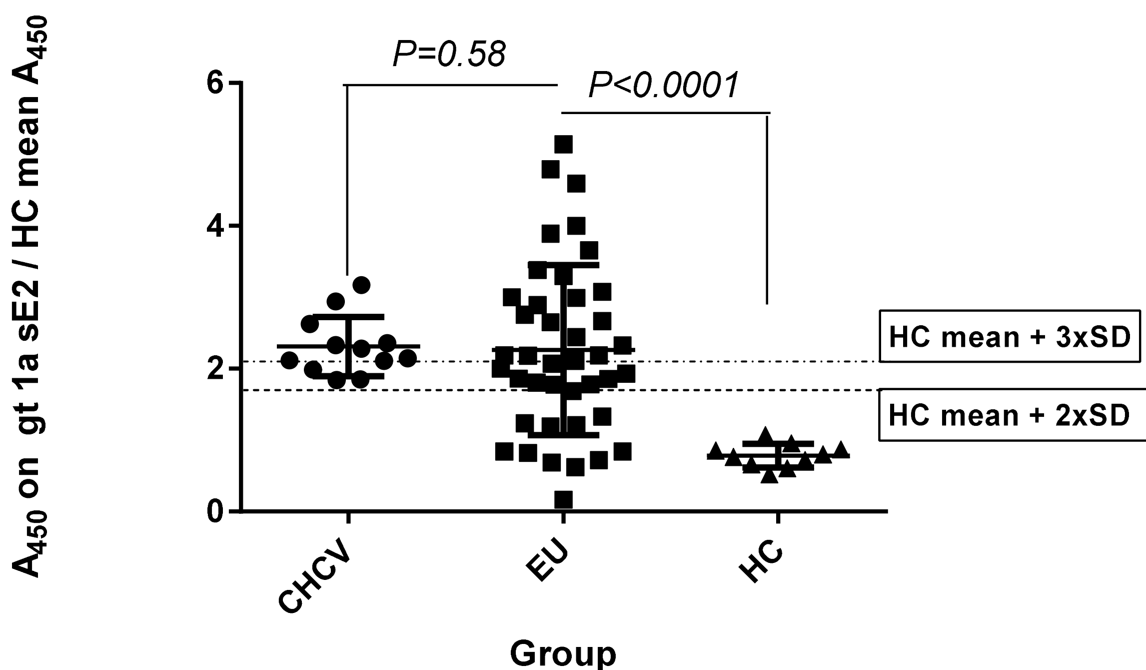


Figure 7.5 IgM responses to soluble gt 1a E2 are evident in EU individuals.

IgM binding to purified gt1a sE2 protein was determined for serum from CHCV, EU and HC individuals. Serum from each individual was diluted 1:50 in high Tween PBSTM (PBSTM with 0.5% Tween) and tested using a modified ELISA protocol developed as described in the text and in Materials and Methods. Statistical differences between the groups were calculated using the Wilcoxon rank sum test. Mean and standard deviations are shown.

7.5 Pseudoparticle neutralisation by IgG from exposed uninfected subjects

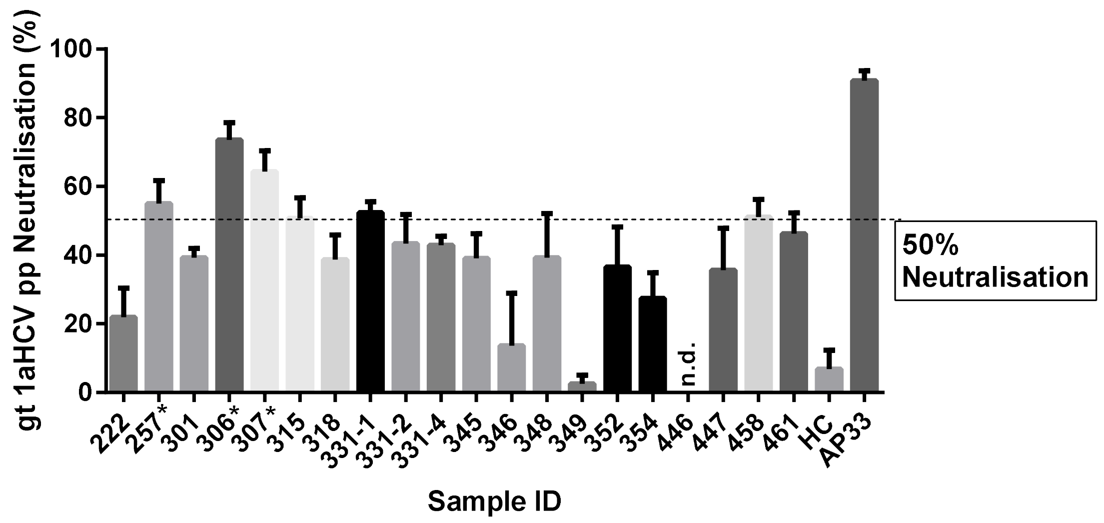
In a subset of EU individuals displaying significantly elevated E1E2 binding compared to HC on ELISA (higher than 2 standard deviations above the HC mean absorbance), HCV pseudoparticle (HCVpp) neutralisation assays were conducted as previously described (119) and detailed in Materials and Methods. Briefly, purified subject IgG was added to 40 µl of HCVpp-containing medium prepared as described in Materials and Methods. Purified IgG was screened for neutralising effects at a concentration of 400 µg/ml apart from a small number of individuals for whom serum was scarce, where this was reduced to 200 µg/ml. The mouse monoclonal anti-E2 antibody AP33 (258) and HC IgG were included as positive and negative controls, respectively. Virus neutralisation was defined as 50% reduction in HCVpp infectivity as measured in relative light units (RLU) using a Chameleon II plate reader. Ability to neutralise both gt 1a and gt 3 was tested. For those samples with apparent neutralizing activity, further neutralisation assays were conducted using serial dilutions of subject IgG where sample volume allowed.

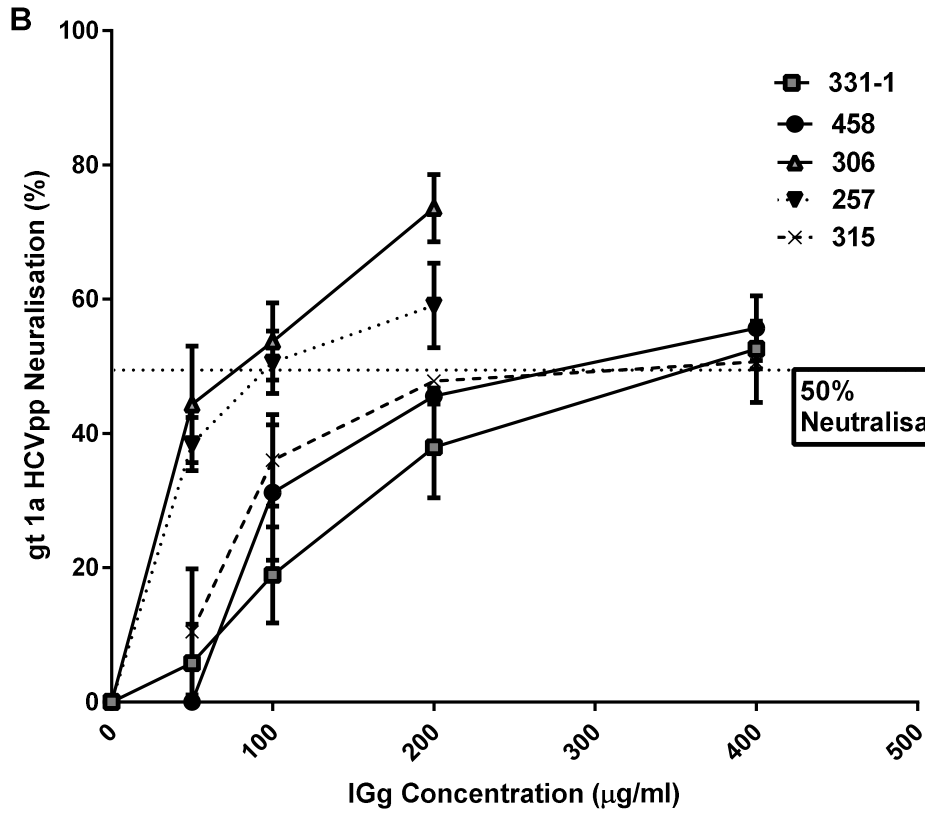
Six of the EU subjects tested (257, 306, 307,315, 331-1 and 458) showed ability to neutralise gt 1a HCVpp by 50% or more at a purified IgG concentration of 400 µg/ml (Fig. 7.6a). IC_{50} values were between 400 and 200 µg/ml for subjects 315, 331-1 and 458, whereas subjects 257 and 306 had IC_{50} values between 100 and 50 µg/ml (Figure 7.6b). While subject 307 appeared to have significant neutralising ability when tested at 200 µg/ml, there was insufficient sample to test serial dilutions of IgG in a neutralisation assay.

Three EU individuals were able reduce infectivity of gt 3a HCVpp by 50% or greater (257,306 and 307) with one further gt 1a neutralising subject reducing gt 3a infectivity by 40% (EU 458) (Fig. 7.6c). Two further individuals (461, 301) showed evidence of a weaker neutralisation effect against both gt 1a and gt 3a HCVpp, consistently reducing infectivity by 40%.

7.6 Duration of antibody detection over time

For 5 EU individuals, serial samples from time points separated by a period of at least 1 year were available. Purified IgG from these individuals was tested on the E1E2 GNA capture ELISAs as detailed in section 7.3.1. Four of these individuals displayed absorbance levels equivalent to HC levels and remained at this level on subsequent testing (Fig. 7.7a). One individual with significant E1E2 reactivity and neutralising responses was studied serially (331), in initial and subsequent samples, detectable responses were present over time but with a diminution in strength at later time points which may reflect the waning of antibody concentration with time following exposure (Fig. 7.6b).


A
gt 1aHCV pp Neutralisation (%)
100
80
60
40
20
0
50%
Neutralisation
n.d.
222
257*
301
306*
307*
315
318
331-1
331-2
331-4
345
346
348
349
352
354
446
447
458
461
HC
AP33
Sample ID


B
gt 1a HCVpp Neuralisation (%)
100
80
60
40
20
0
331-1
458
306
257
315
50%
Neutralisation
0
100
200
300
400
500
IGg Concentration (μg/ml)

C

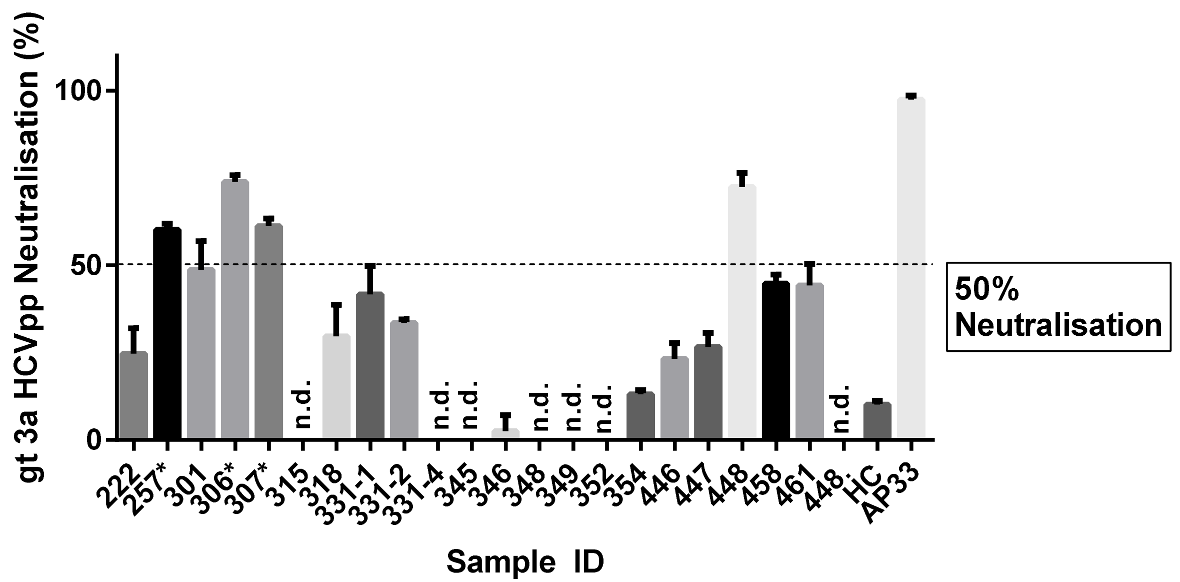


Figure 7.6 Neutralization of HCVpp by EU purified IgG.

