Host and Viral Factors that determine the clinical outcome of Hepatitis C Virus Genotype 3a Infection

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Abstract

HCV infects 170 million persons worldwide and is a serious global health problem. Genotype-3a is the dominant genotype in newly diagnosed infections within the UK and has a high response rate to interferon therapy, with up to 70% patients achieving a sustained virological response (SVR). The reason(s) for this are unknown; therefore the aim was to assess host and viral factors that determine treatment outcome of subtype-3a infection.

Full-length subtype-3a viral sequence analysis identified 2 novel regions of hypervariability within E2 - HVR495 and HVR575, that are subject to positive selection pressure. A 5 amino-acid insertion found only in subtype-3a and a putative glycosylation site were contained within HVR575. These data suggest that HVR495 and HVR575 may serve as major antigenic sites in subtype-3a HCV infection. Successful treatment of chronic subtype-3a infection was not associated with pre-treatment quasispecies diversity and complexity, PePHD, HVR495 or HVR575 sequence. Different patterns of quasispecies variation were observed in patients that failed treatment.

Subtype-3a specific CD8+ T-cell responses in chronic infection target non-structural proteins, in contrast to pre-dominant genotype-1 core-specific CD4+ T-cell responses. SVR was associated with a decline in subtype-3a specific and non-specific T-cell responses, and also total lymphocyte counts, which all recovered after treatment. These data do not support the theory that clearance of subtype-3a is associated with an enhancement of antiviral T-cell responses. Overlapping peptides detected a greater number of subtype-3a T-cell responses compared with peptides representing putative predicted CD8 epitopes. Therefore subtype-3a HCV is distinct from genotype-1 in terms of genome sequence, effect of treatment on quasispecies and subtype-3a specific T-cell responses, further emphasising the importance in understanding this distinct subtype.

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Diolch i bawb.

Collaborations

Chapter 6 “Detection of subtype-3a T-cell responses using class-I HLA-associated putative epitopes” was collaborative work between Silvana Gaudieri’s laboratory, at the Royal Perth Hospital and Murdoch University, Perth, Australia and myself. HCV viral sequencing of subtype-3a patients was performed by Silvana Gaudieri and myself. The identification of polymorphic sites and the association with HLA-alleles using statistical methods were performed by Silvana Gaudieri’s laboratory. I performed the prediction analysis of putative epitopes using computer-based software and the assessment of the resulting epitopes in T-cell ELISpot assays.

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Abbreviations

aa	Amino acid
AIC	Akaike information criterion
ALT	Alanine aminotransferase
APC	Antigen presenting cell
ARFP	Alternative reading frame protein
BEAST	Bayesian evolutionary analysis sampling trees
BSA	Bovine serum albumin
cDNA	Complementary DNA
CMV	Cytomegalovirus
ConA	Concanavalin-A
CTL	Cytotoxic T lymphocyte
DAA	Direct acting anti-virals
DCs	Dentritic cells
DMSO	Dimethyl sulfoxide
dN	Non-synonymous mutation
dN/dS	Rate of non-synonymous to synonymous mutations
dNTP	deoxynucleoside triphosphate
dS	Synonymous mutation
dsRNA	Double stranded RNA
E.coli	*Escherichia coli*
EBV	Epstein-Barr virus
E1	Envelope protein 1
E2	Envelope protein 2

eIF2-α	Eukaryotic initiation factor 2 α
ER	Endoplasmic reticulum
F	Frameshift protein
FCS	Fetal calf serum
FEC	Influenza virus/Epstain-Barr virus/Cytomegalovirus
Geno	Genotype
GTR	General time reversable
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HD	Hamming distance
HIV	Human immunodefiency virus
HLA	Human leukocyte antigen
HVR	Hypervariable region
ICS	Intracellular cytokine staining
IFN	Interferon
igVR	Intergenotypic variable region
IL	Interleukin
IPS-1	Interferon-β promoter stimulator-1
IRES	Internal ribosomal entry site
IRF	Interferon regulatory factor
ISDR	Interferon sensitivity determining region
ISG	Interferon stimulated genes
IVDU	Intravenous drug use
JAK/STAT	Janus kinase/signal transducers and activators of transcription
LCMV	Lymphocytic choriomeningitis virus

LSEC	Liver sinusoidal endothelial cell
MHC	Major histocompatability complex
NFκB	Nuclear factor κB
NK	Natural killer cells
NKT	Natural killer T cells
NonSVR	Relapse and non-responder patients
NS	Non-structural
NR	Non-responder
OAS	2'5' oligoadenylate synthetase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed-death receptor 1
PEG	Polymer polyethylene glycol
PEG-IFN	Polymer polyethylene glycol - interferon
PePHD	PKR-eIF2-α phosphorylation homology domain
PKR	Protein kinase R
RdRp	RNA-dependent RNA-polymerase
REL	Relapse
RIG-I	Retinoic acid inducible protein-I
RNA	Ribonucleic acid
RNase L	Ribonuclease
Rx	Treatment
SD	Standard deviation
sdH_2O	Sterile distilled water

SE	Shannon entropy
SFU	Spot forming units
SIV	Simian immunodeficiency virus
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
SSCP	Single stranded conformational polymorphism
STAT	Signal transducers and activators of transcription
SVR	Sustained virological response
TAP	Transporter associated with antigen processing
TCR	T cell receptor
Th	T helper
TIM-3	T-cell immunoglobulin domain and mucin domain 3
TLR	Toll-like receptor
Tm	Melting temperature
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
TRIF	TIR-domain-containing adaptor protein-inducing interferon-beta
TW	Treatment week
UK	United Kingdom
UNTR	Untreated
US	United States of America
UTR	Untranslated region

Chapter 1 - Introduction

1.1 Hepatitis C Virus

1.1.1 Prevalence

Hepatitis C virus (HCV) infects 170 million persons worldwide and is a serious global health concern resulting in liver cirrhosis, hepatocellular carcinoma and morbidity (World Health Organisation, 1999). HCV infection occurs parenterally through contaminated blood transfusion and blood products, administration of medicine via non-sterile injection and intravenous drug use (IVDU). Due to the introduction of sensitive HCV detection assays in 1992, the risk of infection through contaminated blood-products and blood transfusion is now extremely low (Lauer and Walker, 2001). The main route of transmission within Western countries e.g. the United Kingdom (UK) and United States (US) is via IVDU. Despite these known transmission routes, for many individuals a risk factor cannot be determined (HPA, 2008).

The global prevalence of HCV infection differs greatly, from less than 1% in countries such as the UK and Australia, to 15-20% in Egypt (World Health Organisation, 2002). The number of persons chronically infected with HCV within the US is estimated to be 2.7 million and approximately 142,000 persons in the UK (HPA, 2008). However these numbers may be underestimations of the actual infection prevalence, as infection can remain undiagnosed for many years due to the lack of symptoms.

1.1.2 Hepatitis C Virus Structure

HCV is a RNA virus and the only member of the hepacivirus genus belonging to the flaviviridae family of viruses. The flaviviridae family also includes Yellow fever and Dengue virus (Robertson et al., 1998). HCV is 40-60nm in diameter containing a positive

sense genome, approximately 9.6 kb in length (Lauer and Walker, 2001). The genome is translated into a single open reading frame encoding a polyprotein of 3010-3033 amino acids (aa) (Figure 1.1). The polyprotein is cleaved co- and post-translationally by viral and host proteins into structural proteins core, envelope-1 (E1) and envelope-2 (E2) and 7 non-structural (NS) proteins NS2, NS3, NS4a, NS4b, NS5a and NS5b. The HCV genome also contains two untranslated regions (UTR) at the 5' and 3' ends that are important for viral replication and translation (Figure 1.2) (Dustin and Rice, 2007).

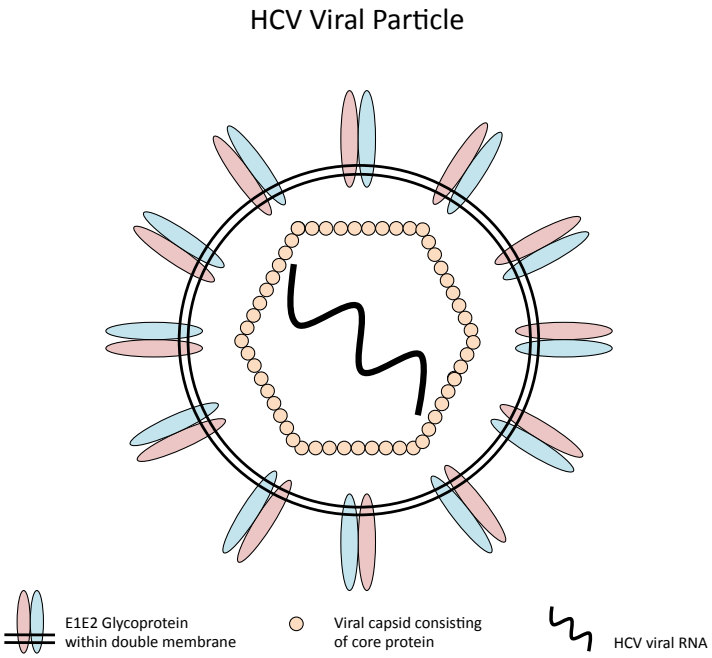


Figure 1.1 Hepatitis C virus particle
Representative diagram of a hepatitis C virus particle. The single strand RNA genome is contained within a capsid consisting of core proteins. The double membrane contains heterodimers of E1 and E2 glycoproteins.

Each HCV protein plays a role in the virus lifecycle however specific functions are not clear for all proteins. The core protein forms the viral nucleocapsid and is involved in the initiation of viral translation (Penin et al., 2004) (Figure 1.1). An additional protein of unknown function, termed the F (frameshift) or alternative reading frame protein (ARFP) is also translated due to a ribosomal frame shift during the translation of core (Walewski et al., 2001). Surrounding the nucleocapsid is a double membrane consisting of E1E2 heterodimers (Figure 1.1). The p7 protein forms a cation-channel, crucial for viral infectivity and replication (Griffin et al., 2003). Cysteine protease NS2 cleaves the junction between p7-NS2 and NS2-NS3. NS3 encodes both a serine protease and RNA helicase (Bartenschlager et al., 1993). The NS3 protease together with its co-factor NS4a, cleave the remaining junctions between the non-structural proteins (Penin et al., 2004), whilst the C-terminus NS3 helicase unwinds double-stranded RNA. NS4b is a hydrophobic protein that attaches to and alters the ER membrane to form a membranous web to assemble a replication complex that is essential for viral replication. The function of NS5a phosphoprotein remains unclear, however it is known to form the replication complex with NS4b. The NS5b encodes the RNA-dependent RNA-polymerase (RdRp) that replicates HCV RNA (Liang et al., 2000, Dustin and Rice, 2007).

1.1.3 Genotypes

HCV can be divided into 6 major genotypes based on sequence similarity between genomes, and are designated by numbers e.g. 1, 2, 3. Genotypes share 70% sequence homology at the nucleotide level, and each genotype can be further divided into multiple subtypes that share up to 80% nucleotide sequence homology. Each subtype is designated by a lower case letter e.g. 1a, 1b, 2a (Simmonds et al., 2005).

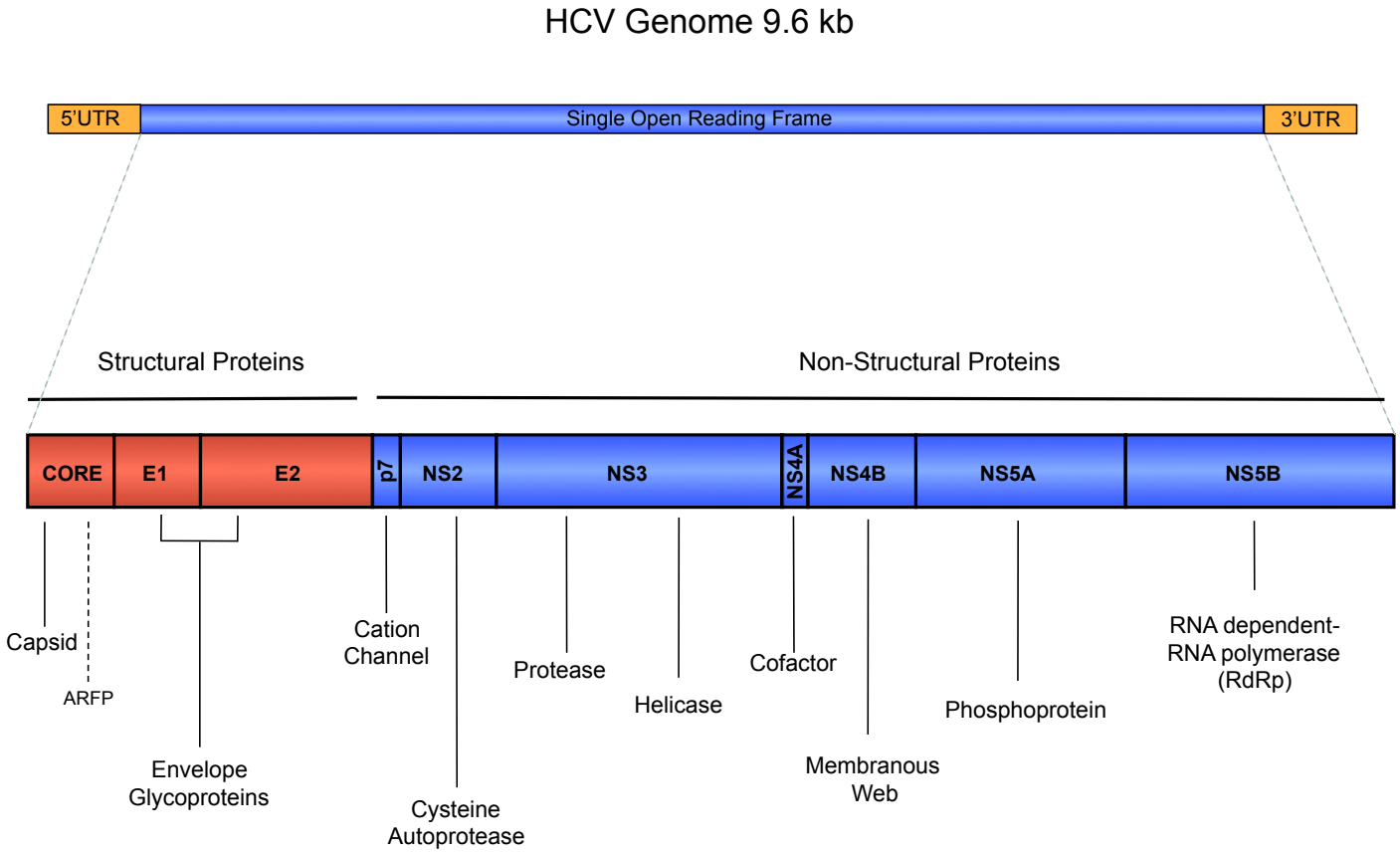


Figure 1.2 HCV genome and polyprotein
HCV genome indicating open reading-frame and untranslated regions (UTR) at the 5' and 3' ends. The single polyprotein is shown and the location of each protein. The structural proteins are indicated in red, and non-structural proteins indicated in blue. The known function of each protein is also indicated.