Virus pseudoparticle neutralization assays were performed using HCVpp bearing E1E2 derived from gt 1a strain H77 (A and B) or (C) gt 3 HCV. (A and C) Percentage reduction in HCVpp entry after incubation with 400 μg/ml test IgG is plotted as evidenced by luciferase reading at 72 hours compared to no IgG control. For those individuals with low sample volume and evidence of strong neutralising activity on initial screen were tested at 200 μg/ml (indicated by *). Individuals with low sample volume and no evidence of neutralisation on initial screen were not tested further (n.d.). (B) Percentage reduction in HCVpp entry after incubation with reducing concentrations of IgG as shown. The traditional 50% cut off is shown. The results from at least 3 separate experiments were plotted as above. Error bars represent SEM.

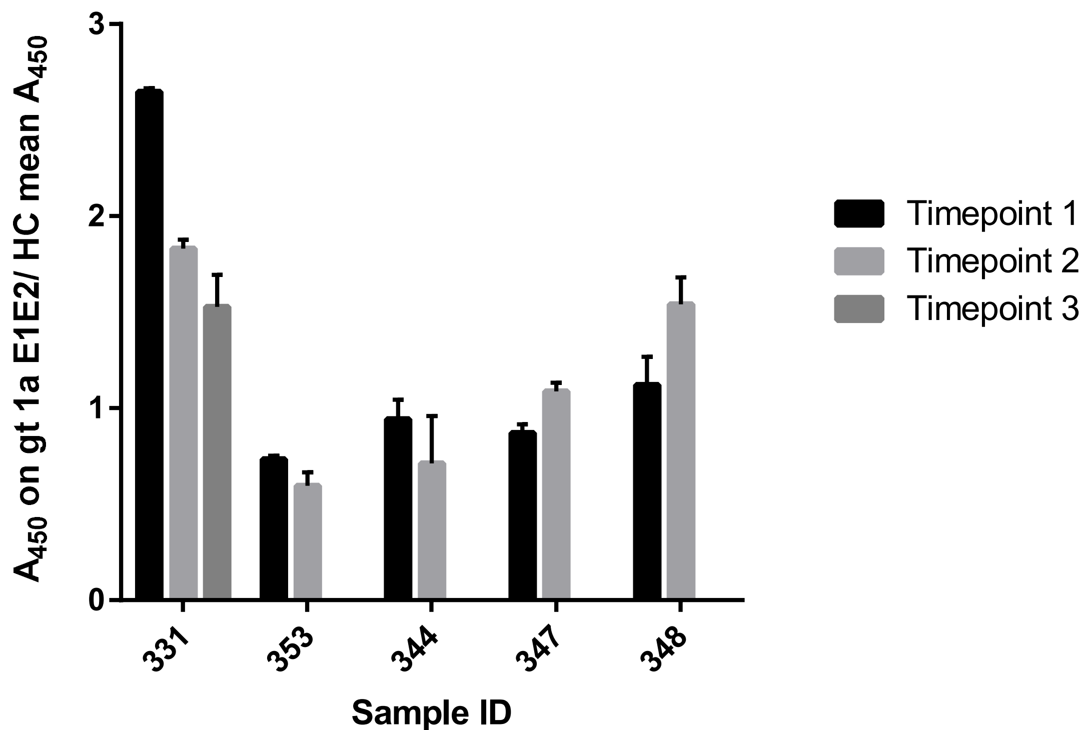


Figure 7.7 Presence of IgG directed at gt 1a E1E2 over time.

Samples from the same individuals taken at time points separated by at least 1 year were tested in the gt 1a GNA capture ELISA as described previously. Absorbance relative to HC mean is plotted. The results of two independent experiments are shown. Error bars represent the SEM.

7.7 Envelope protein binding and neutralisation across multiple assays

While the assays described above have not been externally validated for ‘diagnosis’ of HCV exposure, an accepted diagnostic ELISA cut-off value of 2 times the control mean was used as guide to determine a positive result. Alternatively HC mean plus 2 or 3 times standard deviation can be used. Using a absorbance cut-off of ≥ 2 times the HC mean value as indicating a significantly positive result, a total of 20/42 (48%) EU showed evidence of IgG reactivity to HCV envelope E1E2 proteins on either the GNA-capture ELISA or IgG gt 1a purified soluble E2 (sE2) binding ELISA (Table 7.2). Four of the 7 EUs with the

highest level of binding to gt 1a E1E2 lysate (≥ 2 times the HC mean) also had significant binding to sE2 displaying absorbance readings ≥ 2 times the HC mean with 2 more showing above average binding to sE2 but below this cut off value. Seven further individuals with significant responses against sE2 showed elevated reactivity against gt 1a lysate but the absorbance attained did not reach the cut-off value. Only four samples showed significant binding to sE2 alone.

As our HC group clustered tightly around the mean, using a less stringent positive cut off value of HC mean +2xSD produced even more positive results in the EU group. Samples which reached this cut off but did not reach an absorbance value of above 2 times the HC mean are indicated in a lighter shade in Table 7.2. In total 20 (48%) of EUs had an absorbance value of HC + 2x SD on the gt 1a GNA ELISA assay, 11 (26%) on the gt 3 GNA ELISA and 25 (65%) on the sE2 assay. In the gt 1a GNA-capture ELISA the value of 2x HC mean was equivalent to HC mean plus 3x SD (2.0 compared with 2.01). All samples neutralizing at the 50% level had binding on at least one ELISA assay at the ≥ 2 times the HC mean level.

Table 7.2 EU binding to and neutralisation of E1/E2 across the various assays used.

EU ID	x HC gt1a E1E2 ELISA	xHC gt3a E1E2 ELISA	XHC E2 ELISA	Neutralisation >50%		Neutralisation >40%	
				Gt 1	Gt 3	Gt 1	Gt 3
246	1.2	1.3	1.9				
306	2.2	1.5	2.3	Y	Y	Y	Y
307	1.8	1.2	2.4	Y	Y	Y	Y
315	1.3	1.1	2.5	Y		Y	
318	1.4	2.5	1.3				
320	1.4	1.2	0.9				
324	1.8	1.4	1.2				
331	2.6	1.7	3.9	Y		Y	Y
333	1.3	1.2	0.8				
352	1.0	1.7	2.6				
353	0.7	1.1	1.5				
354	1.8	1.2	3.5				
447	1.9	1.0	2.2				
461	2.6	1.5	1.7			Y	Y
466	1.0	0.8	1.1				
270	1.3	1.6	1.0				
298	1.2	1.4	0.8				
301	1.8	1.5	2.9			Y	Y
303	1.3	0.9	1.3				
304	0.7	1.1	1.5				
309	1.7	0.9	1.3				
319	0.9	0.9	1.8				
222	2.2	1.1	0.7				
326	1.2	1.2	1.7				
332	0.9	1.0	1.8				
344	0.9	1.1	0.8				
345	1.1	1.2	9.1			Y	
347	1.1	0.8	2.0				
350	0.5	1.7	0.9				
444	1.0	1.3	1.9				
445	1.6	2.4	0.8				
446	2.5	1.2	2.4				
458	2.1	3.9	1.7	Y		Y	Y
459	0.6	0.8	2.0				
469	0.8	0.6	2.9				
257	4.0	1.7	3.2	Y	Y	Y	Y
290	1.9	1.4	0.7				
294	1.2	1.2	1.0				
346	1.6	1.3	1.3				
348	1.5	1.2	2.2				
349	1.6	1.6	2.1				
456	0.9	0.8	0.9				

Legend for Table 7.2: Values for ELISA are absorbances as multiples of the HC mean for each assay are shown. In neutralization columns, Y indicates HCVpp infectivity was reduced by >50% or >40%, respectively, in the presence of 400 μg/ml of purified EU IgG. Values in dark grey reach the stringent specified cut off of >2 times HC mean (or 50% neutralisation). Values in light grey are between HC mean plus 2 standard deviations and 2 times HC mean absorbance (or 40% neutralisation for HCVpp assays). As only selected samples were tested in the neutralization assays, a black box indicates the sample was not tested.

7.8 Epitopes targeted by neutralising IgG from EUs

In order to determine which epitopes were being targeted by EU sample IgG with E1E2 binding and neutralising ability, competition ELISAs were performed. These tested the ability of IgG from those neutralising EUs with sufficient sample available to compete for E2 binding at the same site as with a panel of well characterized monoclonal antibodies (mAbs) targeting known epitopes. Three of these (HC-1, HC-11, and CBH-7) recognize amino acid residues critical for the interaction of E2 to CD81 (329-332) (see Materials and Methods and Chapter 4). Briefly, PBSTM containing no IgG (control) or purified test IgG at 200 μg/ml in PBSTM was incubated with a gt 1a sE2 coated plate for 2 h before being washed. Subsequently biotinylated mAbs were added to the plate, incubated and washed. Presence of biotinylated mAb was detected using Streptavidin-HRP conjugate followed by addition of TMB substrate. Percentage reduction in absorbance in the test wells compared to the control wells was calculated which gives an indication of test IgG competition for similar epitopes or interference with mAb binding. Although IgGs from chronically infected samples with neutralizing activity tend to compete with antibodies to these regions (see Chapter 4), we did not observe any significant competition in the EU samples (Fig. 7.8). While IgG from these individuals bound pure E2 in these samples at >2x HC samples (Table 7.2) their absorbance values were considerably weaker than CHCV individuals which bound

to viral glycoproteins at >20 times the strength of HC samples (Fig 7.3). This difference in quantity of anti-E1E2 antibody may also have contributed to the lack of detectable competition as this assay was developed for use with samples from HCV-infected individuals.

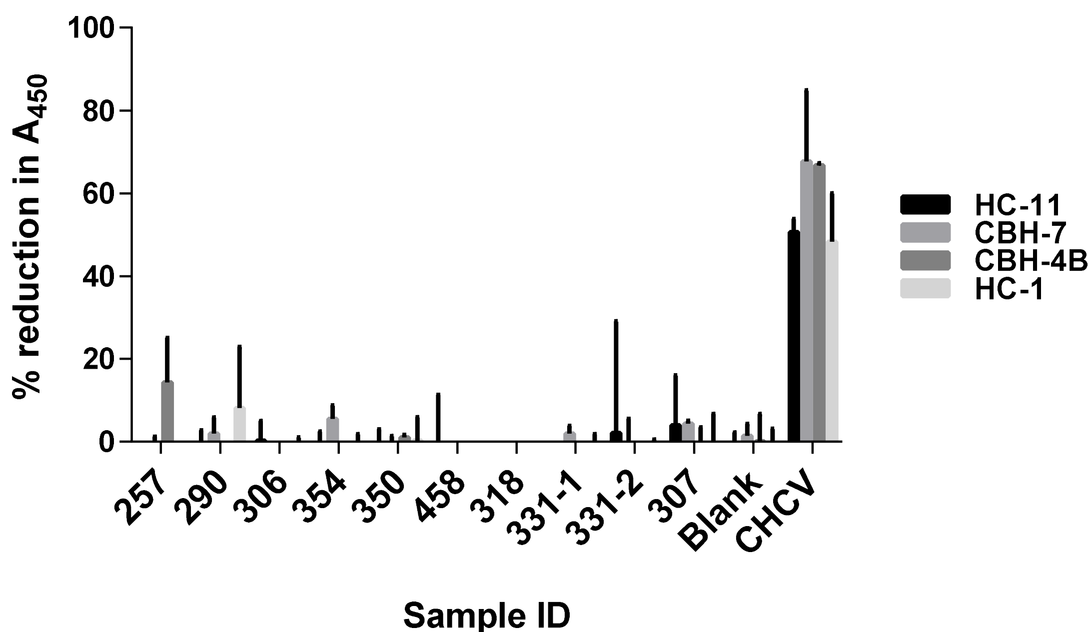


Figure 7.8 EU IgG fails to compete with conformational antibodies to known epitopes for binding to the gt 1a sE2.

IgG from EU individuals with neutralising ability in the HCVpp system were selected for testing by competition ELISA to determine competitive binding with monoclonal antibodies to conformational epitopes on E2 (Materials and Methods). Two samples from chronically infected individuals with known neutralizing activity were also included as positive controls. Mean percentage reduction in absorbance of the monoclonal antibodies was calculated and plotted. Significant competition would be expected at a level of 50% inhibition. The results shown are the means and SEM from two independent experiments due to limited quantity of serum.

7.9 Chapter 7 Discussion

Individuals who are recurrently and frequently at high risk of HCV exposure through sharing injection equipment, but who remain uninfected by conventional testing, represent an ideal cohort to explore mechanisms of natural protection from HCV infection. While in other viruses such as HIV, where resistance may be conferred by mutations in host entry proteins, no variants in CD81 have been identified in HCV exposed uninfected cases (333-335). It is therefore likely that such individuals have immune-mediated features which enable them to resist infection.

This study is the first to report evidence of anti-envelope antibodies in an exposed uninfected cohort. Although HCV-specific T cell responses have been demonstrated in EU individuals it remains unclear if those have a functional role in protecting from infection in these individuals or are merely a marker of viral exposure. It has been shown that HCV-specific T cell responses tend to wane rapidly on cessation of injection drug use (92, 324). The presence of an anti-envelope humoral response provides further confirmation that this EU group has indeed been exposed to HCV virions.

In this cohort, anti-E1E2 IgG responses have been detected at levels significantly higher than controls in almost half of EU cases with anti-E1E2 IgG binding at levels >2 times healthy controls in at least one assay observed in 20 of the 42 EU cases. Although such a cut-off is arbitrary and risks false positive (and negative) results, all individuals with neutralising activity show binding around this level supporting use of this value as a threshold for presence of a significant quantity of antibody.

Anti-envelope antibodies pose one possible mechanism by which these subjects may have resisted infection. This is further supported by the *in vitro* evidence for viral neutralization with IgGs from a subset of individuals significantly reducing cellular entry by HCVpp. IgGs from 6 of the cohort were able to reduce HCV pp entry into hepatocytes in vitro by 50% at IC50s ranging from 75-400 µg/ml. In order to compare this figure with other studies it is helpful to consider these values relative to the concentration of IgG in human serum. As the concentration of IgG in human adult serum ranges from 700-1600 mg/dl, the IC50 concentrations from these individuals equate to a serum dilutions of approximately 1:25-1:100. This is comparable to the strength of neutralising responses observed in in those with acute HCV infection during viral clearance (336).

Individuals who have resolved acute infection show reduction in the strength of detectable NAb responses over a period of months to years. However NAb responses may be restored in subsequent episodes of infection aiding more rapid viral control (113). One individual in the EU cohort, 331, had detectable NAbs which persisted responses over a period of at least a year. In the EU cohort, the presence of anti-envelope humoral responses was more pronounced in individuals with the greatest likelihood and frequency of HCV exposure suggesting that such anti-envelope antibody responses may need ongoing priming by exposure to HCV to be sustained.

IgM is usually a marker of acute exposure to an antigen. The majority of EU subjects tested had evidence of significantly higher anti-HCV E2 IgM than healthy controls. This would support ongoing intermittent exposure to envelope proteins as might be expected through the high risk injecting practices of the cohort. Interestingly, the strength of these responses was equivalent to those seen in

CHCV individuals with ongoing exposure to HCV antigens and the precise role of IgM in resisting infection in the EU cohort and other groups warrants further study.

Whilst a neutralising effect was demonstrated using EU serum, the regions of the viral glycoprotein targeted by their antibodies have yet to be defined. As there is no significant competition of antibodies targeting E2 epitopes known to be involved in cell entry, this raises the possibility that novel epitopes, potentially with E1 binding sites, are involved. However, the lack of competition may simply reflect lower avidity of antibodies in the EU cohort. As avidity of binding increases with duration of infection (337) it is conceivable that the EU antibody responses are directed at the CD81 binding regions tested but are 'competed off' by the conformational mAbs derived from chronically infected individuals. It is also possible that the EUs, in common with most acutely infected individuals, develop antibodies predominantly targeting the HVR-1 region of the E2 protein. Alternatively, as E2 amino acid sequences are highly diverse, these individuals may raise antibodies to a local envelope sequence which has structural differences at key CD81 binding regions, resulting in antibodies which have only weak cross-reactivity with the H77 sequence at these sites. Future work could involve exploring reactivity of the antibodies in EU individuals to locally derived envelope sequences and overall breadth of their anti-envelope antibodies. In addition, further information about anti-HCV responses could be gained from studying the B cell receptor populations in such individuals.

7.10 Chapter 7 Summary

- There is evidence of HCV envelope-specific humoral immune responses in this cohort of IDUs who are at high risk of exposure but remain uninfected by conventional testing (EU).
- Levels of anti-HCV envelope IgG appear to correlate with number of lifetime injections.
- Some EU individuals produce neutralising anti-envelope antibodies which may contribute to host immunity. It is unclear whether this is at a level sufficient to provide protection from subsequent infection.
- IgGs from EU individuals that are able to neutralise HCVpps do not appear to compete with commonly targeted CD81 binding sites on E2 and may therefore recognise novel epitopes, alternatively this could simply represent antibodies targeting these sites with lower affinity binding.
- Many of the EU cohort have IgM directed at the HCV E2; in some this is at the same level as found in chronically infected individuals.
- These data complement previous reports of HCV-specific T cell responses (96, 98), and upregulated innate immune responses in EU populations (97). Together, these studies provide robust evidence that such individuals have been exposed to HCV, but are resistant to developing established infection.

8 Impact of HCV infection and cryoglobulinaemia on the B cell repertoire and functional response

8.1 Introduction

While the main clinical impact of HCV infection is on the liver, it has various extrahepatic effects and is closely associated with clonal B cell pathologies. The antibody response to HCV is atypical compared to most other viruses. Although neutralising antibodies are produced, their appearance is delayed (308), in addition, unlike many chronic infections, anti-HCV IgM remains elevated (327). There is growing evidence that HCV can affect B cells in a number of ways: infection and replication within B cells can occur (338) altered expression of molecules involved in B cell regulation have been described in HCV infection (339), finally HCV is the most common cause of the benign clonal B cell disorder, mixed cryoglobulinaemia (MC).

MC is characterised by precipitation of auto-reactive antibody aggregates on serum cooling to 4°C which can be redissolved at 37°C. Precipitated antibody complexes consist of auto-reactive antibodies (usually IgM) targeting the fixed (Fc) region of host IgG (also known as Rheumatoid factor-like activity) (6). This can lead to a clinical syndrome including renal failure and a small proportion of affected individuals progress to develop B cell lymphoma (182).

The prevalence of mixed cryoglobulinaemia in HCV infected populations varies widely between geographic regions. While the classical vasculitis syndrome is thought to affect 5% of those with chronic HCV infection (340), one meta-analysis suggested a cryoglobulin prevalence of 44% in chronically infected patients, with the highest prevalence reported in Southern Europe and North America (341,

342). There are no published studies reporting the rate in Scottish HCV infected populations.

The exact pathogenesis of cryoglobulinaemia in HCV is incompletely understood. As it tends to resolve on viral clearance some have suggested HCV proteins induce cryoglobulinaemia by acting as a molecular mimic of the IgG Fc portion (142). Similar gene usage in clonal cryoglobulinaemic B cells and B cell Receptor (BCR) binding to HCV envelope proteins raises the possibility that envelope proteins may induce the autoreactive antibodies seen in cryoglobulinaemia. This is also supported by high levels of somatic hypermutation in the BCRs of IgG Fc binding B cells, suggesting their affinity for this molecule is antigen driven (343). In contrast, some researchers have found HCV infection to be associated with expansion of a naïve subtype of B cells (47, 183) and the IGHV1-69 BCR gene commonly expanded in MC is similar to the BCR on immature B1 cells which have polyreactivity and do not undergo somatic hypermutation (183).

In a healthy B cell population there are hundreds of BCR gene classes which are expressed at varying levels, giving a potential of $>10^{14}$ unique BCR combinations(344). Conversely in clonal B cell disorders, this expression becomes restricted to fewer more dominant gene classes (263). The recent development of Next Generation Sequencing (NGS) technology where the sequence of individual fragments of DNA within a population can be determined has revolutionised the ability to search for patterns in immune cell populations in healthy and diseased individuals (263, 345). Previously such detailed analysis would have taken years with clonal techniques.

I aimed to use such techniques in collaboration with the Sanger Centre, Cambridge to complement other means of analysing the B cell response to HCV. I

hypothesised that chronic HCV infection exerts a pathological effect on the B cell population which may be related to the development of cryoglobulinaemia and may have an effect on functional antibody responses. I used samples from the CHCV cohort in an attempt to answer the following questions:

- How common are cryoglobulinaemic antibodies in the Scottish CHCV cohort and do they have any clinical associations?

- Does chronic HCV infection or asymptomatic cryoglobulinaemia alter B cell population diversity and overall gene usage?

- Are HCV envelope proteins the targets of cryoglobulinaemic antibodies?

- Does the presence of asymptomatic cryoglobulinaemia or BCR clonality have any association with restricted function of the anti-HCV humoral response?

8.2 Prevalence of asymptomatic cryoglobulinaemia in a British cohort of individuals chronically infected with HCV and its association with cirrhosis.

A cohort of individuals chronically infected with HCV (CHCV) were recruited alongside healthy controls (HC) from HCV treatment centres in Glasgow. Clinical information and samples were collected as described in Materials and Methods. Samples for cryoglobulin testing were available for 75 HCV infected patients (36 Genotype 1, 39 Genotype 3) and 24 healthy controls. Blood from each patient was collected, clotted and centrifuged at 37°C before cooling for 7 days at 4°C and observed for appearance of a cryoprecipitate. If a cryoprecipitate was observed then this was confirmed by dissolution on heating to 37°C and reappearance on cooling. Of the HCV infected individuals, 31% (23/75) had detectable cryoprecipitates. No cryoglobulin was detected in the HC samples.

Samples containing cryoglobulins were washed with an ice-cold saline solution and submitted to the diagnostic laboratory at Gartnavel General Hospital for typing and detection of rheumatoid factor activity (see Materials and Methods).

Sufficient cryoprecipitate was available for typing in 10 of the positive samples. Six were characterised as Type 2 (monoclonal and polyclonal band) and the remainder type 3 (oligo or polyclonal bands). Rheumatoid factor was activity was checked in 8 of these patients assay and was positive in 3 (38%) and equivocal in 1.

Clinical associations are listed in Table 8.1. A significant association between cryoglobulinaemia and liver fibrosis was observed (Fig. 8.1a, *P*=0.002 by Wilcoxon

rank sum test). Also, a significantly lower median viral load was seen in the cryoglobulin positive group (Fig. 8.1b *P*=0.026).

There was no difference in the prevalence of IDU or diabetes in those with cryoglobulins. No individual symptom was statistically associated with cryoglobulin detection. There was no significant association between IL28B genotype and presence of cryoglobulinaemia (*P*=0.27 by Fisher's exact test, Table 8.1).

Table 8.1 Clinical associations with cryoglobulinaemia.

Clinical Parameter	Cryoglobulin Positive (n=23)	Cryoglobulin Negative (n=52)	*P* value
Male (%)	14 (60)	41 (79)	0.27
Age (median yrs and range)	45 (28-65)	44 (28-68)	0.73
Genotype 1 (%)	12 (52)	28 (54)	0.97
Genotype 3 (%)	11 (48)	24 (46)	0.97
Cirrhosis (%)	12 (52)	12 (24)	**0.019**
Renal impairment	1(4)	0 (0)	0.28
Viral load (median IU/ml)	2.3×10^5 (7.6×10^3 – 7.1×10^6)	1.1×10^6 (5.5×10^3 – 1.4×10^7)	**0.026**
IDU (%)	15(65)	37(71)	0.25
Diabetes (%)	1(4)	3(6)	1.00
IL28B Genotype CC	7 (30)	12 (19)	0.57
Fatigue (median score/10)	5	5	0.52
Sensory Symptoms (%)	6 (26)	13 (25)	1.00
Rash (%)	3 (13)	1 (2)	0.08

Clinical features of the CHCV cohort tested for cryoglobulinaemia were compared between those with evidence of cryoglobulins and those without. Renal failure was defined as estimated creatinine clearance (<60ml/min). *P* values were calculated using Fisher's exact test for categorical variables and Wilcoxon rank sum test for ordinal variables.

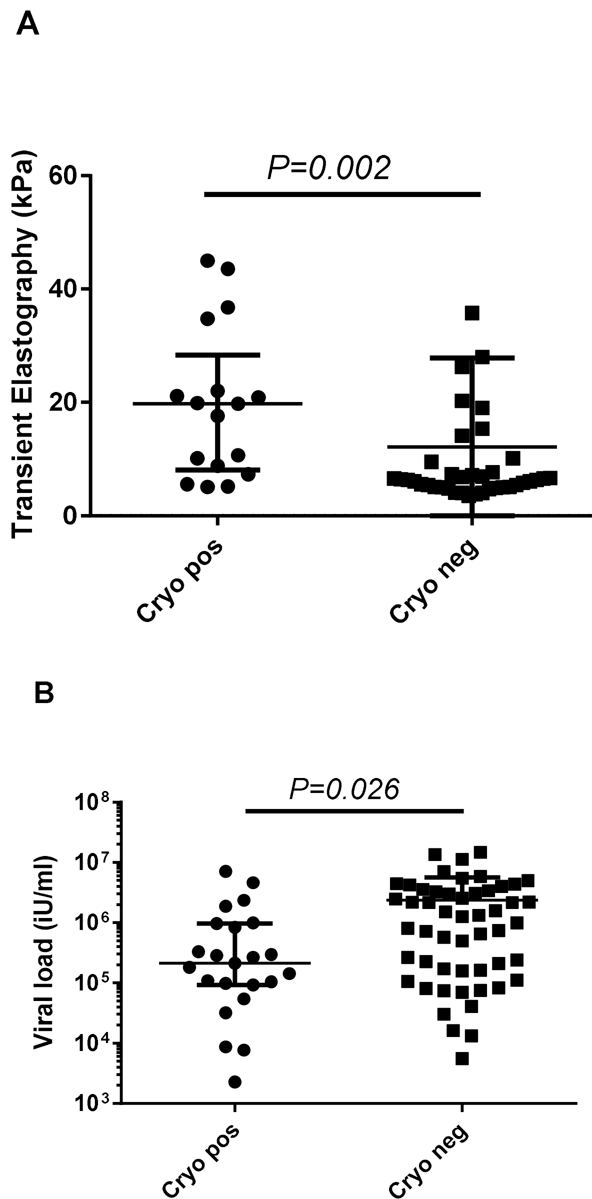


Figure 8.1 Association of liver fibrosis and viral load with cryoglobulin status.