HCV genotypes have a geographical distribution with subtypes-1a and -1b found mainly in Western countries, genotypes-2 and -4 found in Africa, genotypes-3 and -6 in Asia and genotype-5 in Syria and Southern Africa (Nguyen and Keeffe, 2005, Antaki et al., 2009b, World Health Organisation, 1999). However subtype-3a is becoming increasingly prevalent in Europe and is now the dominant genotype in newly diagnosed infections within the UK (HPA, 2008). More than 90% of newly diagnosed infections in the UK occur in intravenous drug users, and a greater proportion of individuals with subtype-3a infection have a history of drug abuse compared with genotype-1 infection (Mihm et al., 1997, Shev et al., 1995). Additionally the increasing number of younger individuals diagnosed with HCV subtype-3a infection may reflect tramission via intravenous drug use (IVDU) (HPA, 2008, Mihm et al., 1997, Pawlotsky et al., 1995).

The severity of HCV infection also differs with each genotype. Patients infected with subtype-3a infection have significantly more steatosis than patients with subtype-1a or genotype-4 infection (Mihm et al., 1997, Tsochatzis et al., 2007). Patients with subtype-1b are more likely to develop fibrosis and ultimately hepatocellular carcinoma (Mihm et al., 1997, Harris et al., 2000, Zein et al., 1996). Genotype-2 has been associated with a less severe liver disease compared with genotype-1 (Sartori et al., 1996). Unfortunately data on infection severity is limited for genotypes-5 and -6 (Antaki et al., 2009a).

1.1.4 Infection of the Liver

HCV is a non-cytopathic virus infecting between 7-20% of hepatocytes (Liang et al., 2009). The liver contains a diverse combination of immune cells including Kupffer cells, plasmacytoid and myeloid dendritic cells (DCs), and lymphocytes such as natural killer (NK) cells, natural killer T (NKT) cells and T-cells (Calne and Davies, 1994). The liver receives blood from the intestine, and the constant presence of antigen creates an

environment that can maintain self-tolerance and immunity to infection. The presence of HCV is detected by Toll-like receptors (TLR) located on the various immune cells such as Kupffer cells, liver sinusoidal endothelial cells (LSECs) and DCs, allowing the local activation of circulating naïve T-cells that pass through the sinusoids (Racanelli and Rehermann, 2006, Racanelli and Manigold, 2007).

1.2 Natural Course of Infection

1.2.1 Acute Infection

The first 6 months of HCV infection is termed the acute phase. HCV RNA is detectable in the blood within 7-21 days of infection (Farci et al., 1991) with peak RNA levels detected at 6-10 weeks (Abe et al., 1992b, Beach et al., 1992, Alter et al., 1995). Diagnosis during the acute phase is rare as symptoms rarely present clinically. When symptoms are detected they include jaundice, malaise, nausea, and are usually mild. These symptoms present 2-12 weeks after exposure (Orland et al.), with jaundice occurring in less that 20% of those infected (Alter et al., 1992). Liver damage is indicated by elevations in the alanine aminotransferase (ALT) levels and usually detected 4 weeks after infection (Orland et al., 2001). Development of fulminant hepatitis during the acute phase is very rare (Farci et al., 1996).

Spontaneous resolution of infection during the acute phase occurs in only 20% of persons, whilst the remaining 80% will progress to chronic infection (Alter et al., 1999) (Figure 1.3). Poorly controlled viremia during the acute phase can predict the progression of infection to chronicity (Cooper et al., 1999, Missale et al., 1996), such as a pattern of initial viral control followed by viral rebound (Gerlach et al., 1999, Thimme et al., 2001, Thimme et al., 2002).

Factors that determine resolution or persistence of infection are not completely understood. However spontaneous clearance is more likely in persons that exhibit symptoms, in females, younger patients, shorter infection duration, and controversially in persons with non-genotype 1 infection (Gerlach et al., 2003, Micallef et al., 2006, Martinot-Peignoux et al., 1995, Gerlach et al., 1999, Kamal et al., 2001, Kamal et al., 2004, Alter et al., 1999, Giuberti et al., 1994, Villano et al., 1999).

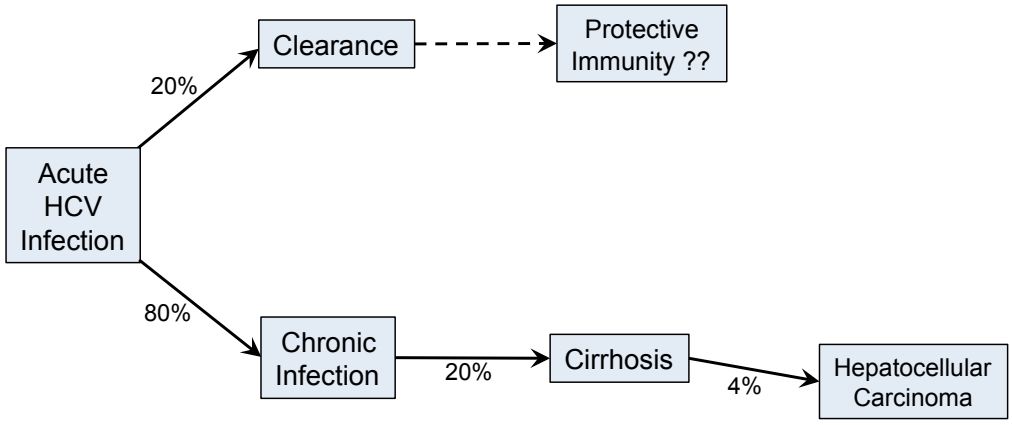


Figure 1.3 Outcome of HCV infection
Diagram shows the outcomes of HCV infection with only 20% of persons achieving viral clearance during the acute phase. It is unclear if clearance of HCV infection results in immune protection against further infection (indicated by dashed line). Eighty percent will be chronically infected, that can result in liver cirrhosis in 20% of chronically infected persons, of which 4% will develop hepatocellular carcinoma.

1.2.2 Chronic Infection

By definition, HCV infection progresses to chronicity after 6 months of infection. Spontaneous resolution of chronic infection is rare (Alter et al., 1992). Symptoms are uncommon once chronic infection is established, therefore if not detected within the acute phase, infection may remain undetected for many years. Progression of chronic disease is

slow. Many persons will ultimately develop fibrosis, 20% of persons will progress to liver cirrhosis and up to 4% of persons with liver cirrhosis are at risk of developing hepatocellular carcinoma per year (Figure 1.3). However rates of disease progression can differ between individuals (Lauer and Walker, 2001) and are affected by host factors such as age at infection, gender, alcohol consumption, HCV genotype, and co-infection with hepatitis B virus (HBV) (Zarski et al., 1998) or human immunodeficiency virus-1 (HIV-1) (Soto et al., 1997).

1.3 Host Response To HCV Infection

1.3.1 Acute phase

Innate Immunity

Successful clearance of HCV infection involves all aspects of the host immune response. The initial response is orchestrated by the innate immune system that is nonadaptive and does not produce long-term protective immunological memory. An important factor of the innate response is the ability of macrophages to discriminate between self and pathogen. This ability controls the subsequent release of its pro-inflammatory cytokines and chemokines. Macrophages are the first cells to encounter a pathogen, followed by neutrophils. These cells recognise and engulf pathogens, resulting in the release of a variety of toxic products such as antimicrobial peptides and nitric oxide to help kill the engulfed pathogen by phagocytosis. Macrophages also release chemokines and cytokines, such as interleukin (IL)-1, IL-6, IL-12 and tumor necrosis factor (TNF)-α, to set up a state of inflammation (Delves and Roitt, 2000).

During the replication of HCV double-stranded RNA (dsRNA) is produced and is detected by retinoic acid inducible protein-I (RIG-1), which interacts with interferon-β promoter stimulator-1 (IPS-1) at the mitochondrial membrane (Figure 1.4). IPS-1 activates the downstream signalling pathways of NFκB (nuclear factor κB) and interferon regulatory factor-3 (IRF3), both of which stimulate the expression of type I interferons - IFN-α, and IFN-β (Meurs and Breiman, 2007, Dustin and Rice, 2007). IFN-α/β act in an autocrine and paracrine manner, binding to the IFN-α/β receptor to activate the JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathway resulting in the up-regulation of over 100 IFN-stimulated genes (ISG) (Dustin and Rice, 2007, Peters, 1996).

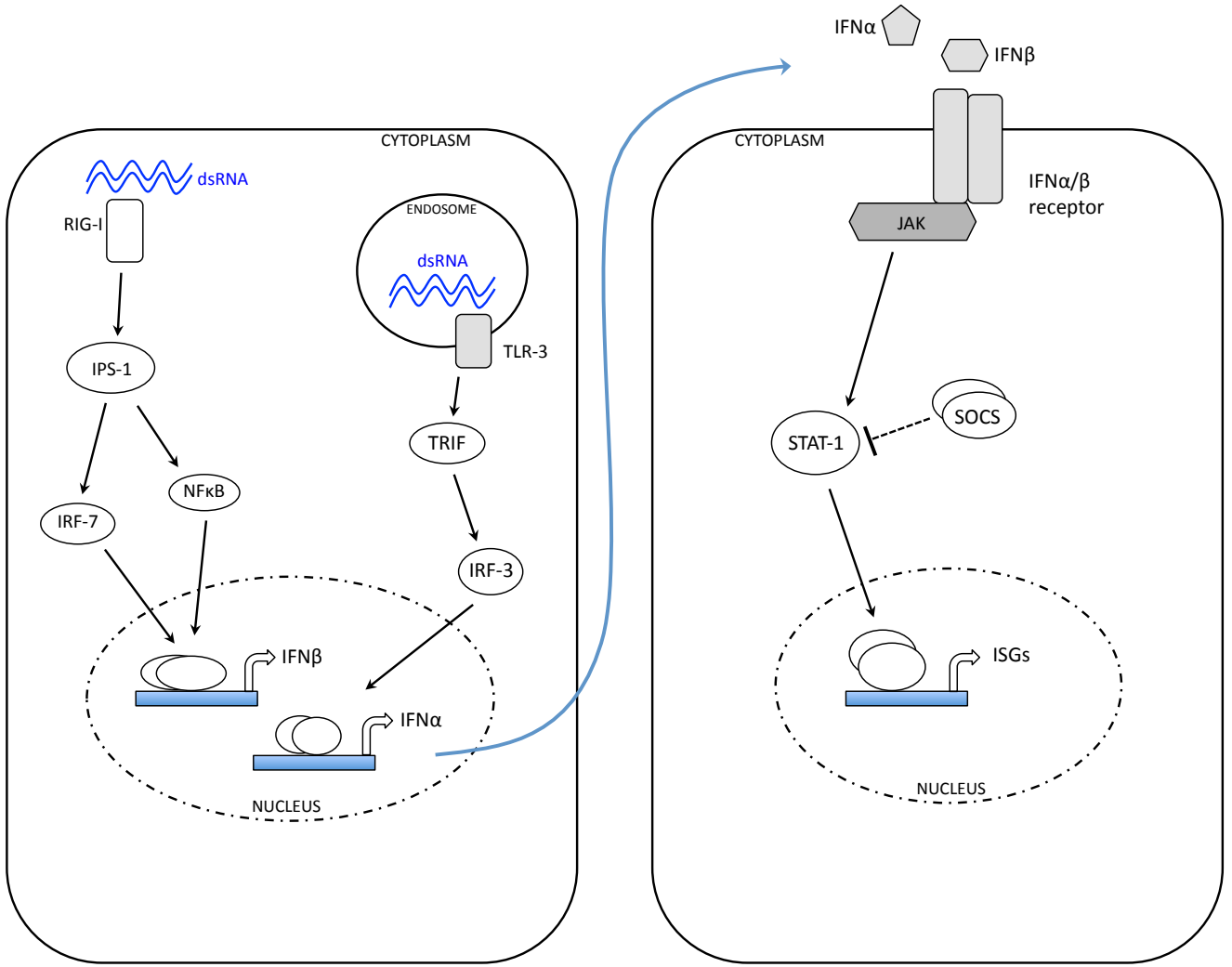
Virally infected cells are also detected by TLR-3 of macrophages and DCs that activates the NFκB signalling pathway through TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN-β). This results in transcription of proinflammatory cytokines and chemokines e.g. IL-1 and tumor necrosis factor-α (TNF-α) (Meurs and Breiman, 2007).

The up-regulation of ISGs induces an "antiviral state" within the infected cell and neighbouring cells also (Samuel, 2001). ISGs that are upregulated include 2'5' oligoadenylate synthetase (OAS), ribonuclease (RNase L) and protein Kinase R (PKR). PKR detects double-stranded RNA and inhibits viral translation by phosphorylating the eukaryotic initiation factor 2α (eIF2α).

The release of type-I IFNs also results in the activation of other innate immune cells such as NK cells, macrophages and DCs. Detection of pathogens by macrophages and DCs results in the production of co-stimulatory molecules that enable them to act as antigen-presenting cells to T lymphocytes, inducing an adaptive immune response when the innate immune response is evaded, bypassed or overwhelmed (Delves and Roitt, 2000).

Immature DCs can stimulate both CD4+ and CD8+ T lymphocytes. DCs capture antigens by phagocytosis and display antigenic peptides onto major histocompatibility complex (MHC) class II molecules to activate CD4+ T cells (Sallusto et al., 1995, Cella et al., 1997, Pierre et al., 1997, Turley et al., 2000). DCs also process viral peptides that are taken up from apoptotic cells or synthesized within the cell to be presented on MHC class I molecules to stimulate CD8+ T-cells (Cella et al., 1999, Albert et al., 1998). The cytokines produced by innate immune cells such as NKT cells and DCs determine whether a Th1 or Th2 response is produced (Godfrey and Kronenberg, 2004, Munz et al., 2005). The release of IL-12 results in the induction of T-cells with T-helper 1 (Th1) phenotype, whilst IL-4 production results in a Th2 T-cell response (Peters, 1996).

Figure 1.4 Innate immune pathway
Schematic showing the pathways involved in the innate immune response. The intracellular presence of HCV dsRNA is detected by RIG-I that activates the IRF-3 and NFκB pathways through IPS-1 to induce the production of IFN-β. dsRNA is also detected within endosomes through TLR-3, to activate TRIF and stimulate IFN-α through translocation of IRF-3 to the nucleus. Secreted IFN-α/β bind to the IFN-α/β receptors to activate the JAK-STAT pathway. ISGs are induced by the translocation of STAT-1 to the nucleus. dsRNA – double stranded RNA; RIG-I – retinoic acid inducible protein-1; IPS-1 – interferon-β promoter stimulator-1; NFκB – nuclear factor κB; IFN – interferon; TLR-3 – Toll-like receptor 3; TRIF – Toll/IL-1 receptor domain containing adaptor inducing IFN-β; IRF-3 – interferon regulatory factor-3; JAK-STAT – Janus kinase/signal transducers and activators of transcription; ISGs – interferon stimulated genes.



Adaptive Immunity

CD4+ and CD8+ T-cells

HCV-specific T-cells can be detected from 4-8 weeks after the onset of infection (Thimme et al., 2001, Cox et al., 2005a, Woollard et al., 2003). The delay in the onset of these T-cells may be due to the maturation of naïve T-cells into effector T-cell populations (Lanzavecchia and Sallusto, 2002) or to the late induction of HCV-specific CD8+ T-cells (Shin et al., 2011). It is also unclear whether this delay affects liver infiltrating T-cells or may provide an advantage in establishing HCV infection (Shoukry et al., 2004).

Activation of T-cells involves the binding of the T-cell receptor (TCR) of naïve T-cells to their specific peptide-MHC complex on DCs. The simultaneous binding of co-stimulatory molecules CD28 to B7 (Turley et al., 2000, Caux et al., 1994, Sallusto and Lanzavecchia, 1994), amplifies TCR signalling to facilitate the initial phase of T-cell activation (Lenschow et al., 1996, Chambers and Allison, 1997, Viola et al., 1999).