A) A comparison of liver fibrosis, as determined by Transient Elastography readings (Fibroscan kPa), with the cryoglobulin status (positive, pos or negative,neg) of individuals in the CHCV cohort using a Mann Whitney U test. Where cirrhosis had been confirmed on prior biopsy but no fibroscan was available, a value of 12.5kPa was assumed. Where there was no histological evidence of cirrhosis and no fibroscan reading, the sample was excluded. B) Viral load between the two groups was compared using a Mann Whitney U test. Median and interquartile range are shown.

8.3 HCV infection and asymptomatic cryoglobulinaemia does not appear to affect overall BCR diversity but is associated with longer CDR3 length and may exert small changes on the BCR population

To investigate any effect that HCV infection and cryoglobulinaemia may have on the diversity of the B cell population, 6 HCV gt 1-infected cryoglobulin-positive patients, 6 gt 1-infected cryoglobulin-negative patients and 6 healthy controls were selected for next generation sequencing (NGS) analysis of the B cell repertoire.

PBMCs were isolated from these individuals as previously described, RNA extracted and cDNA libraries generated using a pool of primers to the BCR region. These processes were conducted by me and Dr Mark Robinson with further details in Materials and Methods. In addition, for 3 individuals in each group, cDNA libraries were generated by Dr Mark Robinson using IgM- and IgG-specific primers designed by Dr Rachael Bashford Rogers (RBR) at the Sanger Centre, Cambridge. The BCR sequences from the cDNA samples were then amplified using RT-PCR and prepared for NGS analysis as described in Materials and Methods. Briefly, PCR products from the RT-PCR reactions were purified using Agencourt® Ampure® XP beads (Beckman Coulter) , then A-tailed and adaptor ligated using the Kappa Real-Time Library Amplification kit (KAPA Biosystems) and NEBNext® Multiplex Oligos primers for Illumina (NEB). These libraries were then sequenced using a MiSeq SystemDesktop sequencer (Illumina). The resulting sequence file was transferred to RBR for analysis of BCR gene usage using bioinformatics software she had developed as described in Bashford-Rogers *et al* (2015)(263). Quality control information for the samples is shown in Table 8.2 (prepared by RBR, Sanger Insititute, Cambridge).

Table 8.2 Quality of sequencing information for BCR next generation sequencing.

Clinical group	Chain	Mean number of raw reads	Mean read depth after filtering	Mean read length (bp)	Mean % reads retained after filtering
Cryo neg	IgG	549822.0	370375.3	240.3	67.4
Cryo pos	IgG	430123.3	294819.7	242.6	70.5
Healthy	IgG	605537.3	397034.0	239.1	65.5
Cryo neg	IGH	251582.3	174317.2	244.6	68.5
Cryo pos	IGH	176042.2	128472.8	247.3	72.8
Healthy	IGH	328644.7	238283.3	245.9	72.4
Cryo neg	IgM	541148.0	354505.0	242.5	65.5
Cryo pos	IgM	445399.3	296493.3	245.4	68.7
Healthy	IgM	628945.0	403285.0	243.8	63.5

Samples were prepared for NGS as described in Materials and Methods. Table prepared by Dr Rachael Bashford-Rogers at the Sanger Institute, Cambridge showing quality control data from NGS sequencing of the cohorts.

8.4 Diversity of IGHV sequences across clinical groups

An in-house programme designed by RBR within the Sanger lab was used to calculate several different measures of sequence diversity within the BCR populations for each of the patients. These measurements were as follows:

- *The vertex Gini index:* measurements of overall clonality.
- *The cluster Gini index:* measurements of cluster size heterogeneity.

- *The largest cluster size (percentage)*: to distinguish between samples with different maximum cluster sizes.

- *The sum of the largest two cluster sizes (percentage):* measurement to incorporate the second largest cluster size, which may distinguish between samples with secondary rearrangements.

- *The percentage of unique BCRs in the largest cluster*: to distinguish between samples with different levels of SHM (where present) in the largest cluster.

- *The percentage of sequences representing the most frequently observed BCR sequence*: to distinguish between samples with or without dominant BCR sequences.

- *The percentage of sequences representing the first and second most frequently observed IGHV-J rearrangement:* measurement to distinguish between samples with specific rearrangements, irrespective of the largest cluster sizes.

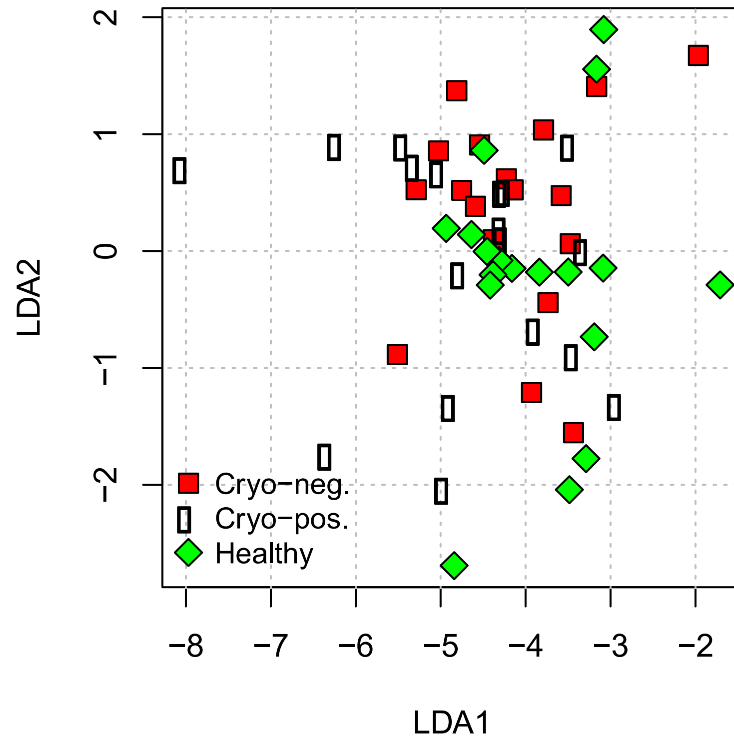
- *The ratio of the number of unique CDR3 sequences to unique full length BCR sequences*: as the CDR3 length is shorter than the full length BCR sequence, but B-cells sharing the same CDR3 sequence are likely to originate from a single B cell precursor, then lower ratios of unique CDR3 sequences to unique full length BCR sequences suggests lower B-cell clonal complexity.

In particular, Gini measures of diversity along with absolute cluster size and proportion of reads in clusters compared to total number of reads have previously

been used in research of clonal B cell populations in the context of B cell malignancies (263). These measurements were further analysed and interpreted in collaboration between myself, RBR, and Dr Mark Robinson.

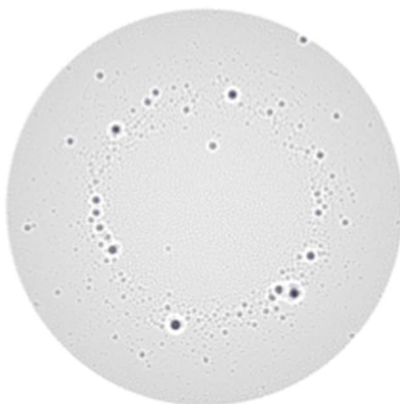
Linear discriminant analysis (a generalisation of Fisher's linear discriminant) was conducted by RBR and revealed none of these measures were able to discriminate between the three clinical groups. Such analysis is increasingly being used to analyse features of NGS output (346). There was no evidence of abnormally large clusters in the cryoglobulin positive group or of difference in overall BCR diversity between chronically infected HCV patients with and without cryoglobulinaemia and healthy controls as measured using a number of diversity indices (see Fig. 8.2a). In addition, there was no significant evidence of abnormal clusters in B cell heavy chain sequence diversity suggesting that significant clonal expansion had not yet occurred in these individuals. An example of the distribution of BCR gene clusters seen can be visualised as shown in Figure 8.2b. When we compared measures of diversity between those with HCV infection (cryoglobulin-positive and -negative) and healthy control groups there were no significant differences. However, there was a trend to lower levels of diversity in the HCV infected IgM specific BCR group. This was particularly seen using the Cluster Gini Index (Fig. 8.2c, P=0.05) suggesting that the presence of HCV may exert a small effect on the IgM BCR population.

A



B

Healthy 1 IGH
161,559 total sequences
76% sequences > 1 read
0.69% maximum cluster size



Cryo-negative 1 IGH
161,212 total sequences
74% sequences > 1 read
1.06% maximum cluster size

Cryo-positive 1 IGH
89,124total sequences
39% sequences > 1 read
0.12% maximum cluster size