The activation of T-cells is further facilitated by DCs through the production of cytokines including IL-12, IL-18 and IFN-α that drive Th1 polarization of CD4+ T-cells, which produce IL-2 and IFN-γ to stimulate CD8+ cytotoxic T-cells (Macatonia et al., 1995, Cella et al., 1999, Trinchieri, 1998). CD4+ helper T-cells are responsible and essential for the efficient regulation of the antiviral immune response. CD8+ cytotoxic T-cells destroy infected cells through perforin-granule exocytosis and release antiviral cytokines such as IFN-γ and TNF-α to inactivate intracellular virus (Guidotti et al., 1994).

T-cell responses and the anti-viral response within the liver, results in inflammation and subsequent release of liver enzymes. Released enzymes such as ALT are detectable in the blood and used as a marker for hepatic injury.

HCV-specific T-cell responses are important in determining whether HCV infection is spontaneously resolved or progresses to chronicity. Spontaneous resolution of acute HCV infection has been associated with strong HCV-specific CD4+ and CD8+ T-cell responses. These responses have been demonstrated to target a broad range of HCV proteins in both humans and chimpanzees (Grakoui et al., 2003, Rahman et al., 2004, Diepolder et al., 1995, Gerlach et al., 1999, Missale et al., 1996, Cooper et al., 1999). The importance of T-cells in HCV clearance is further supported by studies in the chimpanzee model, whereby antibody mediated depletion of CD4+ or CD8+ T-cells resulted in the prolonged presence of viremia, and a decrease in magnitude and breadth of HCV-specific T-cell responses .

In addition, studies of T-cell responses in HCV patients have found an association of spontaneous clearance or persistence of HCV with the carriage of specific human leukocyte antigen (HLA) molecules. A study of Irish women infected by a single source of contaminated anti-D immunoglobulin, demonstrated that HLA-B*27, HLA-A*03, and HLA-Cw*01 were associated with spontaneous viral clearance (Shoukry et al., 2003). Spontaneous clearance of HCV infection has also been associated with the carriage of HLA-B*57 (McKiernan et al., 2004). Other HLA class I and class II alleles have also been found to be associated with either HCV clearance or progression (Table 1.1). Protection conferred by a specific HLA allele, can also be specific for HCV genotype e.g. HLA-B*27 confers protection in a genotype-1 epitope NS5b 2841-2849, but not in genotype-3 infection which contains 3 amino acid differences within this epitope (Thio et al., 2002).

Table 1.1 Studies of HLA alleles associated with HCV infection outcome

HCV Infection	HLA	Study
Clearance	A*03	(Neumann-Haefelin et al., 2010)
	A*1101	(McKiernan et al., 2004)
	B*07	(Thio et al., 2002)
	B*27	
	B*57	(McKiernan et al., 2004)
	Cw*01	(Thio et al., 2002)
	Cw*0102	(McKiernan et al., 2004)
	DRB1*0101	(Thio et al., 2002) (McKiernan et al., 2000)
	DRB1*1101	(Alric et al., 1997) (Minton et al., 1998)
	DQB1*0301	(Thursz et al., 1999) (Alric et al., 1997) (Minton et al., 1998)
	DQB1*0501	(Thursz et al., 1999)
Persistence	A*2301	(McKiernan et al., 2000)
	B*08	(Thio et al., 2002)
	Cw*04	(McKiernan et al., 2004)
	DRB1*03011	(Thio et al., 2002)
	DRB1*0701	(McKiernan et al., 2000)
	DQB1*0201	(Fanning et al., 2001)

Studies have demonstrated that HCV-specific T-cells in acutely infected patients are functional and have proliferative capacity with an activated phenotype (McKiernan et al., 2000, Lechner et al., 2000a, Shoukry et al., 2003). Although it is unclear whether an immunodominant T-cell response to a specific HCV protein is associated with spontaneous resolution (Thimme et al., 2001, Diepolder et al., 1995).

Despite the numerous studies supporting the role of HCV-specific T-cells in the clearance of acute infection, detectable cellular responses have been observed in many acutely infected patients that progress to chronicity. This suggests that HCV-specific T-cell responses are not sufficient to clear HCV infection in all acutely infected patients (Lauer et al., 2004) (See 1.5 Ineffective Immune Response).

Interleukin-28B / Interferon-lambda3

The importance of host factors in determining infection outcome has been strengthened by a recent importance of interferon-lambda3 (IFN-λ3) or IL28B allele status. Two single nucleotide polymorphism (SNP) sites close to the IL28B gene have been identified in several large genome-wide association studies (GWAS), that have strong associations with clearance of HCV infection, both spontaneous resolution of acute infection and sustained virological response (SVR) of chronic infection (Lechner et al., 2000b, Thomas et al., 2009, Tanaka et al., 2009, Suppiah et al., 2009, Rauch et al., 2010, Ge et al., 2009, Montes-Cano et al., 2010). GWAS involve the study of a large number of individuals to associate a complex disease with polymorphic markers i.e. SNPs throughout the host genome. However GWAS specifies risk-SNPs and not risk-genes, and further analysis is needed to identify the genes involved. An important consideration in assessing the results is the power of the GWAS, defined as its probability to detect a casual variant. The most crucial determinant of power to detect the casual SNP is the study size (Tillmann et al., 2010).

Ge *et al.* (Spencer et al., 2009) first identified an association between IL28B status and treatment response by assessing more than 1600 individuals that were part of the IDEAL study investigating the effectiveness of HCV treatment in genotype-1 infection (Ge et al., 2009). The GWAS found that individuals homozygous for the protective allele (CC) were three times more likely to achieve HCV clearance than patients with the risk allele (TT). Many studies have also found the same associations in different patient populations, and in both genotype-1 and -4 infections (McHutchison et al., 2009, Suppiah et al., 2009, Rauch et al., 2010). The role of IL28B status is less clear in genotype-2 and -3, and has been found to be predictive of successful treatment, possibly due to the higher treatment response rates of these genotypes (Ge et al., 2009, Mangia et al., 2010, McCarthy et al.,

2010). An association has also been found for spontaneous clearance of acute infection in different patient populations (Moghaddam et al., 2011, Thomas et al., 2009, Rauch et al., 2010, Montes-Cano et al., 2010).

These associations are further supported by studies demonstrating that IL28B mRNA expression was higher in SVR compared with nonresponders (Tillmann et al., 2010, Suppiah, 2009). Due to differences in associations across genotypes, it suggests that the virus itself may have genotype dependent effects on the host innate immune response.

The IL28B gene encodes for IFN-λ3. IFN-λs encoded by IL28A/B and IL29 are type III interferons related to type I IFN-α/β. The precise mechanism of action of IFN-λs is not known, although they are known to be similar to type-I IFNs having both anti-viral and immunomodulatory activity (Tanaka, 2009, Marcello et al., 2006, Dai et al., 2009). IFN-λs activates the JAK/STAT pathway through a unique receptor, which results in the expression of ISGs (Jordan et al., 2007b). IFN-λ has been shown to decrease the expression of Th2-type cytokines (Onoguchi et al., 2007). However IFN-λs are produced by fewer cell types compared with IFN-α (Jordan et al., 2007a) and their secretion by pDCs and macrophages suggests a role in innate immune response (Pagliaccetti and Robek, 2010). Both IFN-α and IFN-λ are produced in response to viral infections, although they may upregulate different ISGs, and variants of IFN-λ3 may further alter ISG expression. IFN-λs may therefore play an important role in host innate immunity through the expression of specific ISGs.

The level of ISG expression before commencement of treatment is associated with treatment outcome. In genotype-1 an upregulation of ISG expression was found in Kupffer cells of SVR compared with nonresponders, whereas an upregulation of ISGs were found in hepatocytes of nonresponders compared with SVR patients at pre-treatment (Coccia et al., 2004, Chen et al., 2010, Honda et al., 2010).

Combination treatment of IFN-λ and ribavirin found that IL28B status was a strong predictive value for undetectable virus at treatment week 4 (Sarasin-Filipowicz et al., 2008). However the antiviral effects of IFN-λ were demonstrated to be less potent that IFN-α, and as IFN-α upregulates IFN-λ, there may be an additive effect produced by both IFNs (Muir et al., 2010, Siren et al., 2005, Ank et al., 2006).

It is unclear as yet, how polymorphisms affect the function and specific effects of IFN-λ3. Elucidating these effects will require the identification of IFN-λ3 variants and assessment of the specific functions in vitro.

The discovery of the importance of IL28B occurred at the end of my studies, and unfortunately I was not able to measure IL28B status in this study.

Antibodies

HCV-specific antibodies appear approximately 8 weeks after exposure to HCV infection and are detected in more than 60% of patients (Pagliaccetti et al., 2008, Alter et al., 1992, Tanaka et al., 1993). The initial antibodies target core and NS3, with later responses targeting NS4 and envelope proteins (Chien et al., 1993, Vallari et al., 1992, Mondelli et al., 1994, Barrera et al., 1995).

Neutralizing antibodies are specific to envelope proteins. However an association with spontaneous viral clearance and the presence of neutralising antibodies remains controversial as neutralizing antibodies have been documented in patients that spontaneously resolve and those that progress to chronicity (Hoofnagle, 1997, Pestka et al., 2007, Zibert et al., 1997, Pawlotsky, 2002, Grellier et al., 1997, Kobayashi et al., 1997). The importance of HCV-specific antibodies is also questioned as spontaneous resolution of HCV infection has been documented without seroconversion in humans (Matsuura et al., 1992), chimpanzees (Post et al., 2004) and in patients with a humoral

immune response deficiency, such as agammaglobulinemia (Cooper et al., 1999, Bjoro et al., 1994, Christie et al., 1997).

Seroconversion during HCV infection may cause additional complications within a patient. Infection with HCV is associated with extrahepatic manifestations that can affect up to 40% of patients (Adams et al., 1997); many of these are related to B-cell antibodies. The most common extrahepatic abnormalities are mixed cryoglobulinemia (MC), membranoproliferative glomerulonephritis, Sjögren's syndrome, non-Hodgkin's lymphoma and to some extent porphyria cutanea tarda and lichen planus (Mayo, 2003, Manns and Rambusch, 1999, Nocente et al., 2003). MC is the most common abnormality, seen in up to half of infected persons, with the majority of patients asymptomatic (Tanei et al., 1995). It is characterized by polyclonal B-cell activation and autoantibody production, with the reversible precipitation of cryoprotein complexes (Nocente et al., 2003).

Also seen during HCV infection are liver/kidney microsomal antibodies (LKM). LKM type-1 antibodies recognize a microsomal P450 cytochrome and may be involved in liver damage (Agnello et al., 1992, Muratori et al., 2000). Treatment of HCV with interferon may worsen or induce autoimmune conditions (Manns and Obermayer-Straub, 1997, Manns and Rambusch, 1999). In contrast improvement of clinical and biochemical abnormalities of cryoglobulinaemia has also been shown. However recurrences of these conditions are frequent after treatment (Jacobson et al., 2010, Misiani et al., 1994).

The mechanism in which HCV can induce abnormalities to B-cells in not understood, but may relate to the ability of HCV to replicate with immune cells (Hermine et al., 2002, Okuda et al., 1999).

1.3.2 Protective Immunity

It is unclear if spontaneous resolution of HCV at any stage of infection can protect against re-infection (Zignego et al., 1995, Lai et al., 1994). The presence of HCV-specific T-cells many years after clearance, that in some cases have decresased in magnitude, may be responsible for the decreased persistence after re-exposure to HCV, in both humans and chimpanzees (Bassett et al., 2001, Shoukry et al., 2003, Major et al., 2002, Diepolder et al., 1995, Grüner et al., 2000, Lechner et al., 2000c, Lauer et al., 2004, Takaki et al., 2000).

The presence of neutralising antibodies and their ability to confer protection against HCV re-infection also remains unclear. Viral suppression or clearance is accompanied by a reduction in (or even failure to generate) specific antibodies both in humans and chimpanzees (Farci et al., 1992, Cooper et al., 1999, Beld et al., 1999), which may also play a role in the lack of protection. Neutralising antibodies have been demonstrated to confer protection against homologous but not heterologous strains (Bassett et al., 1998, Farci et al., 1992, Lai et al., 1994, Farci et al., 1994, Prince, 1994b, Prince, 1994a). However, upon re-infection neutralizing antibodies have been associated with a decrease in intensity and duration of viremia (Choo et al., 1994, Major et al., 2002, Mehta et al., 2002).

1.3.3 Chronic phase

CD4+ and CD8+ T-cells

During the chronic phase of HCV infection HCV-specific T-cells are difficult to detect and are observed in approximately 50% of patients (Thomson et al., 2003). Responses are weak in magnitude and frequency (Lauer et al., 2004, Rehermann et al., 1996b, Lechner et al., 2000b, Ulsenheimer et al., 2003) compared with responses to other viral infections,

such as HIV, Epstein-Barr virus (EBV) or cytomegalovirus (CMV) (Hiroishi et al., Urbani et al., 2006, Ogg et al., 1999, Tan et al., 1999, Spiegel et al., 2000).

HCV-specific T-cell responses target only a few proteins or peptides compared to the broad range targeted during the acute phase (Jin et al., 2000). Previous studies have identified dominant CD4+ T-cell responses to core and NS4 proteins in many but not all chronically infected patients (Lauer et al., 2002, Ferrari et al., 1994, Hoffmann et al., 1995, Iwata et al., 1995, Leroux-Roels et al., 1996, Botarelli et al., 1993).

It is unclear why the HCV-specific T-cell responses in chronic infection are weak and narrowly focused, and are ineffective in clearing infection. It is not known whether the lack of HCV-specific T-cell response is the cause of persistent viremia or whether the continuous presence of viral antigen results in an ineffective T-cell response.

Antibodies

The detection of HCV-specific antibodies many years into chronic infection indicates an ongoing B-cell response. However, a study of patients infected from a single source, found that patients that progressed to chronicity had neutralizing antibodies that were only induced late in chronic infection (Lauer et al., 2004). Additionally, HCV-specific antibodies have been demonstrated to continuously change during chronic infection, aiming to adapt to the continually changing E2 protein (Pestka et al., 2007). The significance of HCV-specific antibodies in chronic infection therefore remains unclear.

1.4 HCV Sequence Diversity

1.4.1 Genome Variability

Sequence diversity is not distributed equally throughout the HCV genome. Core protein is the most conserved region amongst genotypes, whilst E2 protein is the most variable, containing a region of 27 amino acids (aa) at the N-terminus designated hyper-variable region 1 (HVR1) (Figure 1.5). Although not as pronounced, other regions of variability within E2 have been described in chronic HCV infection; including a 7 amino-acid variable sequence in HCV subtype-1b infection - HVR2 stretching from amino acids 474 to 482 (von Hahn et al., 2007), and a 9 amino-acid region of variability in HCV genotype-2 (Kato et al., 1992b). A third region (HVR3) of variability has been described, located between HVR1 and HVR2 (Fan and Di Bisceglie, 2001, Troesch et al., 2006, Helle and Dubuisson, 2008) although this is confounded by an analysis that grouped patients of mixed genotypes. A feature of subtype-3a E2 is an insertion of 15 nucleotides that is not found in any other genotype (Law et al., 2008), the nature of which has not been systematically examined.

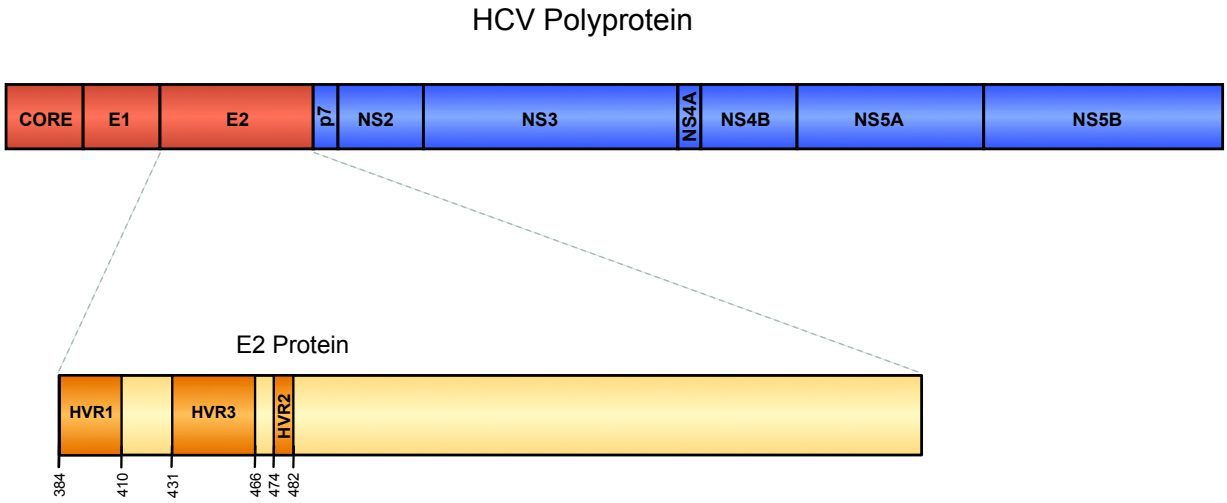


Figure 1.5 E2 hypervariable regions
Diagram showing HCV polyprotein and regions of diversity within E2 protein. Amino acid numbering is relative to the HCV subtype 1a sequence. HVR2 is only found in subtype 1b. HVR1 spans amino acids 384-410; HVR2 spans amino acids 474-482; and HVR3 spans amino acids 431-466.

1.4.2 Causes of HCV Variability

Sequence variability between HCV genotypes is likely due to the existence and evolution of HCV within human populations for at least several centuries in distinct geographical regions and the rapid expansion of certain subtypes e.g. 1a, 1b, 3a, through their introduction into new transmission routes in the 20th century e.g. IVDU (McCaffrey et al., 2007, Simmonds, 2004). Variability within HCV genotypes is due to the extremely high mutation rate – 0.4 x 10^3 to 1.2 x 10^3 nucleotide changes per site per year over the entire genome, due to the lack of proof-reading ability of the NS5b RdRp (Smith et al., 1997, Abe et al., 1992a, Moradpour et al., 2007, Ogata et al., 1991), and also an extremely high virion production, with as many as 10 x 10^{12} new virions produced each day (Okamoto et al., 1992).

Sequence variability can be assessed at a number of levels. Regions of variability that are distinct between genotypes but conserved within individual genotypes are termed inter-genotypic, whereas variable regions within a genotype are termed intra-genotypic. Intrahost variability occurs when regions are variable within the same genotype and also within an individual host. Whilst inter-genotypic variability may be a feature of geographically distinct HCV genotypes, intra-genotypic and intrahost variability may reflect viral regions subject to specific selection pressures and hence important functional implications. Regions of hypervariability within the same HCV genotype, may effectively signal a major target of adaptive immune responses. HVR1 of E2 has been shown to act both as a major target for HCV specific antibodies whilst also containing epitopes to HCV specific T-cells (Neumann et al., 1998).

1.4.3 Quasispecies

Due to the high mutation rate of HCV, the viral population within an individual consists of different but genetically related viral strains, termed quasispecies (von Hahn et al., 2007). A virus that causes chronic infection will evolve within a host to obtain its optimal fitness, and a quasispecies nature enables the fast adaptability of the virus to changing environmental conditions. The optimal fitness of the virus takes into account its recognition by the immune system and also the functional restraint on viral replication (Martell et al., 1992). It also enables different strains to be better adapted to survive within the host e.g. mutations may render viral strains sensitive or resistant to anti-viral drugs, or may alter the ability of the host immune response to recognize the viral sequence i.e. escape mutations (Martell et al., 1992).

1.5 Ineffective Immune Response

The host immune response plays a critical role in determining the outcome of HCV infection. Despite being active and strong during the acute phase, the immune response is incapable in most persons to clear HCV infection. And once chronic infection is established, the immune response is often weak and ineffective.

The virus itself may also play a role in the ineptness of the immune response, as viruses capable of causing persistent infection must evade the immune system either through inducing an ineffective immune response or through mechanisms that allow it to escape efficient immune responses.

1.5.1 Failure of T-cells

Spontaneous clearance of acute infection is associated with strong HCV-specific T-cell responses targeting numerous epitopes. However during the chronic phase, HCV specific T-cell responses are weak and target only a few epitopes. Currently it is unclear whether the inefficient HCV specific T-cell response is the cause or effect of persistent viremia.

HCV-specific T-cell responses in chronic infection are not only weak in magnitude but have been shown to be dysfunctional also. They are unable to produce IFN-γ, have reduced secretion of cytotoxic granules or unable to proliferate, compared with cells specific for other chronic viruses e.g. CMV (Martell et al., 1992, Lechner et al., 2000c, Penna et al., 2007).

The inability to proliferate and produce cytokines may account for the limited responses detected during the chronic phase across all genotypes (Lechner et al., 2000a). However it has been suggested that not all HCV-specific T-cells are dysfunctional and that function may be improved following *in vitro* stimulation (Penna et al., 2007). It has also been suggested that liver damage, measured by ALT levels is indicative of T-cell activity

(Lauer et al., 2002) but may be caused by other cells such as NK cells, or an antiviral effect. Spontaneous clearance of acute HCV infection does not correlate with presentation of symptoms, suggesting that T-cells were able to cause hepatic damage but were inefficient in clearing viral infection (Lechner et al., 2000). A similar association has also been found in acute HBV infection (Rahman et al., 2004). In addition, functional HCV-specific cytotoxic T lymphocyte (CTL) responses in acute infection have been shown to be transient in nature and may rapidly change surface phenotype and antigenic specificity (Maini et al., 1999).

There are many possible explanations for dysfunction of HCV-specific T-cell responses that include T-cell exhaustion, impaired priming by dendritic cells, and suppression of T-cell function by inhibitory molecules or regulatory T-cells.

Stunned

T-cells that are temporarily unable or have a reduced capacity to secrete antiviral cytokines are known to be stunned. If this inability continues long-term then it is known as stunting (Lechner et al., 2000b). The temporary reduction in IFN-γ secretion of HCV-specific CD8+ T-cells has been documented in HCV acute infection (Klenerman et al., 2002, Thimme et al., 2001), possibly due to continual antigen stimulation. However the precise mechanism is not known (Lechner et al., 2000a, Klenerman et al., 2002).

Exhaustion

Exhausted T-cells were first described in the lymphocytic choriomeningitis virus (LCMV) mouse model of viral infection (Lechner et al., 2000c). Infection of mice with a LCMV strain known to cause persistence resulted in exhausted virus specific CD8+ T-cells, due to continuous stimulation by high levels of antigen (Moskophidis et al., 1993).

Exhaustion results in a series of changes in both cellular function and phenotype that can occur to both CD4+ and CD8+ T-cells. As infection continues, cells progressively lose effector cytokine function, firstly IL-2, then TNF-α and Granzyme-B, and finally IFN-γ, rendering them incapable of mounting an effective antiviral response. The amount and length of antigen stimulation are critical in determining the development of exhausted T-cells as continued infection can result in severely exhausted T-cells that are at risk of being deleted (Moskophidis et al., 1993, Yi et al., 2010, Klenerman and Hill, 2005). Exhausted T-cells have been documented in HCV infection (Wherry et al., 2003, Gruener et al., 2001, Radziewicz et al., 2007, Urbani et al., 2006). Penna *et al*. found exhausted HCV specific CD8+ T-cells in chronic HCV infection that were defective in cytolytic capacity including the production of TNF-α, granzyme-A and IFN-γ with ineffective de-granulation (Penna et al., 2007).

Lack of CD4+ T-cell help

CD4+ T-cells regulate the adaptive immune response and a deficiency in CD4+ T-helper cells would subsequently affect the CD8+ T-cell response (Penna et al., 2007, Battegay et al., 1994). In the chimpanzee model, the absence of CD4+ T-cells by antibody depletion resulted in longer duration of infection and the emergence of escape mutations in CD8+ T-cell epitopes (Matloubian et al., 1994).

Failure in Priming

The failure of T-cells to clear HCV infection has also been attributed to improper priming. Priming of a naïve T-cell involves the binding of the TCR with the peptide-MHC complex on the surface of antigen presenting cells (APCs). To amplify the TCR signal and facilitate the initial phase of activation, binding of B7 molecules on the APC

and CD28 on the T-cell surface is also required. This interaction together with cytokines produced by the APC drive the polarization of the naïve T-cell to a Th1 or Th2 response (Shoukry et al., 2003).

Improper priming may be due to a defect of APCs, such as the presence of HCV within APCs (Lanzavecchia and Sallusto, 2000) or the ineffective activation of APCs by NK cells (Bain et al., 2001). NK cells are important in innate immunity and in the control of HCV in that they are potent activators of dendritic cells. However their activity may be inhibited by E2 protein (Jinushi et al., 2004, Tseng and Klimpel, 2002). Whilst lack of co-stimulation during T-cell priming, may cause inactivation and apoptosis of HCV-specific T-cells (see Tolerogenic Environment of the Liver) (Crotta et al., 2002).

The inability of the immune response to clear HCV infection has been associated with the presence of a Th2 rather than Th1 response (Bertolino et al., 1998, Reiser et al., 1997), as a Th2 response results in the production of antibodies through the release of IL-4, IL-5 and IL-10, instead of the more beneficial Th1 response that releases IFN-γ and enables the killing of viral infected cells (Tsai et al., 1997). CD4+ T-cell responses have been shown to have either a Th1 or Th0 phenotype in patients that spontaneously resolve infection, whereas a Th2 phenotype, resulting in mainly humoral responses have been observed in patients that progress to chronic infection (Banchereau and Steinman, 1998). A switch from Th2 to Th1 response can be achieved with the administration of treatment such as ribavirin, commonly used in treatment of HCV infection (see 1.6 Treatment of HCV Infection).

Regulatory T-cells

The responses of CD4+ and CD8+ T-cells are regulated by a subset of T-cells known as regulatory T-cells (Tregs). Tregs are responsible for the suppression of immune responses

and may suppress HCV-specific T-cells resulting in the weak responses detected in chronic infection. Increased frequencies of Tregs have been detected in chronically HCV infected patients compared with spontaneous resolvers or normal controls (Tsai et al., 1997, Cabrera et al., 2004). Tregs have also been shown to suppress the proliferation of HCV-specific CTLs, with an increase in CD8+ T-cells after *in vitro* depletion of Tregs (Sugimoto et al., 2003, Boettler et al., 2005, Rushbrook et al., 2005, Mills, 2004).

Escape Mutations in T-cell Epitopes

T-cells recognize epitopes that are bound to MHC molecules resulting in the initiation of a T-cell response. Residues within the epitope which are crucial for MHC or TCR binding (anchor residues) may be modified by mutations, decreasing the epitope binding affinity for the MHC or TCR (Cabrera et al., 2004, Bertoletti et al., 1994). An altered epitope that retains an ability to bind the MHC and TCR may anergize or antagonize the T-cell response to the original (wild-type) epitope (Klenerman et al., 1994, Rehermann et al., 1996). Such mutations may also adversely affect the ability of new T-cell clones to expand (Rehermann et al., 1996). Amino acid mutations outside epitopes can also affect antigen presentation by affecting the processing of viral proteins to produce epitopes. Mutations may affect residues essential for proteosomal degradation or the transport of viral antigens into the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP), and therefore inhibit epitope presentation on the surface of the cell.

A mutation that abolishes epitope recognition and is said to have "escaped" the immune response, and may result in its selection over the wild-type residue due to decreased recognition by the immune system (Klenerman and Zinkernagel, 1998). Escape mutations within HCV-specific CD8+ T-cell epitopes during acute infection are associated with HCV persistence in humans (Bowen and Walker, 2005b, Chang et al.,

1997, Bowen and Walker, 2005a, Cox et al., 2005a, Tester et al., 2005, Timm et al., 2004) and also in chimpanzee models (Neumann-Haefelin et al., 2006, Grakoui et al., 2003, Erickson et al., 2001). Immune pressure exerted by CD8+ T-cells results in mutations within and flanking epitopes and less often in CD4+ T-cell epitopes (Weiner et al., 1995, von Hahn et al., 2007).

Nevertheless, an escape mutation may also be beneficial to the host, if the resulting mutation results in a less infectious virus or ineffective replication (Fuller et al., 2010). The lower viral fitness may allow the immune system to eliminate the virus or result in delayed disease progression (Salloum et al., 2008). However escape mutations that result in reduced viral fitness (Martinez-Picado et al., 2006) may ultimately restore fitness through additional mutations termed compensatory mutations. These mutations may occur within or flanking the epitope and have been described in the protective HLA-A*03 NS3 1080-1088 T-cell epitope (Salloum et al., 2008). Compensatory mutations may take a long time to develop, such as in HIV infection, thereby providing the host with a prolonged period of protection (Crawford et al., 2007, Dazert et al., 2009).

A weakened CD8+ T-cell response, observed when CD4+ T-cell help is insufficient results in CD8+ T-cells inefficient at viral elimination but are still able to exert pressure on the virus resulting in escape mutations (Fitzmaurice et al., 2011, Weiner et al., 1995, Wang and Eckels, 1999, Grakoui et al., 2003, Chang et al., 1997, Cooper et al., 1999).

Tolerogenic environment of the Liver

HCV viral antigens are constantly presented within the liver environment (Giuggio et al., 1998). As the liver is tolerogenic, the continual presence of antigens may cause the suppression of CD8+ T-cell responses as they become tolerized or even deleted (O'Farrelly and Crispe, 1999, Qian et al., 1997). Loss of T-cell effector functions may

also be a result of the ineffective activation of naïve CD8+ T cells by hepatocytes that present antigen but do not have co-stimulatory molecules (Nuti et al., 1998, Bertolino et al., 1998).

HCV-specific T-cells may also be sequestered in the liver (Nuti et al., 1998, He et al., 1999), accounting for the low frequency of CD8+ T-cells in peripheral blood (Valiante et al., 2000, Hiroishi et al., 1997, Rehermann et al., 1996, Cerny et al., 1995, He et al., 1999, Shirai et al., 1994, Battegay et al., 1995).

1.5.2 Antibodies

HCV-specific antibodies appear within 8 weeks after infection. HVR1 is the most common region targeted due to its location on the surface of the E2 protein (Wedemeyer et al., 2002, van Doorn et al., 1995). However the importance of HVR1-specific antibodies is unclear as the appearance of antibodies does not correlate with the resolution of acute infection in all patients (Op De Beeck et al., 2004, Pestka et al., 2007, Zibert et al., 1997, Pawlotsky, 2002, Grellier et al., 1997, Kobayashi et al., 1997). HVR1 has a high mutation rate enabling the virus to escape the humoral response rendering neutralising antibodies ineffective (Matsuura et al., 1992, Shimizu et al., 1994, Thimme et al., 2006, Scotta et al., 2008, Gremion and Cerny, 2005). The outcome of HCV has been predicted by sequence changes in the HVR1 occurring simultaneously with the appearance of anti-HCV antibodies (Kanto and Hayashi, 2006). However it has been suggested that HVR1 acts as a decoy to the immune response, by focusing the humoral response on a specific protein that continually escapes and ultimately ineffective on viral eradication (Farci et al., 2000).