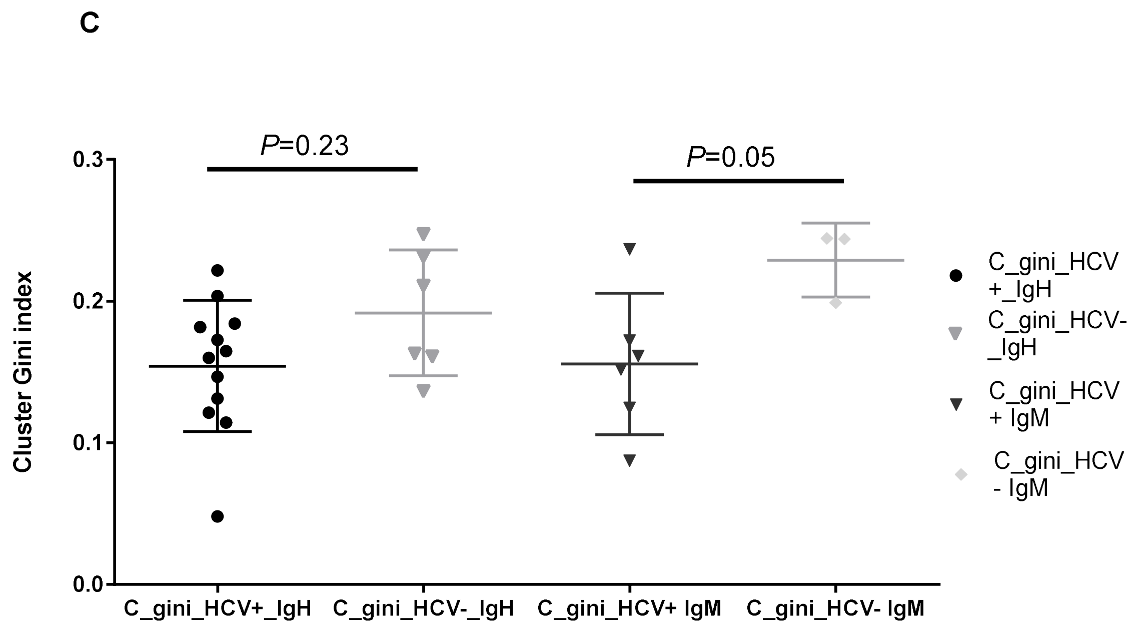


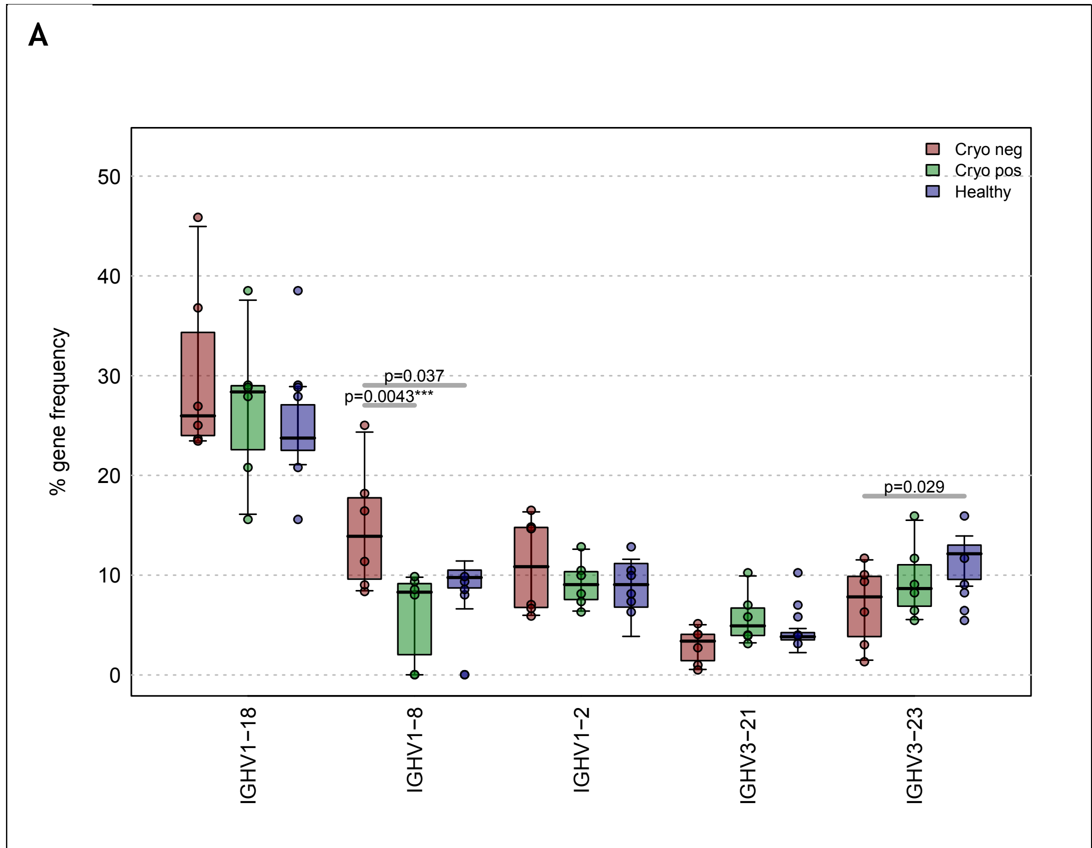
Figure 8.2 BCR diversity measures across clinical groups.

A) Linear discriminant analysis (LDA) performed on repertoire structure measures of the BCR sequences generated by NGS sequencing. This technique employs a generalisation of Fisher's linear discriminant. This statistical method determines if a combination of features can be used to sort two classes of subjects in a linear fashion. This graph was prepared in SPSS by RBR. Nine BCR population diversity and structural features were analysed (*the Vertex Gini Index, Cluster Gini Index, % of reads in largest cluster, % of reads in largest two clusters, % of unique sequences in largest cluster, % of reads of highest observed sequence, % of sequences with the highest observed VJ gene usage, % of sequences with the 2nd highest observed VJ gene usage, ratio of unique CDR3s sequences to total reads)*. The values for each patient group for the first and second LDA dimensions (LDA1 and LDA2) are shown on the graph above. There is no clear separation between the three clinical groups using B-cell sequencing architecture as shown by the distribution of samples projected in the two LDA dimensions. B) Graphical depiction of relative size of clusters seen for one patient in each clinical group. Within the circle, each dot is scaled to represent an individual cluster of reads. Larger dots indicate larger clusters. C) Scatter plot of Cluster Gini index for HCV-positive and -negative individuals for total BCR heavy chain sequences and for IgM specific heavy chain BCR sequences. *P* value was calculated using the Wilcoxon rank sum test. Mean and SD are shown.

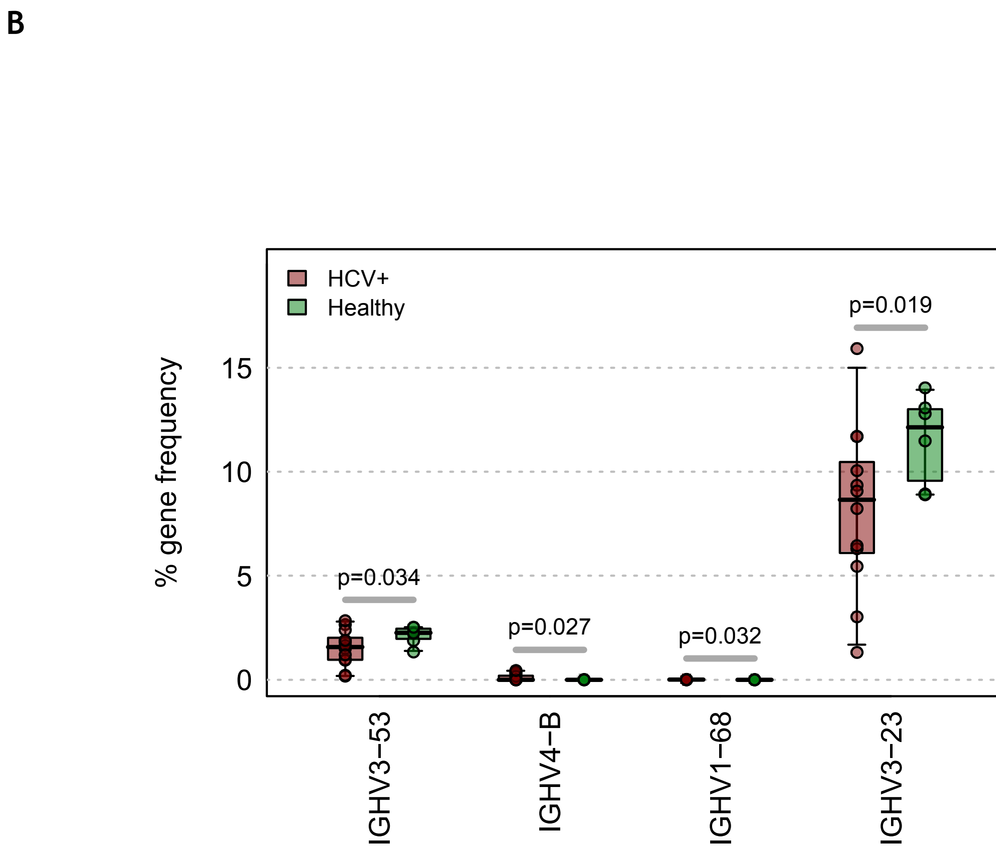
8.5 HCV infection, cryoglobulinaemia and IGHV gene usage

To determine if particular genes were used in increased frequency in either HCV infection or cryoglobulinaemia, the proportion of usage of each IGHV gene class for individuals in each clinical group were compared. This analysis revealed only one significant difference in gene usages between cryoglobulin-positive and -negative groups (Fig. 8.3a) with IGHV 1-8 appearing at a higher frequency in HCV infected cryoglobulin-negative patients than in cryoglobulin positive patients (P=0.004), however there was no significant difference between either HCV infected group and healthy controls when multiple testing was accounted for using the Bonferroni correction. There also appeared to be a trend to lower IGHV 3-23 expression in those with HCV infection but this was not statistically significant after correcting for multiple testing (Fig. 8.3b).

A



B



C

IGVH 1-69 gene usage

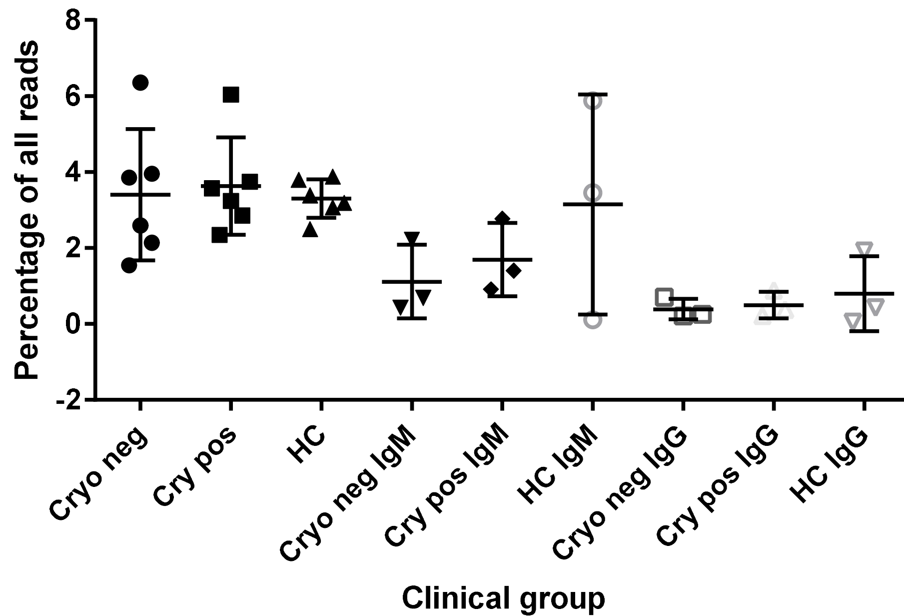


Figure 8.3 Individual BCR gene usage across clinical groups.

Sequencing data from the BCR libraries were analysed by RBR using an in-house programme to identify which genes were represented in each individual sample and what proportion of the total number of reads came from each gene. These proportions were compared for different groups as follows: A) Percentage of gene frequency across clinical groups for individual genes comprising more than 5% of total number of reads. B) Percentage of gene frequency between HCV-infected and uninfected individuals where $p<0.05$. C) Percentage of total reads in IGCH 1-69 class across clinical groups – no comparisons reached significance therefore *P* values not shown. *** denotes statistically significant different gene frequencies after accounting for multiple testing using the Bonferroni correction. *P*-values were determined using the Mann–Whitney U test. Figures A and B were prepared by RBR and Figure C by me. Mean and SD are indicated on the graphs.

As cryoglobulins usually have a monoclonal IgM component and there is evidence of abnormal IgM levels in chronically HCV infected individuals (327), the balance of IgM and IgG within the different BCR gene types was examined. Analysis of the balance of IgM and IgG BCR types within different IGHV genes showed small differences in the distribution of IgG and IgM receptors for many gene types in all three clinical groups although the majority of these were not statistically significant on correction for multiple testing, with only IgH gene 3-30 showing a significantly lower proportion of total gene usage in the IgM BCRs compared to the IgG BCRS (*P*=0.0003 Table 8.3). There were no significant differences in the balance of IgG/IgM usage in these genes in the healthy control population.

When BCR gene usage was compared separately for IgM genes, only IGHV 1-24 expression showed a significant difference, being higher in individuals infected with HCV compared to healthy controls (Fig. 8.4; *P*=0.0083).

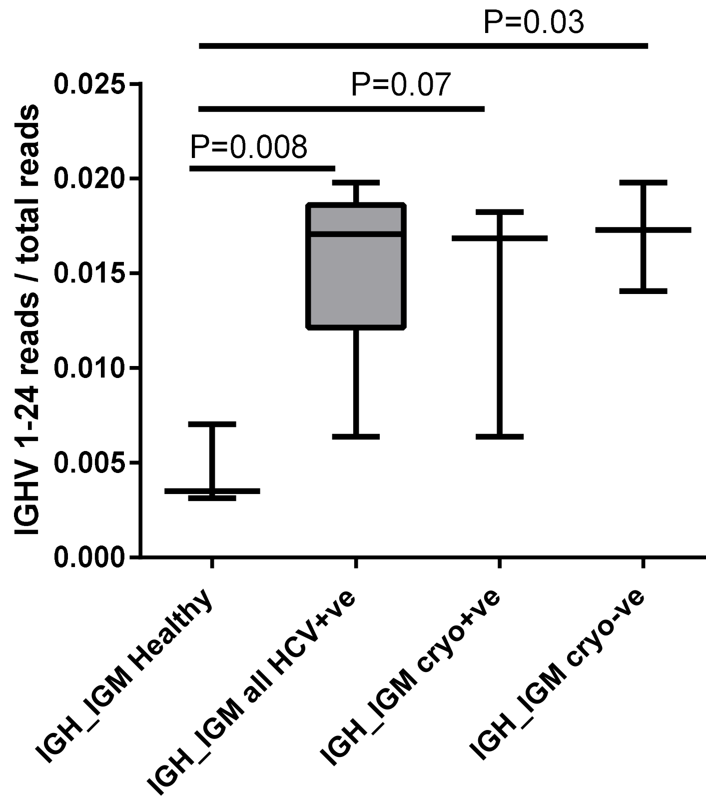


Figure 8.4 Proportion of reads for IgM class IGHV 1-24 between clinical groups.

Reads for individual BCR libraries specific for IgM type receptors were determined as described previously. The proportion of reads for each gene in cryoglobulin-positive individuals, cryoglobulin-negative individuals and all HCV-positive individuals were compared to the proportion of reads in the healthy control (HC) group using a student's t test (unpaired). The bars shown indicate mean and range for all individuals within each clinical group for the sequencing output obtained from a single experiment as described in Section 8.3 and Materials and Methods.