1.5.3 Viral Factors

Ineffective clearance of HCV infection may also be due to the ability of the viral proteins to evade or subvert the immune response (Ray et al., 1999). Viruses have two main mechanisms to disrupt the immune response - i) interference of signalling pathways involved in cytokine production or response, and/or ii) mimicking cytokines and their receptors (Katze et al., 2002, Wieland and Chisari, 2005, Taylor et al., 1999, Hu et al., 2005). The initial response of the immune system is the secretion of the IFN α/β, therefore disruption of this pathway would be of great benefit in establishing persistent infection.

HCV Proteins

HCV proteins can interact with host cellular proteins, affecting their function and potentially abrogating a signalling pathway. During the innate immune response NS3-4A protein abrogates the expression of IFN-α/β by inhibiting both RIG-1 and TLR-3 pathways, through cleaving IPS-1 and TRIF, respectively (Figure 1.6) (Dustin and Rice, 2007). The inhibition of ISG expression is affected further by inhibition of the JAK/STAT pathway, as HCV core and NS3/4A proteins induce the production of suppressor of cytokine signalling-3 (SOCS-3) that binds and inhibits STAT-1 protein, an integral protein of the JAK-STAT pathway (Thompson and Locarnini, 2007, Samuel, 2001, Bode et al., 2003).

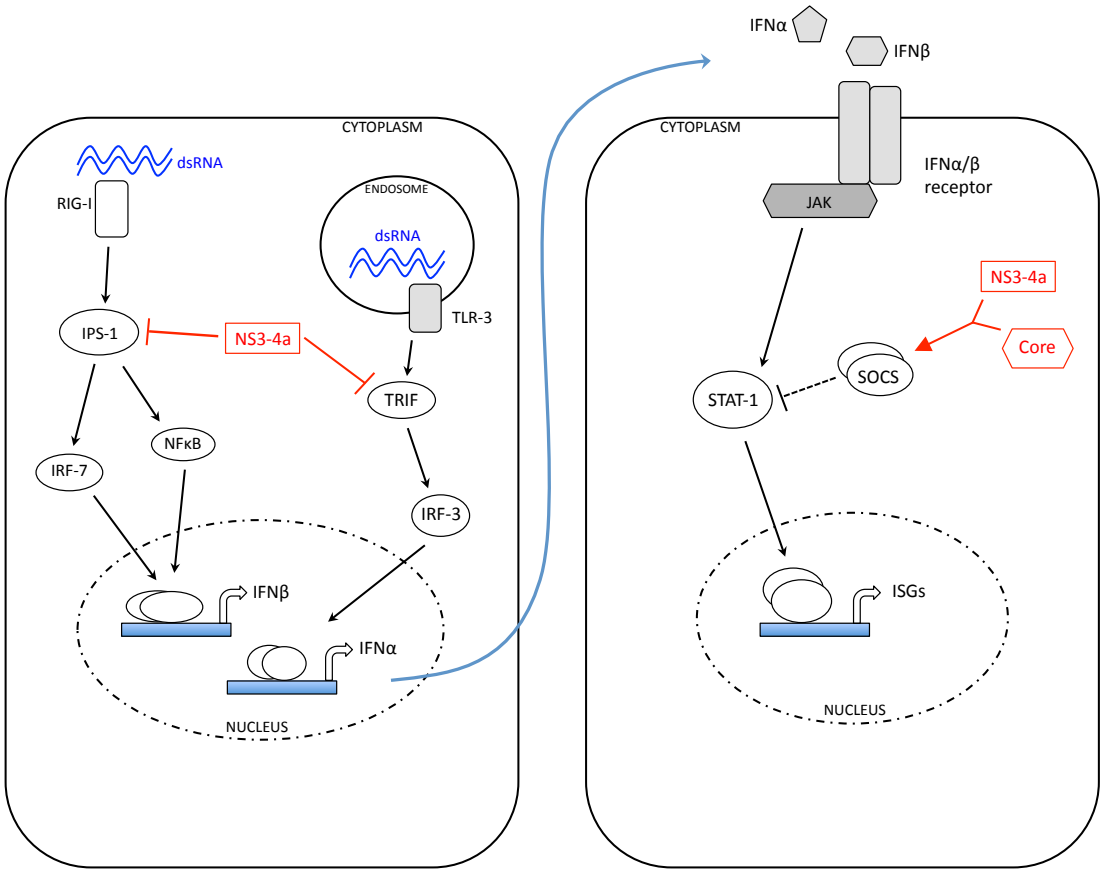


Figure 1.6 HCV inhibition of innate immune system signalling pathways
Schematic of innate signalling pathways inhibited by HCV proteins. Both RIG-I and TLR-3 pathways are inhibited through the inhibition of IPS-1 by NS3-4a protein. The JAK/STAT pathway that results in the transcription of ISGs, is inhibited through the upregulation of SOCS-3 protein by core and NS3-4a HCV proteins. SOCS is the cellular inhibitor of STAT-1 and therefore downregulates the transcription of ISGs. dsRNA – double stranded RNA; RIG-I – retinoic acid inducible protein-1; IPS-1 – interferon-β promoter stimulator-1; IRF-7 – Interferon regulatory factor-7; NFκB – nuclear factor κB; IFN – interferon; TLR-3 – Toll-like receptor 3; TRIF – Toll/IL-1 receptor domain containing adaptor inducing IFN-β; IRF-3 – interferon regulatory factor-3; JAK-STAT – Janus kinase/signal transducers and activators of transcription; SOCS-3 - suppressor of cytokine signalling-3; ISGs – interferon stimulated genes.

Specific Sequence Motifs

The function of the host dsRNA-dependent protein kinase (PKR) is to inhibit viral translation. Following its activation through the detection of double-stranded RNA, PKR phosphorylates eIF2-α translation initiation factor, resulting in inhibition of protein synthesis and viral replication within the cell (Rehermann, 2009). However HCV proteins E2 and NS5a can inhibit the activity of PKR.

The HCV E2 protein contains a 12 amino acid sequence (amino acids 659-671) at the C-terminus that is similar to the eIF2-α and PKR autophosphorylation site – RSELSPLLLTTT, termed the PKR-eIF2α phosphorylation homology domain (PePHD) (Figure 1.7) (Zilberstein et al., 1978). HCV subtype-1a sequences contain a PePHD sequence that is identical to the PKR autophosphorylation sequence, and has been shown to bind and inhibit PKR *in vitro* (Taylor et al., 1999). Whilst the PePHD of other genotypes contain amino acid mutations within PePHD, and E2 of genotypes-2 and -3 are less efficient in inhibiting PKR acitivity *in vitro* (Sarrazin et al., 2000, Kato et al., 1990). The PePHD sequence may be responsible for the lower treatment failure seen in subtype-3a infection (see 1.8.2 HCV sequences associated with treatment resistance).

PKR phosphorylation site		RSELSPLLLTTT
PePHD	1a	RSELSPLLLTTT
	1b	RSELSPLLL**S**TT
	2a	RS**Q**LSPLL**HS**TT
	2b	R**GQQ**SPLL**HS**TT
	3a	RSE**QH**PLL**HS**TT

Figure 1.7 PePHD sequences of HCV genotypes
The above sequences are the representative sequences from the genotypes indicated. Amino acid differences compared to the PKR phosphorylation domain are shown in bold.

PKR activity is also inhibited by the binding of HCV NS5a protein (Taylor et al., 1999). The NS5a contains the complete PKR-binding domain spanning amino acids 2209-2274,

which contains a sequence termed the interferon-sensitivity determining region (ISDR) (amino acids 2215-2254) (Gale et al., 1998b, Enomoto et al., 1995). NS5a proteins of subtype-1a and -1b infection bind and inhibit PKR *in vitro* (Gale et al., 1998b). Introduction of at least 2 mutations to the wild-type subtype-1b ISDR sequence abolishes the binding to PKR (Gale et al., 1997, Gale et al., 1998b).

Quasispecies

The quasispecies nature of HCV results in a population of viral genomes that are closely related but distinct (Gale et al., 1998a). A higher quasispecies complexity is associated with development of chronic infection (Martell et al., 1992), as it has a greater likelihood of containing viral strains with IFN-resistant or escape mutations (Martell et al., 1992, Marrone and Sallie, 1996).

Alternate Replication Sites

HCV primarily infects and replicates within the liver but has been detected within other cell types such as peripheral blood mononuclear cells, B-lymphocytes, granulocytes and DCs (Martell et al., 1992, Goutagny et al., 2003, Radkowski et al., 2005). It is unclear whether HCV is able to replicate within these alternate sites. Nevertheless these sites may act as reservoirs of potential virus, that can "hide" from the immune system. Hence despite successful clearance of HCV as determined by a PCR negative plasma sample, the virus may remain within the host to re-emerge at a later date (Goutagny et al., 2003).

1.6 Treatment of HCV Infection

The initial treatment of HCV infection involved the administration of IFN-α, known to have immunomodulatory and direct anti-viral effects (Blackard et al., 2006, Le Bon and Tough, 2002), resulting in the upregulation of ISGs, and HLA class I molecules to create an antiviral state (Peters, 1996, Takaoka and Yanai, 2006). IFN-α also results in the activation of macrophages, NK cells and effector T-cells, enhances the maturation of professional APCs and promotes Th1 response (Samuel, 2001, Luft et al., 1998, Brinkmann et al., 1993, Rogge et al., 1997).

The outcome of HCV treatment can be defined as patients that successfully clear virus termed sustained virological responders (SVR) or those that fail treatment as either virological relapse patients (REL), breakthrough or non-responders (NR) (Figure 1.8).

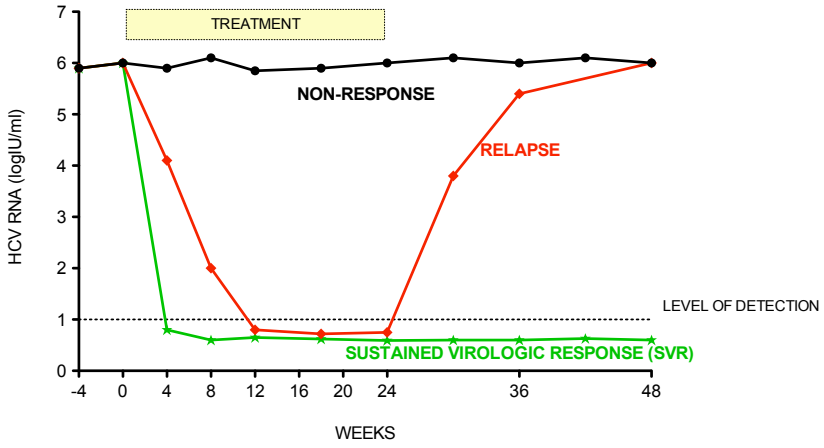


Figure 1.8 Treatment responses based on HCV viral load
Treatment outcomes are shown based on HCV viral load (y-axis) over the treatment period and follow up (x-axis; weeks). Sustained virological response indicated in green, virological relapse in red and non-response in black. Treatment period of 24 weeks is indicated. Limit of RNA detection is shown by dotted line.

Treatment of HCV infection with IFN-α alone achieved only very low SVR rates - 10-20% (Iellem et al., 2000, Thevenot et al., 2001, Liang et al., 2000). The addition of a polymer polyethylene glycol (Poynard et al., 1996) to IFN-α (PEG-IFN-α) improved its

half-life and subsequent SVR rates (Martin et al., 2006, Heathcote et al., 2000, Zeuzem et al., 2000, Lindsay et al., 2001). Current treatment involves weekly administration of PEG-IFN-α combined with daily administration of ribavirin to achieve the highest SVR rates to date - 54% in genotype-1 infection (Bailon, 2001). Ribavirin is a synthetic guanosine nucleotide analogue that resembles guanosine; its precise mechanism remains unclear. However ribavirin has been shown to promote a Th1 cytokine profile (Manns et al., 2001, Tam et al., 1999, Hultgren et al., 1998) whilst suppressing IL-10 production (Martin et al., 1998).

The response to treatment is assessed through measuring HCV viral load within plasma, throughout the treatment period. In SVR, the decline in viral load occurs in 3 phases (Cramp et al., 2000). The initial rapid decline occurs within 24-48 hours (Dahari et al., 2007) and is likely due to the direct effect of IFN-α as shown by mathematical models (Dahari et al., 2007, Layden et al., 2000). A second flat-phase or "shoulder phase" occurs at 4-28 days during which viral decay slows or remains constant, and a third phase possibly due to an enhanced immune response or the mutagenic effect of ribavirin (Layden-Almer et al., 2003, Neumann et al., 1998, Zeuzem et al., 1996, Lam et al., 1997, Herrmann et al., 2003).

1.6.1 Treatment of Acute Infection

The standard treatment for acute HCV infection is IFN-α monotherapy (Dahari et al., 2007). Treatment is usually initiated 12 weeks after initial infection, with early initiation associated with greater SVR rates (Kamal et al., 2004, Wiegand et al., 2006, Corey et al., 2006). SVR rates of 75-98% have been achieved, regardless of genotype (De Rosa et al., 2006, Santantonio et al., 2008, Jaeckel et al., 2001, Gerlach et al., 2003, Corey et al., 2006, McGovern et al., 2009). A low viral load is also associated with SVR, and as lower

levels of viraemia are more common in acute infection, this may be an important factor in these high SVR rates compared with those of chronic infection (Nomura et al., 2004). Despite these high SVR rates, relatively few patients receive treatment during the acute phase, as infection remains undiagnosed due to the lack of symptoms.

1.6.2 Treatment of Chronic Infection

Treatment of chronic HCV infection involves both PEG-IFN-α and ribavirin, with duration and treatment success dependent on the infecting genotype. Patients infected with genotypes-1 and -4, receive 48 weeks of treatment, with SVR achieved in only 50% of patients, whereas those infected with genotypes-2 and -3, are treated for only 24 weeks with up to 70% achieving SVR (McGovern et al., 2009, Fried et al., 2002, Kaplan et al., 2005). The reason(s) for the better response rate for subtype-3a infection is not known. However the younger age and shorter infection durations of genotype-3a patients compared with genotype-1 patients, may be important host factors for treatment success (Manns et al., 2001). Combination treatment of IFN-α and ribavirin is expensive with patients suffering many severe side-effects (Simmonds, 1995, Manns et al., 2001, Strader et al., 2004) therefore a method to predict patients' responsiveness to treatment would be invaluable to avoid unnecessary treatment.

1.6.3 HCV Vaccine

There are currently no vaccines against HCV infection. Difficulties encountered in developing a HCV vaccine include sequence diversity across genotypes and within the genome itself, high mutation rate and practical constraints, such as lack of small animal models (Fried et al., 2002). However clinical trials with prophylactic and therapeutic

vaccines are currently underway in HCV genotype-1 infection, targeting the most conserved regions of the HCV genome (Simmonds, 1995).

1.6.4 HCV Inhibitors

An additional mechanism for viral clearance is to inhibit the activity of HCV proteins essential to the viral life cycle. Drugs that target and inhibit HCV proteins are collectively known as DAA's – Direct Acting Anti-virals and target NS3 helicase, NS3/4a serine protease, NS5a and NS5b RdRp. The majority of the DAA's currently being developed inhibit HCV protease and polymerase proteins (Folgori et al., 2006). NS3/4a protease inhibitors act by preventing the cleavage of the HCV polyprotein, inhibiting viral replication. Polymerase inhibitors act by interfering with the replication activity of the NS5b RdRp either by directly targeting the active site (nucleoside analogues) or by binding to several sites on the polymerase (non-nucleoside) (Asselah et al., 2009).

Due to the emergence of resistance mutations during DAA monotherapy, the aim would be to use DAAs in combination with other inhibitors to target multiple viral proteins, or as part of a triple therapy with PEG-IFN-α and ribavirin (Kwong et al., 2008, Kieffer et al., 2010).

Other therapeutic approaches that are also being considered are the inhibition of host proteins and micro-RNAs, that HCV uses during replication and hence are essential for the HCV life cycle (Zeuzem et al., 2008).

1.7 Effect of Treatment on HCV-specific T-cells

The reason for the lack of detectable and efficient HCV-specific T-cell responses in chronic infection remains unclear and the effect of combination treatment on HCV-specific T-cell responses is controversial. It is thought that HCV-specific T-cell responses are not completely exhausted or deleted, and that therefore the treatment induced decline in viral load may allow the recovery of T-cell responses, as in chronic HBV infection (Webster et al., 2009). However if the presence of T-cell responses albeit weak in magnitude, are in fact driven by continual antigen stimulation, then treatment induced fall in antigen levels may further decrease the number of HCV-specific T-cells, as seen in HIV infection (Boni et al., 1998).

1.7.1 Acute Infection

Treatment of acute infection results in the clearance of HCV in 80-98% of patients (Ogg et al., 1998, Santantonio et al., 2008, Jaeckel et al., 2001). Previous studies of acute infection have shown that treatment significantly increases the frequency and strength of CD4+ HCV-specific T-cell responses in genotype-1 and -4 patients irrespective of outcome (Gerlach et al., 2003, Kamal et al., 2002). However studies have also shown that CD8+ T-cell responses decrease during treatment in SVR patients, without the emergence of new T-cell specificities, and hence successful viral clearance was independent of CD8+ and CD4+ T-cell responses (Kamal et al., 2004, Lauer et al., 2005).

1.7.2 Chronic Infection

In chronic HCV infection the effect of treatment on HCV-specific T-cell responses is also controversial. The majority of previous studies have found that treatment enhances HCV-

specific T-cell responses (Rahman et al., 2004, Missale et al., 1997, Kamal et al., 2002) to be multispecific and strong in magnitude (Caetano et al., 2008, Barnes et al., 2002).

However many studies have not found such an association due to the presence of detectable responses in patients that cleared infection and those that failed treatment (Kamal et al., 2002, Barnes et al., 2002). A lack of association in many studies was due to a heterogeneous effect of treatment on HCV-specific T-cell responses, in that enhanced responses appeared transiently, late during treatment or were not detectable (Missale et al., 1997, Barnes et al., 2002). It has also been shown that T-cell responses during treatment may not be multispecific but preferentially target a single protein such as core (Caetano et al., 2008).

Other studies observed a decrease in HCV-specific T-cell responses during treatment of genotype-1 patients (Barnes et al., 2002, Burton Jr et al., 2008). Burton Jr *et al.* found that HCV-specific CD4+ T-cell responses decreased during treatment, and increased after cessation of therapy in patients that experienced virological relapse suggesting that the treatment induced fall in antigen level resulted in the decrease in T-cell responses. This is further supported by the CMV-specific CD4+ T-cell responses that were unaffected during treatment (Barnes et al., 2009). Barnes *et al.* also found that HCV-specific CD4+ T-cell responses decreased during treatment, in addition to CMV-specific CD4+ T-cell responses (Burton Jr et al., 2008). However this decrease may be due to fall in antigen and the immunosuppressive effects of high-dose IFN in the study, nevertheless the greatest decline was observed in SVR patients suggesting a greater inherent sensitivity to IFN (Barnes et al., 2009).

A study by Caetano *et al.* found that the magnitude of pre-treatment HCV-specific T-cell responses is the determining factor in viral clearance (Barnes et al., 2009), such that in chronic infection, pre-treatment HCV-specific CD8+ T-cell responses were significantly

higher in patients that successfully cleared virus compared with non-responders (Caetano et al., 2008).

Collectively these studies suggest that in many patients, detectable responses during treatment may not be sufficient for successful viral clearance and that treatment does not have a specific effect on HCV-specific T-cells in all patients.

1.8 Predicting Treatment efficacy

It is important to identify factors that predict treatment success or failure, enabling improvements in patients care as a significant number of patients fail treatment that causes many severe side-effects.

1.8.1 Viral Factors

Many viral factors can be used as indicators to help predict treatment outcome. A high viral load is associated with treatment failure of chronic HCV infection (Caetano et al., 2008, Davis, 1994, Tsubota et al., 1994, Yoshioka et al., 1992). Once treatment is initiated, weekly measurements of the viral load can help predict whether the patient will successfully clear the virus. A fall in viral load of greater than 2 logs by TW12, also known as rapid virological response, has been associated with SVR. Whilst the presence of HCV RNA at treatment week 24 in genotype-1 or at treatment week 16 in genotype-3 infection indicates response to further treatment is very unlikely, and treatment is usually stopped (Martinot-Peignoux et al., 1995, Fried et al., 2002, Davis, 2002, Davis et al., 2003, Berger et al., 2000, Strader et al., 2004, Zeuzem et al., 1998).

Quasispecies diversity before treatment has been associated with treatment success, as a highly diverse population would be more likely to contain an IFN resistant strain (Zeuzem et al., 2001, Martell et al., 1992). In support of this hypothesis a high pre-treatment HVR1 quasispecies complexity is associated with treatment failure in genotype-1 infection (Martell et al., 1992, Abbate et al., 2004, Salmeron et al., 2006). Treatment failure in genotype-1 is also associated with the presence of a pre-treatment resistant strain that dominates during the treatment period (Torres-Puente et al., 2008, Farci et al., 2002).

Previous studies of quasispecies have included few subtype-3a infected patients hence the analysis of viral complexity in relation to treatment in subtype-3a infection remains largely unexplored. A single study with an entire subtype-3a cohort found a lower complexity and diversity of quasispecies before treatment in SVR patients (Pawlotsky et al., 1999). Further studies are needed to assess the quasispecies diversity and affect of treatment in additional subtype-3a cohorts. Previous studies are based on the HCV quasispecies detected within the blood, providing an opportunity to track changes in the quasispecies population at multiple timepoints. However as HCV replicates within the liver it is unclear whether quasispecies found in the blood represent the entire HCV quasispecies within the liver, as it has been found that there is a lack of correlation between quasispecies within the blood and the liver (Moreau et al., 2008, Cabot et al., 1997).

1.8.2 HCV sequences associated with treatment resistance

The ability of HCV to evade endogenous and exogenous IFN-α through interrupting multiple steps of signaling pathways has inevitable implications for standard treatment of HCV. Viral sequencing of the HCV genome in patients undergoing treatment has enabled the identification of sequence motifs that are associated with treatment outcome. Viral sequences associated with treatment resistance are the PePHD within E2 protein and the ISDR in NS5A (Maggi et al., 1997).

PePHD sequences have been associated with IFN resistance depending on genotype, such that genotype-1 E2 binds to PKR and is IFN resistant, whereas mutations in subtype-3a PePHD render it less resistant (Enomoto et al., 1995). However an association between PePHD sequence and treatment outcome remains controversial (Polyak et al., 2000, Berg et al., 2000, Sarrazin et al., 2001, Gerotto et al., 2000, Abid et al., 2000, Puig-Basagoiti et

al., 2001, McKechnie et al., 2000). In subtype-3a infection, an association between mutations within the PePHD and treatment outcome has not been found (Squadrito et al., 1997, Cochrane et al., 2000, Sarrazin et al., 2000, Puig-Basagoiti et al., 2001).

The ISDR of NS5a also inhibits PKR activity. Japanese patients with more than 4 mutations within ISDR compared with consensus subtype-1b sequence have been associated with SVR in subtype-1b infection (Afzal et al., 2010, Enomoto et al., 1995, Enomoto et al., 1996, Chayama et al., 1997, Kurosaki et al., 1997). However the significance of this region remains controversial as only few studies of genotype-1 and -3a patients from Europe and USA have found an association (Saiz et al., 1998, Sarrazin et al., 1999, Sarrazin et al., 2000a, Puig-Basagoiti et al., 2001, Berg et al., 2000, Zeuzem et al., 1997, Nousbaum et al., 2000, Hofgartner et al., 1997, Squadrito et al., 1997, Khorsi et al., 1997, Chung et al., 1999, Squadrito et al., 1999, McKechnie et al., 2000).

1.9 Subtype-3a HCV Infection

1.9.1 Importance of Assessing HCV Subtype-3a Infection

The prevalence of subtype-3a infection is increasing and is now the dominant genotype in newly diagnosed infections within the UK (Nakano et al., 1999). However previous studies of both sequence analysis and T-cell responses have predominantly focused on genotype-1, providing little information regarding subtype-3a. As a consequence few published full-length HCV subtype-3a viral sequences are available (Los Alamos HCV database http://www.hcv.lanl.gov). The lack of sequence data of subtype-3a has negative implications for peptide and protein design for assays of adaptive immunity, primer design for sequence analysis, and also vaccine design. It is essential to assess subtype-3a viral sequences and host responses, in addition to other genotypes to provide information on each infecting genotype and enable analysis at comparable levels.

1.9.2 Aims of Study

The aims of this study were to identify both host and viral factors that determine treatment success of subtype-3a infection, such as viral factors e.g. specific sequence motifs that alter the virus' sensitivity to treatment, and also host immune responses e.g. HCV-specific T-cell responses.

1. Assess subtype-3a quasispecies diversity in chronic infection. To assess the affect of combination treatment on quasispecies populations, and determine whether subtype-3a quasispecies behave similarly to quasispecies in genotype-1 infection.
2. Assess subtype-3a specific T-cell responses in chronic infection using subtype-3a specific peptides. To assess the affect of IFN and ribavirin treatment on subtype-

3a T-cell responses, to determine whether these are enhanced during treatment, accounting for the increased treatment response rate.

3. Assess two methods of detecting subtype-3a HCV-specific T-cell responses in chronic infection. The first approach based on population-sequencing that identified polymorphic residues within HLA-associated epitopes, and the second using overlapping peptides representing the non-structural proteins.

Chapter 2 - Materials and Methods

2.1 Cohort and Treatment

2.1.1 Patients

Patients are characterized at the beginning of each chapter. In brief, peripheral blood mononuclear cells (PBMC) and plasma samples were obtained from chronically infected subtype-3a patients from John Radcliffe Hospital, Oxford and Barts and the London NHS Trust, London. Plasma samples from acutely infected subtype-3a infected patients were obtained from San Bortolo Hospital, Vicenza, Italy. Chronically infected genotype-1 PBMC and plasma samples were obtained from patients attending John Radcliffe Hospital, Oxford. PBMC from 10 healthy volunteers were assessed to define a positive cut-off for enzyme-linked immunospot assays (ELISpot) (see Chapter 5).

2.1.2 Treatment and definitions

All patients received combination therapy with pegylated-interferon-α2b (180μg/week) and ribavirin (800-1200mg/day dependent on body weight) for 24 weeks. Local Ethical approval was obtained and all patients gave written informed consent for study participation.

Sustained Virological Response (SVR) – RNA negative during treatment and 6 months after treatment was stopped. Non-responder (NR) – remains RNA positive throughout and following treatment. Relapse patient (REL) – RNA negative during treatment, but RNA positive within the first 6 months of stopping treatment.

2.2 Viral/ Molecular Techniques

2.2.1 Viral RNA extraction from plasma

Plasma was thawed, and 500µls was concentrated by high-speed centrifugation (23,600g for 1 hour) at 4°C. Carefully 360µls of plasma was removed, and pellet was resuspended in the remaining 140µls and viral RNA extracted using QIAmp Viral RNA Mini Kit (Qiagen) according to manufactures instructions. RNA was eluted into 60µls Buffer AVE (Qiagen) with RNasin (40U/µl, Promega). Samples were stored in 20µl aliquots at -80°C.

2.2.2 cDNA Synthesis

RNA (10µls), random primers (500µg/ml, Promega), deoxynucleoside triphosphates (dNTP) mix (25mM each, Bioline) and sterile distilled H_2O (sdH_2O) were mixed and heated to 65°C for 5 mins and chilled immediately on ice. To each sample, First Strand buffer (Invitrogen), dithiothreitol (DTT) (0.1M, Invitrogen) and RNasin (40U/µl, Promega) were added and incubated for 2 mins at 25°C. Superscript II Reverse Transcriptase was added (1µl; 200U/µl, Invitrogen) and samples incubated at 25°C for 10 mins, heated to 42°C for 50 mins and finally 70°C for 15 mins. RNase H (2U; Invitrogen) was added and samples incubated at 37°C for 20mins to remove complementary RNA. Samples were stored at -20°C until needed.

2.2.3 Primers and Primer Design

Primers were designed to be between 18 to 28 nucleotides in length, to contain 50-60% C/G content and to have an annealing temperature of approximately 65°C. Degenerate bases were added if a polymorphic site were included. Primers contained CG at the 3'end where possible and repeated sequences were avoided. Primers were designed to bind to

conserved regions by comparing to full-length and partial subtype-3a sequences available from Los Alamos, to ensure maximum binding efficiency to all patients. A full list of primers can be found in Table 2.1 and Table 2.2. Subtype-3a specific primers included both previously described and in house newly designed primers (Table 2.1 and Table 2.2).

2.2.4 One-Step Reverse Transcription First Round PCR

Reverse-transcription and first round PCR was carried out in a single reaction using the Invitrogen Superscript III One-Step Reverse Transcription – PCR system with Platinum Taq High Fidelity. Viral RNA (2.5μls) was added to primers (10μM each, MWG), Superscript III/Taq polymerase and buffer (Invitrogen), and sterile distilled water to final volume of 25μls. Samples were heated in thermocycler. For first round reactions, primers 277-For and F4-Rev amplified a 4kb product that encoded Core, E1 and E2 structural proteins (Figure 2.1). Primers 2412F and 9192R amplified a 7kb product that encoded non-structural (NS2-5) proteins. RT-PCR cycling conditions were; 55°C for 30min, 94°C for 2min, 39 cycles of 94°C for 15s, 60°C/58°C for 30s (7kb/4kb reactions, respectively), 68°C for 1min/kb, final extension of 68°C for 10min.