Table 8.3 Proportion of IGHV gene type by IgG and IgM BCRs in different clinical groups

	Mean frequencies of genes in each clinical group (ordered by highest frequency) (%)						P-value between IgG and IgM (two sided, paired)	
Gene	Cryo neg IgG	Cryo neg IgM	Cryo pos IgG	Cryo pos IgM	Healthy IgG	Healthy IgM	Cryo neg IgG	Cryo pos IgM
IGHV1-18	6.5791	16.7677	7.0391	23.6382	5.5814	15.4829	0.0418	0.0103
IGHV3-23	25.4340	16.8431	20.9423	12.4331	25.5579	19.1862	0.0251	0.0280
IGHV3-7	13.4253	5.1138	12.3611	4.1190	11.1481	5.1333	0.0304	0.0903
IGHV1-2	7.5508	12.8671	3.4079	12.0451	4.8649	10.0243	0.1369	0.0164
IGHV3-21	4.7625	5.2171	8.6644	6.0331	8.9115	6.3601	0.7030	0.1521
IGHV3-30	5.9533	4.0918	6.6180	3.4523	4.5371	2.9144	0.4151	0.0003
IGHV3-48	4.8587	3.7700	6.2254	3.7283	5.0967	4.1026	0.2320	0.1351
IGHV3-74	6.6098	4.0614	6.0082	2.8530	8.5867	3.5911	0.3619	0.1220
IGHV1-8	2.4874	7.3155	1.4449	4.8679	1.9298	6.0558	0.1093	0.1805
IGHV3-53	3.8098	2.8275	4.2079	3.0231	3.9657	2.7048	0.0241	0.0323
IGHV3-15	2.2556	1.6997	3.1321	1.9126	2.4207	2.0282	0.4738	0.0982
IGHV3-30-3	1.4754	1.6835	2.9581	2.3057	1.3927	1.2400	0.5859	0.1321
IGHV3-33	1.9659	2.3968	2.5384	2.1008	1.7850	2.0045	0.5303	0.3987
IGHV3-11	1.5914	1.3449	1.8829	1.1532	2.0525	1.1283	0.1458	0.2552
IGHV1-69	0.3967	1.1288	0.4935	1.7143	0.8017	3.1642	0.2152	0.0886
IGHV6-1	0.5997	1.1781	0.4123	1.5071	0.4050	0.8599	0.0638	0.0474
IGHV1-24	1.5406	1.7088	0.7125	1.3803	0.3085	0.4549	0.8780	0.1157
IGHV3-64	0.6838	0.7611	1.3314	1.0639	1.1183	1.0106	0.2382	0.6536
IGHV3-66	1.5025	1.2830	1.2832	0.7705	1.6925	1.7046	0.4418	0.4326
IGHV4-59	0.2653	0.9979	0.2391	1.2642	0.2034	1.0689	0.0575	0.0210
IGHV3-43	1.6174	0.9557	1.2319	0.5719	1.9204	0.7432	0.4568	0.1014
IGHV4-34	0.1557	0.5249	0.2430	1.0922	0.1395	0.9459	0.0381	0.0197
IGHV1-58	0.2572	0.7300	0.4797	1.0813	0.2134	0.9922	0.0316	0.0603
IGHV3-20	0.2991	0.3760	0.9562	0.4119	0.1598	0.0804	0.4752	0.3994
IGHV3-72	0.2199	0.1141	0.8924	0.1501	0.5230	0.2326	0.1910	0.1686

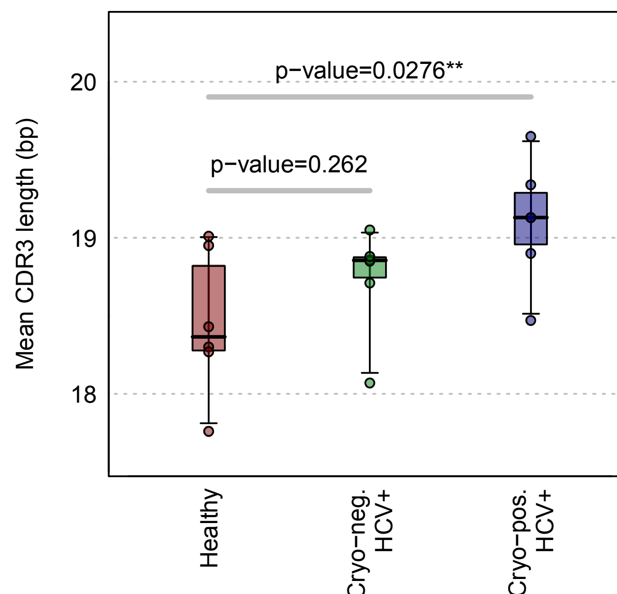
3 cryoglobulin-positive, cryoglobulin-negative and HC individuals underwent NGS sequencing of IgM- and IgG-specific BCR libraries (as detailed in Materials and Methods). For the 25 most frequent genes the percentage representation of each gene in the IgG and IgM populations were compared using a student's t test. Those with a *P* value <0.05 highlighted with those where IgG higher than IgM coloured in orange, IgM higher than IgG coloured in yellow.

8.6 Cryoglobulinaemia and CDR3 length

The sequencing data also allowed us to measure the length of the CDR3 regions. These are the variable region of antibodies and BCRs which enable binding to specific antigens. Variations in CDR3 length have been demonstrated with both chronic viral infections such as HIV and also autoimmune conditions such as Sjorgen’s syndrome (345, 347)

Within our cohorts, the cryoglobulinaemia-positive group had a significantly longer CDR3 length than in healthy controls (p=0.028 Fig. 8.5a). A trend to increase in length was seen in IgM and IgG type BCRs (Figs. 8.5 b and c). HCV-positive cryoglobulin-negative patients also showed slightly longer CDR3 regions than healthy controls but this did not reach statistical significance (0.26) (Fig. 8.5a).

A



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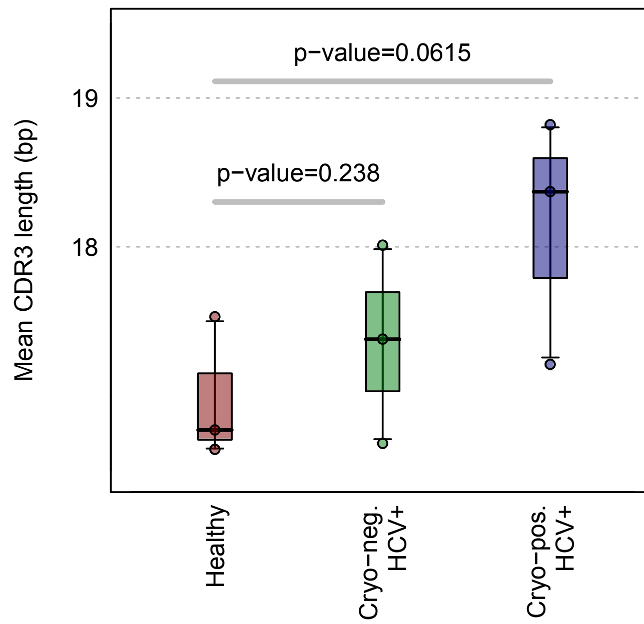

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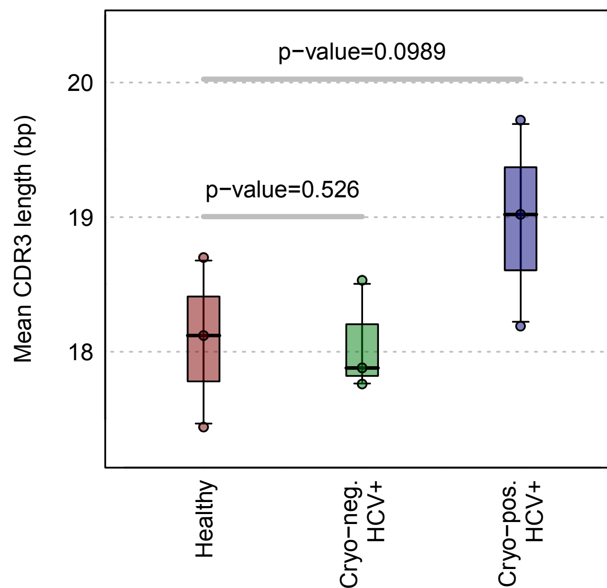


Figure 8.5 Comparison of CDR3 lengths between clinical groups.

CDR3 sequence lengths were calculated for each of the clinical groups using the programme developed by RBR and compared using the Mann Whitney U test. A) All BCR sequences or B) IgG sequences or C) IgM sequences were analysed. ** denotes statistically significant difference after correcting for multiple comparisons. Median and range are shown.

8.7 Asymptomatic cryoglobulinaemia is not associated with restriction of the functional IgG anti-HCV envelope response

As 51 individuals of the CHCV cohort (from which the cryoglobulin-positive and -negative patients were derived) had already been tested for functional IgG responses *in vitro,* I aimed to determine if cryoglobulin status was associated with any difference in antibody function. As described in Materials and Methods and in Chapter 3, serum samples from the CHCV cohort were purified to obtain IgG which was subsequently tested for their ability to neutralise viral particles containing E1/E2 from different HCV strains and ability to bind to the E1/E2 proteins.

8.7.1 ELISA assay for binding to whole E1E2 across different genotypes

As described in Chapter 3, purified IgG was tested in a GNA-capture ELISA for binding to E1E2 glycoproteins from differing sub-genotypes of HCV(the XG panel). Absorbance levels for each individual in the cohort was ranked for each subgenotype tested against. These ranks were added and an overall 'breadth of binding' rank calculated. The ranks in the cryoglobulin-positive and -negative groups were compared (Fig. 8.6a) showing no significant difference between the two groups (P=0.48).

8.7.2 Neutralisation ability across the XG and GT1 panels

A total of 51 HCV positive patient sera were tested for neutralisation activity against 6 different subgenotypes of HCV (the XG panel, see Chapter 3). No significant difference in the number of HCVpp types was seen between MC-positive and MC-negative patients with 4/16 MC-positive patients displaying a

broadly neutralising phenotype (neutralising >3 HCVpp) compared with 13/36 in the MC negative group (*P*= 0.37 by Wilcoxon Rank Sum test, Fig. 8.6b).

Twenty HCV gt 1-infected individuals (5 cryoglobulin-positive and 15 cryoglobulin-negative) were also tested for their ability to neutralise a wide range of gt 1 sequences in the HCVpp system (the intra-gentoype 1 (IG1) panel as described in chapter 3). No difference was seen in the ability to neutralise a panel of 11 G1 pseudoparticles (*P*=0.50 by Wilcoxon Rank Sum,Fig.8.6c).

8.8 Cryoglobulinaemicprecipitate IgM does not bind to E2

Due to previous reports of similar gene usage between BCRs targeting the E2 molecule and those present in HCV associated clonal B cell disorders, I attempted to determine if IgM within cryoprecipitates would bind E2. Serum and cryoglobulinaemic precipitates from 4 gt 1-infected patients and serum from 2 HC samples were tested as described in Materials and Methods. Cryoglobulinaemia precipitates were washed in ice-cold saline and redissolved in PBS at 37°C for 30 minutes. IgM concentration was measured using an IgM quantification ELISA alongside serum (see Materials and Methods). Samples were diluted in warm PBST to obtain a similar concentration of IgM in precipitate solution and dilute serum. These samples were then added to an ELISA plate coated with purified soluble gt 1a E2 (sE2). All ELISA steps up to adding the secondary antibody were performed with pre-warmed reagents in a 37°C room. Relative binding of serum and cryoprecipitate IgM to sE2 was determined. There was no evidence of binding of cryoglobulin IgM to E2 when compared with healthy control IgM, while serum from patients with cryoglobulinaemia demonstrated significant IgM binding to sE2 (Fig. 8.7). To determine if lack of binding was simply due to sequence variability, I also attempted to assess cryoglobulinaemia precipitate binding to lysate

expressing the E1E2 sequence isolated from one of the cryoglobulinaemia positive individuals. However, high levels of background in this assay prevented any meaningful conclusions being drawn (data not shown).

Figure 8.6 IgG responses to a variety of HCV envelope sequences by cryoglobulin status

A) Overall rank of ELISA binding by cryoglobulinaemia status. Individual patient IgG was tested for its ability to bind E1E2 proteins from of 6 differing subgenotypes (the “XG panel”) and ranked according to strength of binding across the panel as described in Materials and Methods. The scatter plot shows the distribution of these ranks between cryoglobulin positive and negative individuals. B+C) Assessment of neutralisation breadth by cryoglobulinaemia status. IgG from patients in the cryoglobulinaemia-positive and -negative cohorts were tested for their ability to neutralise HCVpp bearing (B) E1E2 of 6 differing subgenotypes, from the XG panel and (C) E1E2 of 11 diverse gt 1 envelope sequences, the IG1 panel, as described in Materials and Methods. A reduction in infectivity by 50% was taken as positive neutralisation. The scatter plots show the number of HCVpp strains neutralised to this level by cryoglobulin-=positive and -negative patients. Differences between the groups were analysed using a Wilcoxon Rank Sum test. Median and interquartile range is indicated on the graphs.

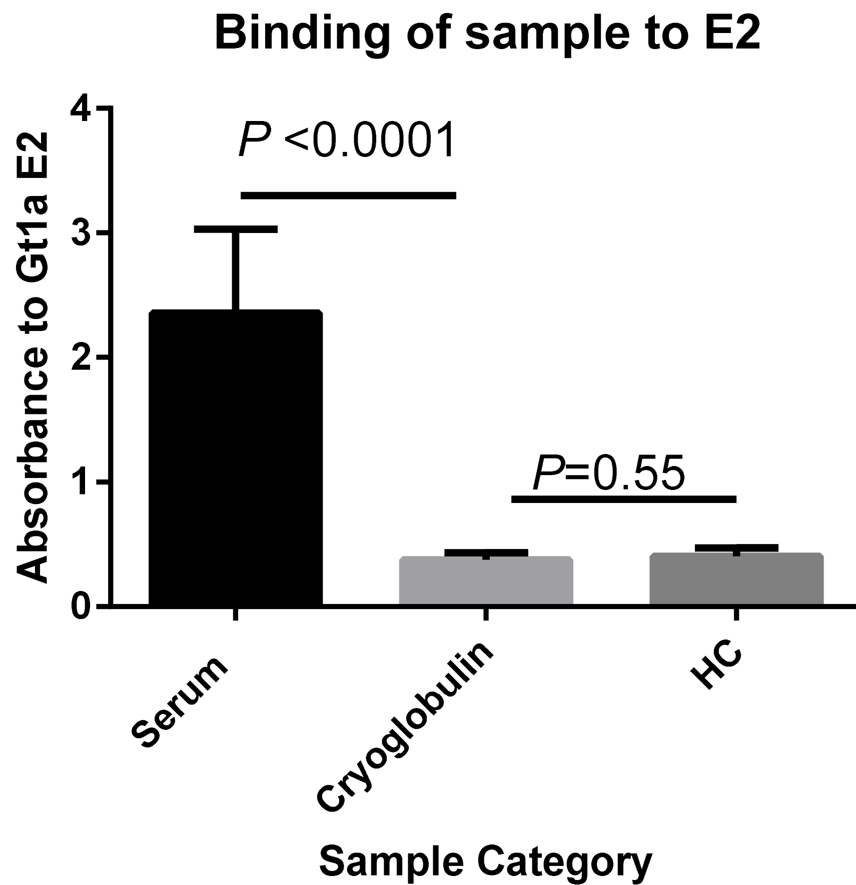


Figure 8.7 IgM binding to gt 1a sE2 by cryoglobulins in solution and dilute serum.

Serum and cryoglobulin samples from the same 4 gt 1 infected individuals were tested as described in Materials and Methods. Briefly, IgM concentrations in the redissolved cryoprecipitate and serum were ascertained using an IgM ELISA. Serum from each patient was normalised to the same IgM concentration as in cryoprecipitate in PBST. These solutions were tested for ability to bind to purified gt 1a sE2 at 37°C in a modified IgM ELISA protocol as described in Materials and Methods. Differences in relative absorbance were compared using the Wilcoxon Rank Sum test.

8.9 Chapter 8 Discussion

8.9.1 Prevalence of cryoglobulinaemia and association with clinical features

Subclinical cryoglobulinaemia has a surprisingly high prevalence of 31% within our UK based cohort of HCV infected patients. Estimated rates of HCV-associated B cell pathologies vary widely depending on geographic region, with the highest prevalences of 40-50% being reported in Southern Europe and some North American series (341, 348). There are no published studies from a Scottish population, with only one London-based study from the UK suggesting a prevalence of 19% in the 1990s (349). A genetic predisposition to MC has been proposed with the HLA types DR5 and DQ3 identified as more likely to develop cryoglobulinaemia in one study, which may go some way to explaining geographical variation (350). Best practice guidelines were followed in collecting cryoglobulinaemia samples in the CHCV cohort, therefore it is likely that the prevalence obtained is a good estimate of the true population prevalence of cryoprecipitates in HCV-infected subjects (262) and asymptomatic cryoglobulinaemia is common in Scottish patients with chronic HCV gt 1 or 3 infection.

In this cohort MC was positively associated with cirrhosis. While many of the patients in this cohort were unsure of their date of infection, a previous prospective study found an association of cryoglobulinaemia with cirrhosis to be independent of estimated duration of infection (351). No positive association with IL28B genotype or HCV genotype was found, however this sample was relatively small and only patients with genotype 1 and 3 were included. There was also a significant trend to lower viral load in cryoglobulinaemia-positive patients, this would fit with previous studies which have suggested that precipitation of virus in

the presence of cryoglobulinaemia may lead to underestimation of the viral load by conventional laboratory methods therefore the presence of cryoglobulins is likely to lead to underestimation of viral load (348).

8.9.2 Clonality, CDR3 length Rheumatoid factor activity and Cryoprecipitation

The finding of no clonal expansion of IGHV genes are in contrast with the observations of other groups. It is possible that, as this cohort is made up of asymptomatic individuals, they represent an earlier stage of disease than those traditionally included in studies of MC. Possible suppression of IGHV 3-23 in HCV-infected individuals is an interesting finding as this antibody class is involved in the response to *Streptococcus pneumoniae*, known to have a more severe course in those with advanced liver disease (352). This would be worth confirming in a larger cohort of cirrhotic individuals.

The significantly different length of the CDR3 region in the cryoglobulinaemic cohort compared to healthy controls is a new finding and contrasts with other autoimmune diseases where pathogenic autoantibodies tend to have a shorter CDR3 (353). However, increased length of CDR3 region has been reported in chronic HIV infection in addition to other chronic viral infections (354). BCRs with longer CDR3 region are often found on B1 cells and derive more closely from the germline sequence. These tend to have a polyreactive phenotype which could explain the presence of autoreactivity seen. This could reflect production of polyreactive, non-specific antibodies by naïve B cells as found by Rosa *et al* (47). Indeed, while significant somatic hypermutation has been demonstrated in some MC studies (343, 355), Charles *et al* (2013), also identified only low to moderate levels of somatic hypermutation in a cohort of MC patients with some clonal B cell

populations being unmutated (183, 343). Other researchers have reported the ability of HCV to activate naïve B cells through CD81-binding (47) and also by stimulating B cell activating factor molecules (184).

One explanation for these findings could be that HCV infection stimulates B cells to produce polyreactive antibodies, over time, as some of these B cells bind antigens – perhaps HCV specific proteins – they undergo somatic hypermutation and the B cells transition from a naïve type to CD27+ memory B cells. Other groups have noted that while naïve and memory B cell populations are expanded in MC, the naïve type is more prone to apoptosis (356, 357). This may result in the clonally expanded memory B cell population observed in more advanced MC by several groups (183, 358).

Traditionally presence of vasculitic cryoglobulinaemia is synonymous with a positive Rheumatoid factor (RhF). In our population RhF positivity was only discovered in a third of patients tested, despite finding a typical pattern of cryoprecipitation made up of polyclonal and monoclonal immunoglobulins. To report a positive RhF a minimum concentration of Fc reactive antibodies must be present in the sample (>15IU/ml for the Seimens assay). If the CHCV cohort represents an early stage of the cryoglobulinaemia process their concentration of RhF may be lower than in textbook cases of cryoglobulinaemic vasculitis. Alternatively, there is evidence that B cells with RhF activity lose this property when reverted back to the germline sequence (343) and that some monoclonal antibodies expressing the typical VH 1-69/Vk3-20 BCR genes did not have RhF activity. It is possible that earlier stages of MC may consist of autoreactive antibodies prior to extensive somatic hypermutations which results in lower avidity binding and a negative RhF assay. Indeed, a recent study of asymptomatic MC found cases with very low levels of RhF activity (359).

8.9.3 The effect of HCV infection on B cell receptor IGHV gene repertoire

The overall diversity of B cell populations is preserved in the presence of HCV infection, even within the cohort of patients with subclinical cryoglobulinaemia there were no detectable abnormally expanded clonal clusters. This suggests that the presence of cryoglobulinaemic precipitate alone does not necessarily equate to the presence of a sizable clonal B cell population.

However, the data from the CHCV cohort suggest that HCV infection may have subtle effects on gene usage within the BCR repertoire. In particular, expansion of some IGHV 1 genes and restriction of some IGHV 3 genes were seen (Fig. 8.3). This would fit with patterns of gene usage previously reported for anti-HCV antibodies, which tend to be in the IGHV 1 class. This is also the predominant IGHV class found in HCV associated B- Cell lymphoma and splenic marginal zone lymphoma (185). Interestingly, expansion of IGHV 1 and restriction of IGHV 3 has been reported previously in HIV associated B cell lymphoma (360). The shift towards IgM usage in some of the genes within this class is also of note as HCV associated MC usually is characterised by monoclonal or oligoclonal IgM (361).

8.9.4 Functional responses

There was no significant difference in breadth of functional antibody responses between MC positive and negative groups. This implies that even if HCV uses molecular mimicry as a decoy mechanism, it does not prevent a broad neutralising response from developing in a proportion of patients, albeit too late to clear infection. Other researchers have also confirmed that HCV infection does not prevent activation of B cells (362).

While all of our cryoglobulinaemic patients tested had raised an anti-envelope protein antibody response, our data suggested the IgM fraction of the cryoglobulin does not easily bind the E2 protein. Until recently, other research groups had also struggled to confirm cryoglobulin IgM binding to the E2 protein. However, the E2 protein has the ability to change conformation, with some flexible regions concealing immunogenic epitopes(363) therefore it is possible that E2 as expressed in HEK lysates is in a different conformation to that which binds cryoglobulinaemic IgM. Since my research was conducted, Dustin *et al* have presented their findings that cryoglobulinaemic antibodies target a conformational epitope on the HCV protein [Antibody specificities in HCV-associated mixed cryoglobulinaemia, Dustin LB, presented at the Fifteenth UK Hepacivirus meeting, May 2017]. This data has not yet been published. . Further exploration of the natural antigenic targets of typically expanded BCR populations in cryoglobulinaemia may help determine why HCV infected individuals do not usually produce a functional antibody response in early infection. Further NGS aided study into the impact of early HCV infection on the BCR repertoire would also provide useful information and warrants further investigation to determine any functional implications.

8.10 Chapter 8 Summary

- Asymptomatic cryoglobulinaemia is common in this chronically HCV infected cohort with a prevalence of 31%.
- Asymptomatic cryoglobulinaemia is associated with higher levels of liver fibrosis. It is also associated with lower measured viral load which is likely to reflect artefactual lower readings due to precipitation of virus.
- Overall BCR diversity does not appear to be affected by chronic HCV infection.
- HCV infection and asymptomatic cryoglobulinaemia is associated with several changes to the B Cell Receptor repertoire, in particular:
  - Lower overall usage of IGHV 3-23
  - Expansion of IgM usage in IGHV 1-24
  - Longer CDR3 regions in cryoglobulinaemia.
- Functional IgG responses are not different between HCV infected cryoglobulinaemia positive and cryoglobulinaemia negative individuals.
- I could not demonstrate any evidence of cryoglobulinaemic IgM binding to HCV E2.

9 Discussion and Conclusions

The field of HCV immunology has made significant progress since initial studies of the anti-envelope antibody response 25 years ago (364), with recent work on the structure of the HCV envelope proteins and targets of broadly neutralising antibodies providing further useful insights (37, 145, 365). The work contained in this thesis contributes to our understanding in a number of ways.

9.1 Defining the breadth of antibody response and its clinical impact

I have demonstrated the presence of broadly neutralising antibodies (bNAbs) in a substantial proportion of chronically infected individuals. As demonstrated in this thesis and elsewhere, detecting bNAbs depends largely on which panel of sequences is used to test for them and the criteria used to define a ‘broad’ response (276). While some individuals in the CHCV cohort described in my work did produce antibodies capable of neutralising a pan-genotypic panel, this ability did not appear to confer any clinical advantage. A ‘within genotype 1’ model of defining breadth did discriminate between groups with clinical features which would logically be associated with a more robust antibody response – younger, non-obese non-cirrhotic individuals. Similar experiments using a historically collected cohort of samples described in Chapter 5 failed to demonstrate a significant association between neutralisation breadth and subsequent development of cirrhosis, therefore it is probable that such an observation may reflect a robust immune response associated with an immune system in generally healthier individuals. Nevertheless, a protective effect of bNAbs against cirrhosis is biologically plausible and cannot be entirely discounted on the findings from this small, heterogeneous cohort.