Table 2.1 Subtype-3a primer sequences

Forward Primers

Location of Primer Binding	Primer Name	Primer Sequence (5'-3')	Tm (°C)	Binding position to Subtype 3a	Reference
UTR	156-For	GGTGAGTACACCGGAATCGCT	61.8	156	(HPA, 2008)
	277-For	CCTTGTGGTACTGCCTGATAG	59.8	277	(Moghaddam et al., 2006)
	286-For	ACTGCCTGATAGGGTGCTTGC	61.8	286	In house
Core	745-For	TACATCCCGCTCGTCGGC	60.5	745	
	887-For	CTTGCTTAATYCATCCAGC	53.4	887	
E1	1082-For	TCARGTAYGTCGGAGCAACCACCGC	67.9	1082	
	1416-For	AGCCTATTACTCCATGCAGGG	62.1	1416	
	1435-For	GGCAACTGGGCCAAGGTCGC	65.5	1435	
E2	2237-For	TCAAGGTGAGGATGTTTGTG	55.3	2237	
	2289-For	CAACTGGACCAGGGGGGAGCG	67.6	2289	
	F4 For	TGGGATGGGCGCTGAAATGGGA	60	2485	(Barnes et al., 2002)
	HCV-2412F2	CACCTCCACCARAACATYGT	57.3	2412	(Gaudieri et al., 2009)
NS2	3018-For	CATCACTAAGCTGCTGATAGC	57.9	3018	In house
NS3	F5 For	ACAGCATACGCCCAGCAAACTAGG	60	3444	(Lucas et al., 2007)
	3892-For	ATCTTTAGGGCTGCTGTGTGC	59.8	3892	In house
	F6 For	GATGAATGTCATGCCCAAGACGCTAC	62	4302	
	4832-For	GCCGAGGTAGACTCGGTACG	63.5	4832	
	F7 For	TGTCTCGTGCGGCTTAAGCCAA	59	5184	
NS4b	5712-For	TGCTGTGGCGTCTCTTATGGC	61.8	5712	
	6076-For	CTGTGCARTGGATGAACMG	56.7	6076	
	F8 For	GGAGGGAGCGGTACAGTGGATGA	63	6083	
NS5a	6573-For	TTGCCCATCACCCAACTACACTCGC	66.3	6573	
	F9 For	TGAGCTAGTGGACGCCAACTTGTTATG	62	7028	(Gaudieri et al., 2009)
	7507-For	GAGTCCGACTCAGAGTCATGC	61.8	7507	In house
NS5b	F10 For	CGAAGTTCGGGTATAGTGCGAAGGA	61	7924	(Gaudieri et al., 2009)
	8500-For	AACACAATCACTTGYTACATCAAGGC	60.9	8500	In house
	8848-For	TCCTGGTTRGGCAACATCATCATGTACGC	65.6	8848	
	8872-For	TACGCGCCTACCATCTGGGTGC	65.8	8872	

Table 2.2 Subtype-3a primer sequences
Reverse Primers

Location of Primer Binding	Primer Name	Primer Sequence (5'-3')	Tm (°C)	Binding position to Subtype 3a	Reference
E1	1105-Rev	ACATGACTGCGTATCGAAGC	57.3	1105	In house
	1207-Rev	GAGGTCTGAACGTGAAGG	58.8	1207	
E2	1585-Rev	ATGTGCCACGAGCCATTGGT	59.4	1585	(Gaudieri et al., 2009)
	1625-Rev	CGGTGTTTATGGACTCATTGC	57.9	1625	In house
	2319-Rev	TCGCTGCGGTCACGATCCTCG	65.7	2319	
	2340-Rev	GAATGCAGCAGCGGATGTTGC	61.8	2340	
NS2	2837-Rev	GTGATAAGGTAAAGAAGC	49.1	2837	
	2982-Rev	ATAAAGCAGGCTTGTTAG	52.4	2982	
NS3	F4 Rev	CTGGGTAGCCGTAGAAAGCACCT	58	3558	(Barnes et al., 2002)
	3904-Rev	GCTACACCTCTGGTGCACACAGC	66.0	3904	In house
	F5 Rev	TAGAATGTGGCACAGTGATGCTGC	58	4438	(Gaudieri et al., 2009)
	4880-Rev	ACCGAGTCAAACATYCCAGACG	61.2	4880	In house
	F6 Rev	GCCATGATGTATTTTGTGATGGGGTGTG	62	5297	
NS4b	5713-Rev	TGAACGCCATAAGAGACGCCACAGC	66.3	5713	
	F7 Rev	GTGACAGTTAGAGAACTCAGCAATG	57	6218	
NS5a	6684-Rev	TTGGCACGGACACTTGAGC	58.8	6684	
	6823-Rev	TCCGGTTCGGGCTCRCANG	63.1	6823	
	F8 Rev	CACAACCTTTGTTTCAGACTCCACCCG	61	7110	
	7578-Rev	TGTCACTAACGGTGGACCAAGAGTCG	66.4	7578	
NS5b	F9 Rev	GTTCTTCGCCATGATGGTGGTTGGAAT	60	8055	(Gaudieri et al., 2009)
	8443-Rev	CAACGGCGATAACCACACTGGGC	66.0	8443	In house
	F10 Rev	TGCCCGATGTCTCCAAGCTCGTA	59	9141	(Gaudieri et al., 2009)
	HCV-9192R	GGAGTGAGTTTGAGCTTGGT	57.3	9192	(Gaudieri et al., 2009)
3'UTR	9550-Rev	AGGTTATTGGGAAGTTTGGGAAGG	61.0	9550	In house
	H3'X45R-3a	ACAGCTAGCCGTGACTAGGGC	63.7	9600	Adapted from (Lucas et al., 2007)

2.2.5 First/Second Round PCR

First or second round PCR was performed using 2.5µls of cDNA, first round PCR product, or RT-PCR product, respectively. PCR reactions were prepared using the Expand High Fidelity PCR System (Roche) according to manufactures instructions, containing sdH_2O, dNTP (25mM each, Bioline), forward and reverse primers (10µM each), buffer and Taq DNA polymerase High Fidelity enzyme. Template was added to mix and placed in thermocycler. Thermocycler programs were dependant on the primers used and manufactures instructions (Figure 2.1). Second round PCR used High Fidelity Taq DNA polymerase (Roche), in nested PCR reactions of approximately 1kb each (Core – 277-For and 977-Re; 600bp. E1 – 745-For and 1585-Rev; 840bp. E2 – 1435-For and 2982-Rev; 1547bp. F4 – F10 primer pairs; Approx 1kb each).

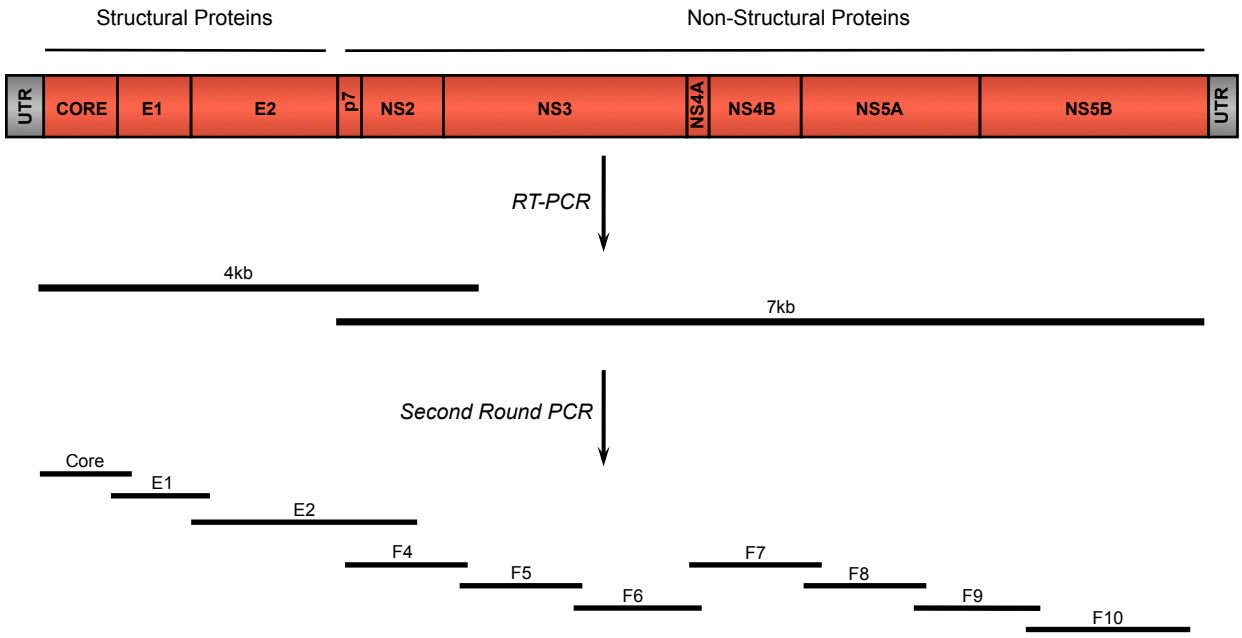


Figure 2.1 Polymerase chain reaction amplification strategy
Diagram shows the strategy followed to amplify varying regions of the HCV genome. RT-PCR – reverse-transcription polymerase chain reaction. Second round fragments were approximately 1000bp.

2.2.6 Gel Electrophoresis and Extraction of PCR products

Positive PCR products of correct size were visualized on an agarose gel and extracted and purified using Qiagen Gel Extraction kit (Qiagen). To each gel fragment, 500µls of Buffer QG was added and heated to 52°C for 10mins or at room temperature to dissolve gel and spun 1300rpm for 1min. A further 500µls of Buffer QG was added to spin column and spun at 1300rpm for 1min. 750µls of Buffer PE with ethanol was added to spin column, incubated at room temperature for 2-5mins and spun at 1300rpm for 2mins. PCR products were eluted into 50µls of sdH_2O and sequenced immediately or stored at -20°C until needed.

2.2.7 Sequencing of PCR products and DNA precipitation

PCR products were sequenced bidirectional with 2nd round PCR primers and additional inner primers using Prism Big Dye (Applied Biosystems) on an ABI 3100 DNA automated sequencer. PCR fragments were precipitated by incubation for 30 mins with sdH_2O, sodium acetate (Sigma) and 100% ethanol. Samples were spun for 1hour at 4000rpm then washed with 70% ethanol.

2.2.8 Cloning of E2 PCR Products

The entire E2 PCR product (1,105 nucleotides) was cloned using TOPO TA Cloning Kit (Invitrogen). Salt solution, TOPO vector and PCR product (4µls) were mixed together on ice, and incubated at room temperature for 20 mins. Samples were transferred immediately to ice and incubated with *Escherichia coli* (*E.coli*) cells on ice for 30 mins. Cells were heat-shocked (42°C) for 30 seconds, and immediately transferred to ice. S.O.C. medium (Invitrogen; 200µls) was added to each vial, and vials shaken

horizontally (200rpm) at 37°C for 1 hour. Cells were spread onto pre-warmed selective plates (ampicillin 100μg/ml) and incubated overnight. Thirty colonies were selected and grown overnight in 1ml LB broth containing kanamycin (1μl/ml). Plasmid DNA purified using Montage Plasmid Miniprep Kit (Millipore) according to manufacturers instructions. Plasmid DNA was eluted in 50μls sdH_2O and stored at -20°C until needed.

2.2.9 EcoRI Digestion

EcoRI enzyme (New England Biolabs) digestion identified positive clones, which were sequenced bidirectionally using M13For (5'-TGTAAAACGACGGCCAGT-3'), M13Rev (5'-CAGGAAACAGCTATGACC-3') and additional internal primers. Sequences were aligned and edited using Sequencher 4.8 (Gene Codes Corp.) software.

2.2.10 Sequence Analysis

Sequences from each primer were aligned and edited using Sequencher 4.8 (Gene Codes Corp.) software. Final sequences were exported as fasta format and aligned using Se-Al (v2.0 a11; http://tree.bio.ed.ac.uk).

2.3 Sequence Analysis

2.3.1 Nomenclature

The amino-acid nomenclature conforms to the system proposed by the Los Alamos database group (Yanagi et al., 1997). Amino-acids are numbered relative to genotype 1a H77 and subtype-3a insertions are designated with a lower case letter.

2.3.2 Shannon Entropy and Hamming distance

A mathematical measure of entropy was used to evaluate sequence diversity. Entropy values for each amino-acid position were calculated using the Shannon Heterogeneity In Alignments Tool v1.0 (http://evolve.zoo.ox.ac.uk/software). This program computes the Shannon (Kuiken et al., 2006) information entropy score, *E*, for each codon as follows:

$$E = -\sum_{i=1}^{n} p_i \log_n p_i$$

where p_i is the proportion of sequences that contain residue i at the codon in question. In this analysis there are n=21 types of residue (20 aa, plus the STOP codons).

Hamming distance was assessed at each timepoint, at nucleotide and amino acid level using Shannon Heterogeneity In Alignments Tool v1.0 (see above).

2.3.3 Positive Selection analysis

The program CODEML was used to identify amino-acids that had undergone positive selection ((Shannon, 1948); http://abacus.gene.ucl.ac.uk). The maximum likelihood method implemented in the CODEML program fits various models of codon evolution to sequence data connected by a phylogenetic tree and considers selection pressures at individual codon sites. The models of codon evolution assume different distribution of non-synonymous to synonymous (dN/dS) values among codons. CODEML detects

selection by calculating the ratio of non-synonymous to synonymous nucleotide substitution (ω). The first step of analysis involves a comparison of two models - M7 (neutral model) and M8 (positive selection model). M7 assumes selection is either neutral (ω = 1.0), or negative (due to the deleterious result of random mutation, ω <1.0). M8 is an extension of M7, by the addition of an extra category of positively selected site, with ω >1.0. Selection at a codon is signified by a ω value that is significantly greater than 1. As advised by Yang *et al.*, a likelihood ratio test between M7 and M8 models was performed in order to test for the presence of selected sites (χ^2 distribution with 1 degree of freedom and $p \leq 0.05$) (Yang et al., 2000). Both M7 and M8 models incorporate multiple dN/dS value distributions (10 classes for M7, and 11 classes for M8 due to the additional category of positive selection), to achieve continuity. As these two models are nested their likelihoods can be compared by using a likelihood ratio test. If M8 rejects M7 (at the 0.05 level, using chi-square test with degree of freedom = 2), this indicates that some sites are under positive selection (and hence contain a class of codons with ω >1.0). The sites under positive selection are identified in the second step of the detail of M8 analysis. The confidence of codon sites with ω >1.0 is determined by posterior probability calculation using empirical Bayesian approach of M8. Therefore, only the sites with posterior probability > 95%, and ω >1.0, are described.

2.4 T-cell Assays

2.4.1 Isolation of peripheral blood mononuclear cells from whole blood

PBMC were isolated from whole blood by density gradient (Lymphoprep) and centrifugation at 2200 rpm for 25 mins at room temperature. The PBMC layer was carefully removed and placed in media RPMI 1640 (GibcoBRL) containing penicillin (100U/ml), streptomycin (100μg/ml) and L-glutamine (2mM) and washed 3 times. Cells were resuspended in 10mls R10 media containing 10% heat-inactivated fetal calf serum (FCS), penicillin (100U/ml), streptomycin (100μg/ml) and L-glutamine (2mM) and counted using Quava Cell Count.

2.4.2 Freezing PBMC

PBMC were frozen at 10 million cells per vial. Each 10 million cells were resuspended in 500μls of 'freezing mix' containing equal amounts of dimethyl sulfoxide (DMSO) and RPMI, and 500μls of FCS. All reagents and vials had been chilled on ice and vials were placed immediately into a chilled isopropanol container and placed in -80°C freezer for fast freezing. Cells were then transferred to liquid nitrogen for long-term storage.

2.4.3 Thawing PBMC

RPMI containing penicillin (100U/ml), streptomycin (100μg/ml) and L-glutamine (2mM), R10 and CTL supplement wash (GibcoBRL) were warmed to room temperature. Thawing media was prepared by diluting CTL wash supplement 1:10 in RPMI medium, plus DNase (50U/ml). Vials were thawed in 37°C waterbath and immediately transferred to 15ml falcon tube. Thawing media was added gradually to avoid osmotic shock. Cells were washed twice in RPMI. Cells were resuspended in R10 and rested until needed.

2.4.4 Interferon (IFN)-gamma Enzyme-linked Immunospot (ELISpot) Assay

PBMC responses were tested by IFN-γ (Mabtech) ELISpot assays, as previously described (E. Barnes et al. 2009). 96-well ELISpot plates (Multiscreen HTS, Millipore) were coated with 100μl/well of mouse anti-human interferon-γ monoclonal antibody (Mabtech; final concentration 5μg/ml). Plates were incubated overnight at 4°C. Plates were washed with 200μl/well with sterile PBS. R10 (200μls) was added to each well and plates incubated at 37°C and 5% CO_2 for at least 2 hours.