Exploring the impact of HCV infection on the composition of receptors in circulating B cells is a novel way to explore "breadth" of response. Next generations sequencing is increasingly being used to examine the evolution of BCR populations in different disease states. While the study detailed in Chapter 8 did not focus on determining which BCR combinations were involved in antibody profiles with a broad functional response, it is clear that HCV infection has some impact on the balance of BCR gene usage and antibody types. NGS BCR sequencing is now being used in other disease to gain new insights into the immune response and would be a powerful tool to explore in depth the evolution of the B cell response to acute infection and vaccination in addition to discovery of novel bNAbs (344, 366).

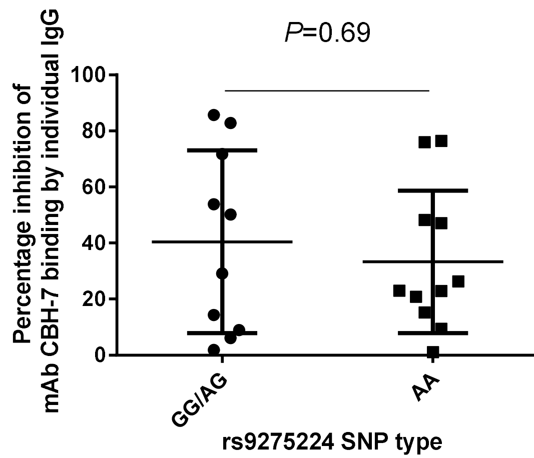
9.2 Why do some individuals develop a bNAb response and others do not?

In addition to the association with clinical features associated with a bNAb within genotype response described in Chapter 3, I have also identified several molecular characteristics of such responses. ELISA-based antibody competition assays described in Chapter 4 have confirmed that a broad "within genotype" response is associated with ability to target two distinct immunodomains on the envelope protein, immunodomain B targeted by mAb HC-11 and immunodomain C targeted by mAb CHB-7. The ability to compete with these two mAbs was also displayed by patient ET who spontaneously cleared a relapse of HCV infection following IFN based treatment. While several sequences within the IG1 HCVpp panel were resistant to both antibodies, this combination, in addition to other antibodies, may have a synergistic effect to overcome escape mutations and better control levels of hepatocyte infection. It is likely that any successful vaccine with an antibody component would need to elicit a response to these epitopes.

Host genetics, in particular, *rs9275224* SNP typing has also demonstrated a strong association between favourable gt 1 neutralisation profiles which will be an important consideration in developing and testing future vaccines. However, there is no association between presence of a favourable SNP and ability to target the HC-11 or the CBH-7 epitope (Fig. 9.1). Additionally, one of the broadest IG1 neutralising CHCV samples (1003) had an unfavourable (AA) SNP type. Another study has identified other HLA types which are associated with specific envelope protein polymorphisms in gt 3 infected subjects(279) .This suggests that while many factors can influence developing a bNAb response to gt 1 envelope sequences, the underlying mechanisms behind such a response are complex. From a vaccine perspective it is also encouraging in that I have not identified any absolute barriers to mounting a bNAb response.

The viral sequences individuals are exposed to may also play a part in determining the breadth of response. The findings described in Chapter 4 have confirmed some previously described escape mutations from antibodies to the immunodomains involved in neutralisation and also identified some potentially novel amino acid mutations conferring resistance to neutralisation. It is possible that exposing individuals to key epitopes containing common escape mutations may elicit antibody responses capable of neutralisation even when these escape mutations are present. Indeed, the work to identify the anti-immunodomain D HC-84 antibodies was based on identifying broadly neutralising antibodies in the presence of a domain B escape mutation (124). Some escape mutations require a number of amino acid substitutions to maintain viral fitness (291). Generating antibodies which target epitopes where escape has a fitness cost may contribute to a successful vaccination strategy, further study of the fitness cost of the escape mutations identified in Chapter 4 would help clarify any such regions.

A



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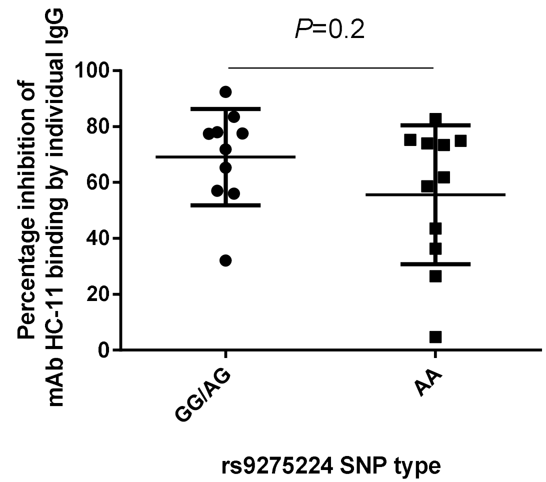

Figure 9.1 Ability of individual IgG from the CHCV cohort to inhibit binding of mAbs CBH-7 (A) and HC-11 (B) by rs9275224 SNP type

Inhibition of mAb binding to sE2 coated plates by IgG from gt 1 infected individuals in the CHCV cohort was determined as described in Chapter 4 and Materials and Methods. SNP typing was conducted as described in Chapter 3 and Materials and Methods. The inhibition of mAb binding between SNP types was compared using the Wilcoxon Rank Sum test. Mean and SD are shown.

9.3 Cross-genotypic versus genotype-specific broad responses

If it was desirable to generate a vaccine only effective against gt 1 HCV then further study of the envelope epitopes which raised the broadest antibody responses against the IG1 or similar panels would be critical in its development to optimise the initial breadth of antibody response. However, in the era of DAAs, the geographical regions most in need of a vaccine are not predominantly infected with gt 1. Therefore, developing a pan-genotypic vaccine would be ideal. Some individuals in the CHCV cohort were able to raise an antibody response which reduced infectivity of HCVpps by greater than 50% for all genotypes tested. In general, those CHCV individuals with a broad IG1 neutralising response also had broad XG neutralising activity (Chapter 3, Fig. 3.7). It is also accepted that genotype is a poor predictor of neutralisation profile. From re-analysis of the CHCV data presented in Chapter 3 it is evident that those infected with gt 1 HCV (therefore exposed to gt 1 envelope proteins) were generally better at neutralising HCVpp of all genotypes and not inferior to individuals infected with gt 3 at neutralising the gt 3 HCVpp (P=0.83, Fig. 9.2). Previous work studying differences between innate immune responses in gt 1 and gt 3 infected individuals in the CHCV cohort showed marked differences in the pathways upregulated between the genotypes (367). It is possible that gt 3 infection may not induce as robust an immune response as gt 1. This provides some hope that an effective gt 1 vaccine may provide adequate protection against other genotypes. However, it is likely that in non-genotype 1 envelope sequences, there may be additional variations in key epitopes which may confer resistance to neutralisation by cross-reactive antibodies. Adapting the techniques and tools used in this work to explore anti-

envelope responses in other genotype infections will be crucial to optimising a pan-genotypic vaccine.

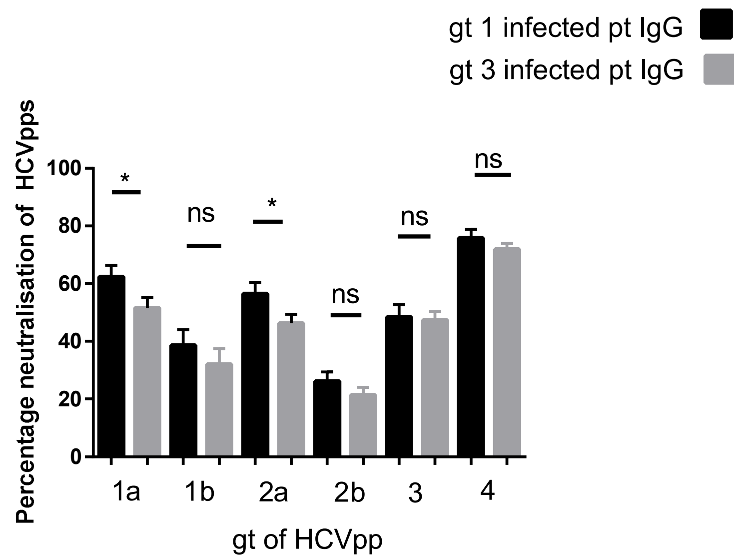

Figure 9.2 Ability of CHCV individuals infected with gt 1 and gt 3 HCV to neutralise HCVpp bearing envelope proteins of different genotypes.

IgG from the CHCV cohort was tested for its ability to neutralise HCVpps bearing envelope proteins of different genotypes in the XG panel as described in Materials and Methods. Here the mean percentage neutralisation of each HCVpp by individuals infected with gt 1 HCV and those infected with gt 3 HCV is plotted. Error bars represent SEM. Statistical comparisons were conducted using the Wilcoxon Rank Sum test, * indicates a *P* value of <0.05. Values shown are the results of 3 independent experiments.

9.4 Early and durable antibody responses

Some researchers have suggested that once individuals have been exposed to a viral antigen, this antigen and the early responses to it determine the nature of subsequent immune responses to similar antigens. This is because antigenic exposure results in the development of memory B cells. There is a tendency for these to be reactivated in the event of exposure to a similar but not identical antigen, rather than activation and affinity maturation of a different set of B cells. This is known as repertoire freeze and has been documented in influenza and dengue viruses (368-370). This may be advantageous to a virus such as HCV which is able to rapidly evolve, in that the host response may consist of persistently activated B cells with a low affinity for the mutated antigen and therefore ineffective at neutralisation. In light of this, an effective vaccine may have to elicit bNAb from the first dose.

If early antibody responses are key to viral control, then exploring the IgM anti-HCV response may give helpful insights into why only a minority of individuals resist or clear infection acutely. IgM is more difficult to work with than IgG due to its less specific binding properties. However, in HCV it remains elevated for longer than would be expected for a chronic infection (326) in addition to being involved in the pathological condition cryoglobulinaemia (183). Conversely, the onset of anti-envelope IgG responses is often delayed (371). In the experiments conducted using the Exposed Uninfected cohort samples (Chapter 7) IgM anti-E2 responses were detected at similar levels to in chronically infected individuals (Fig. 7.5). Further characterising the IgM response in acute HCV infection would be useful in determining if it is beneficial for viral clearance or whether a prolonged IgM phase is a reflection of pathological effects of HCV.

The duration of responses is also key in designing an effective vaccine. In general antibody levels to HCV are known to reduce after clearance, indeed this is demonstrated in Chapter 6 as the potency of neutralising antibodies reduces 10-fold in 1 month following viral clearance (Fig. 6.7). The EU cohort described in Chapter 7, show persistence of antibody responses despite absence of infection, although higher risk of regular exposure to HCV is linked to concentration of anti-E1E2 IgG. Therefore optimisation is likely to be necessary to ensure an ongoing neutralising response which does not require a 'booster' vaccine following the initial vaccination period. Encouragingly, patient ET showed a typical 'boost' response following relapse with a recognised strain of HCV (Chapter 6) which suggests that a rapid reactivation of the anti-envelope antibody response is possible in the event of re-exposure.

9.5 Conclusions

Through examining the breadth of antibody binding and neutralisation of differing strains of envelope proteins in the HCVpp system in clinical cohorts, it is clear that some individuals when exposed to or infected with HCV produce a polyclonal bNAb response. The data presented has identified that host genetics and the ability to target multiple neutralising epitopes on the envelope protein are associated with such responses, although resistance mutations to bNAbs do exist *in vivo*. In addition, the presence of bNAbs is associated with lower levels of liver fibrosis. I have also identified a potentially novel role for NAbs in resisting infection in a highly exposed cohort and in spontaneous resolution of viral relapse post-treatment. Finally, the use of next generation sequencing technology in the study of B cell receptors in HCV infection has been demonstrated with the possibility of yielding many future insights into the generation of bNAb responses.

9.6 Further work

I would like to expand this work further in future. Particular questions raised during my research which I would like to explore, given more time, would be:

- Do bNAbs have a role to play in progression of liver disease beyond cirrhosis to liver decompensation or development of hepatocellular carcinoma?

- What is the relationship between infecting viral strain, development of bNAbs and clearance of infection? Are there specific sequences associated with bNAb production, in particular, do certain sequences induce antibodies targeting the key epitope regions in immunodomain B and C?

- Further analysis of the impact of the polyclonal nature of bNAb responses, which combination of antibodies provides the broadest antibody response?

- Can an antibody response raised against gt 1 also provide protection against a wide range of sequences from other genotypes, particularly gt 3 and gt 4? If not, which parts of the envelope protein needs to be targeted for a bNAb against these genotypes.

- What is the relationship between host genetics and a bNAb response in gt 3 and other genotype infections

- Which BCR sequences are involved in antibodies targeting various immunodomains of the E2 protein and how does the BCR repertoire evolve in acute HCV infection and exposure.

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