PBMC were prepared at $4x10^6$/ml, 50μls plated in duplicate (200,000/well). Antigens were added - HCV peptides (3μg/ml final concentration), cytomegalovirus lysate (CMV) (0.05μg/ml, Chiron), concanavalin-A (ConA) (Sigma, 5μg/ml), Influenza, Epstein Barr virus and CMV CD8+ epitopes (Yang et al.) (3μg/ml final concentration, BEI resources), DMSO (3μg/ml final concentration, Sigma), and media alone served as negative controls. Plates were incubated at 37°C and 5% CO_2 for 18-20 hours. Elispot plates were washed 7 times with phosphate buffered saline (PBS; GibcoBRL)/Tween20 (Sigma) (200μls/well, Sigma). Biotinylated mouse anti-human IFN-γ antibody (Mabtech) was diluted 1:2000 in 5% Bovine serum albumin (BSA; Sigma)/PBS and 50μls added to each well and incubated at room temperature for 3hours. Plates were washed. Alkaline phosphatase-conjugated anti-biotin antibody was diluted 1:750 in 5% BSA/PBS and 50μls added to each well, and incubated at room temperature for 2hours. Plates were washed and BCIP/NBT substrate solution (Pierce) was added to each well (50μl) and incubated at room temperature for 7mins. The reaction was stopped by washing plates with deionized water. Plates were left to air dry.

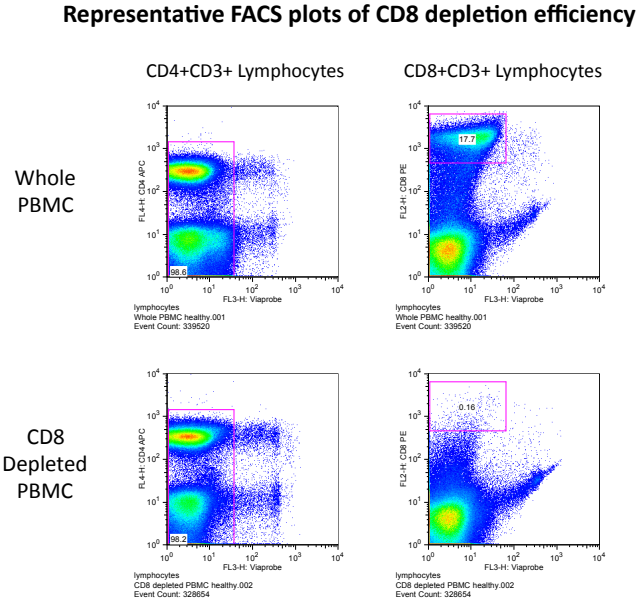
Spot-forming units (SFU) were counted using an automated ELISpot plate reader (AID). ELISpot assays using subtype-3a peptides, were performed in 12 healthy subjects to

define a positive cut off for the assay based on the following calculation; mean number of SFU/10^6 PBMC in test wells minus negative control plus 3 x standard deviation.

2.4.5 CD8 Depletion of Whole PBMC

To determine the restriction of the T-cell response, CD8+ T-cells were depleted from PBMC using magnetic bead separation (CD8 Dynabeads, Invitrogen) following manufacturers instructions. The CD8 depletion efficiency was determined by FACS analysis and ELISpot assay with PBMC from 2 healthy individuals. The average depletion of CD8 T-cells obtained was 97% (representative FACS analysis, Figure 2.2a). The CD8 negative PBMC fraction was used in IFN-γ ELISpot assays and representative ELISpot response of CD8 depleted and whole PBMC are shown in Figure 2.2b.

a.



b.

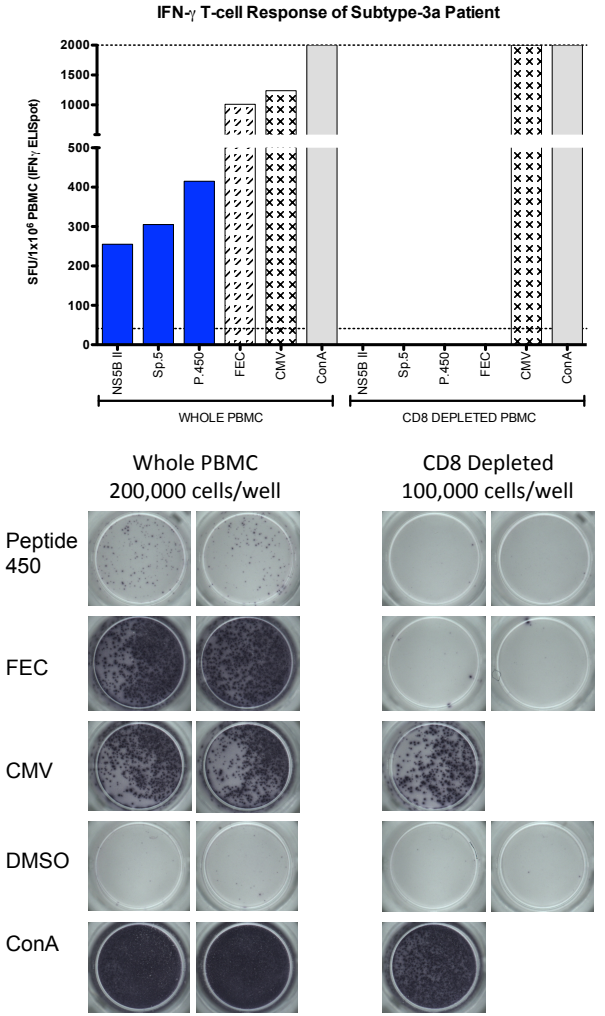


Figure 2.2 CD8 depletion of whole PBMC

a. Representative FACS analysis of CD8 depletion. Upper plots show whole PBMC and lower plots show CD8 depleted PBMC. Plots show that CD4+ CD3+ lymphocytes remain unaffected, whereas CD8+ CD3+ lymphocytes are depleted by 99%.

b. ELISpot responses of representative subtype-3a patient. Upper graph shows the IFN- T-cell responses to NS5B-II pool, subpool 5 and the individual peptides contained within the subpool for whole PBMC and CD8 depleted PBMC. Lower panel shows the ELISpot wells for peptide 450, control antigens – influenza/EBV/CMV (HPA), CMV lysate and Concanavalin A, and DMSO used as negative control. Number of PBMC used are also indicated. CD8-depleted cells expressed as SFU/million CD8-depleted PBMC.

2.5 Peptides and Peptide Design

2.5.1 Subtype-3a Screening Peptides

To generate a representative peptide set, full-length viral sequencing was performed (amino acids 1-2929) on 18 genotype-3a patients with chronic infection (Accession numbers; GQ356200-GQ356215, GQ356217, JF509175-JF509177). Peptides were designed to be 15-19 amino acids in length in order to detect both CD4+ and CD8+ HCV specific T-cells, (overlapping by 11 amino acids, n=460, Mimotopes, Australia). Subtype 3a peptides with the following amino acids at the C-terminal – glycine, proline, glutamic acid, aspartic acid, glutamine, asparagine, threonine, serine, cysteine, were either shortened or lengthened until a tolerated amino acid was at the C-terminal, as these amino acids have been shown to affect peptide presentation (HPA, 2008). If the peptide was shortened or lengthened but the C-terminal position was still occupied by an un-tolerated amino acid, the original 18-mer peptide was retained. Peptides were grouped into 10 pools corresponding to the individual viral proteins as follows (Table 2.3):

Table 2.3 Subtype-3a peptide pools

Peptide Pool	Amino acids (aa)	Number of peptides
Core	1-191	29
E1	192-383	28
E2	384-752	57
p7&NS2	753-1032	41
NS3 protease	1033-1359	49
NS3 helicase	1349-1663	48
NS4	1664-1978	46
NS5A	1979-2430	72
NS5B I	2431-2726	45
NS5B II	2716-3021	45

2.5.2 Genotype-1 Peptides

Subtype-1b J4 overlapping peptides (15-19aa) were obtained from BEI resources (www.beiresources.org/). Peptides were also adapted to remove any untolerated amino acids at the C-terminal, see subtype-3a peptide design. However differences in peptide sets may be due to sequence variation resulting in different untolerated amino acids at the

peptide C'terminus. Also due to sequence differences between subtype-3a and subtype-1b such as amino acid insertions, this may result in differences in detectable T-cell responses. However as the polyprotein sequences are first divided into individual proteins before peptide design, the amino acid insertions in one protein will not affect the peptides of the remaining downstream proteins.

2.5.3 Screening Peptide Strategy

When a positive response to a peptide pool was identified, PBMC were tested with smaller subpools each containing 6-10 peptides. A positive response to a peptide subpool, was further tested with individual peptides from the subpool, to identify the peptide responsible for the IFN-γ response. Once positive responses to individual peptides were identified, CD8 depletion ELISpots were performed to determine if the response was CD4+ or CD8+ restricted (see 2.4.5 CD8 Depletion of Whole PBMC).

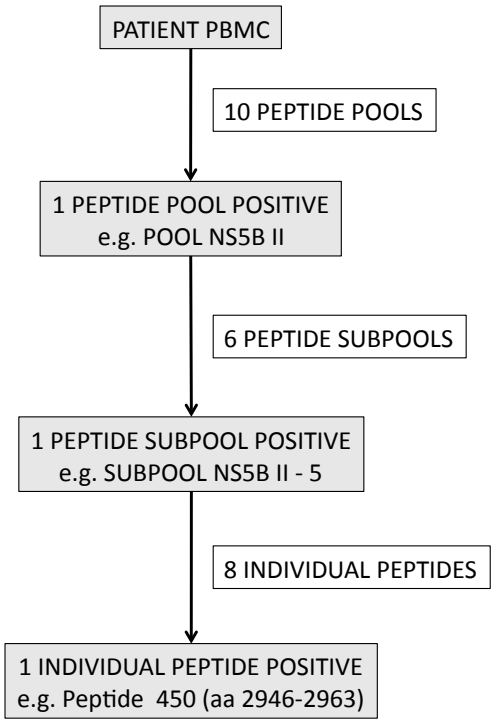


Figure 2.3 Strategy to identify responses to individual peptides

Chapter 3 - Full-length characterisation of hepatitis C Virus subtype 3a reveals novel hypervariable regions under positive selection during acute infection.

This work is based on published manuscript (See Appendix I).

Abstract

HCV subtype-3a is a highly prevalent and globally distributed strain that exhibits particular phenotypic characteristics including a relatively high rate of response to interferon therapy. In spite of this, detailed genetic analysis of this subtype has rarely been performed.

Full-length viral sequence analysis in 18 patients with chronic HCV subtype-3a infection was performed and assessment of genomic viral variability in comparison to other HCV subtypes. Two novel regions of intra-genotypic hypervariability within the E2 of HCV subtype-3a, were identified - HVR495 and HVR575. A 5 amino-acid insertion found only in subtype-3a and a putative glycosylation site were contained within HVR575. These novel regions are subject to positive selection pressure, sometimes very early in acute infection. Whilst HVR575 has been previously recognised as a site of inter-genotypic variation (Draenert et al., 2004), the identification of this region as a hypervariable site within subtype-3a, and as a site under early selection pressure leading to variability within the same host, has not been previously described. Together, these data suggest that HVR495 and HVR575 may serve as major antigenic determinant sites in subtype-3a HCV infection. Further definition of the functional significance of these regions will have important implications for vaccine studies that aim to induce cross-reactive or genotype-specific E2 antibodies.

3.1 Background

HCV infection is a major global health issue leading to persistent viral infection in the majority of those infected and is associated with progressive liver disease, cirrhosis, and hepatocellular carcinoma (McCaffrey et al., 2007). Six major genotypes and multiple subtypes have been identified. HCV subtype-3a infection is now the most common subtype in new infections within the United Kingdom, although it is globally distributed and frequently associated with intravenous drug use.

The classification of HCV viral strains by genotype and subtype has proven informative, in terms of the epidemic and evolutionary history of the virus and also in terms of clinical outcomes. In particular, the response rates to current therapy and the prevalence of hepatic steatosis (McCaffrey et al., 2007) are significantly higher for HCV subtype-3a than for genotype-1 infections. The reasons for this are not understood, but may relate to viral genetic and phenotypic differences between strains, to differences in the hosts' ability to exert an effective immune response against particular viral sequences, or a combination of both factors.

To date, detailed assessment of the HCV genome has largely focused on HCV genotype-1 with only a handful of full-length HCV subtype-3a viral sequences currently (2009) published and available within the major HCV database (Los Alamos; http://hcv.lanl.gov/components/hcv-db/combined_search/searchi.html) (McCaffrey et al., 2007).

Distinct subtype-3a characteristics may be due to viral sequence therefore viral genomic variability was assessed at a number of different levels. Firstly inter-genotypic variability describes genomic regions that are conserved within the same subtype but are distinct between subtypes. Secondly, intra-genotypic variability is defined as regions of viral variability within the same genotype or subtype. Finally, intra-host variability occurs

when regions of viral genome are variable within the same subtype and also within a host. Whilst inter-genotypic variability may simply be a feature of geographically distinct HCV subtypes, intra-genotypic and intra-host variability may reflect viral regions subject to specific selection pressures, with important functional implications.

To assess the extent of variability of HCV subtype-3a genome, near full-length viral sequence was sequenced from 18 chronically infected patients and clonal sequence analysis was performed in chronic and acutely infected patients.

3.2 Chapter Specific Materials and Methods

3.2.1. Patients

Plasma samples were obtained and immediately stored at -80°C from 40 treatment naïve patients with chronic HCV infection; 18 with subtype-3a, 12 with subtype-1a and 10 with subtype-1b (John Radcliffe Hospital, Oxford), and 4 patients with acute subtype-3a infection sampled at multiple time-points longitudinally (San Bortolo Hospital, Vicenza, Italy). Acute HCV was defined as an ALT >1000 IU/ml with detectable HCV RNA, in the presence of specific risk factors for acute HCV infection (3 patients gave a history of recent intravenous drug use and one had recent orthopaedic surgery) and the absence of any other cause of an acute hepatitis. In 2/4 patients the development of HCV antibody seroconversion was also demonstrated. Local ethical approval was obtained and all patients gave written informed consent for study participation. The clonal sequencing of genotype-1 patients were performed by Vicki Fleming and Bodo Schulenburg.

3.3 Results

3.3.1 Cross-sectional analysis of full-length subtype-3a viral genomes

Full-length viral genomic sequences were obtained from 18 treatment-naïve patients with chronic HCV subtype-3a infection. A mathematical measure of entropy (Shannon entropy score) was used to evaluate the sequence diversity of the full-length sequences from the 18 patients. Entropy analysis of these aligned sequences showed, as expected, that the regions of highest variability (defined here as entropy scores >0.4) were predominantly located in the genomic region coding for the envelope proteins, particularly E2 (Figure 3.1 upper panel). Further analysis of the E2 region of subtype-3a revealed three distinct regions of genomic variability including the hypervariable region-1 (HVR1) at the N-terminal of E2 (known to be present in all HCV genotypes), and two further regions. The first of these named HVR495 spans amino-acids 495-501 and is 7 amino-acids long. The second named HVR575, which represents amino-acids 575-578e and is 9 amino-acids long (Figure 3.1 lower panel).

3.3.2 Comparative analysis of E2 HCV subtype-3a with other viral subtypes

To assess whether HVR495 and HVR575 were found in other HCV genotypes, E2 sequences were determined in-house in 22 patients with genotype-1 infection (12 subtype-1a and 10 subtype-1b). In addition, 85 patients with full-length genomic sequences – including 18 patients with each of the HCV subtypes-1a, -1b, 9 patients with subtype-2a, 15 patients with subtype-4a and 15 patients with subtype-6a, were randomly selected from the Los Alamos and euHCVdb databases after exclusion of related and synthetic sequences (Figure 3.2). These sequences were aligned within each subtype. The summed entropy scores for each amino-acid position of each subtype showed that

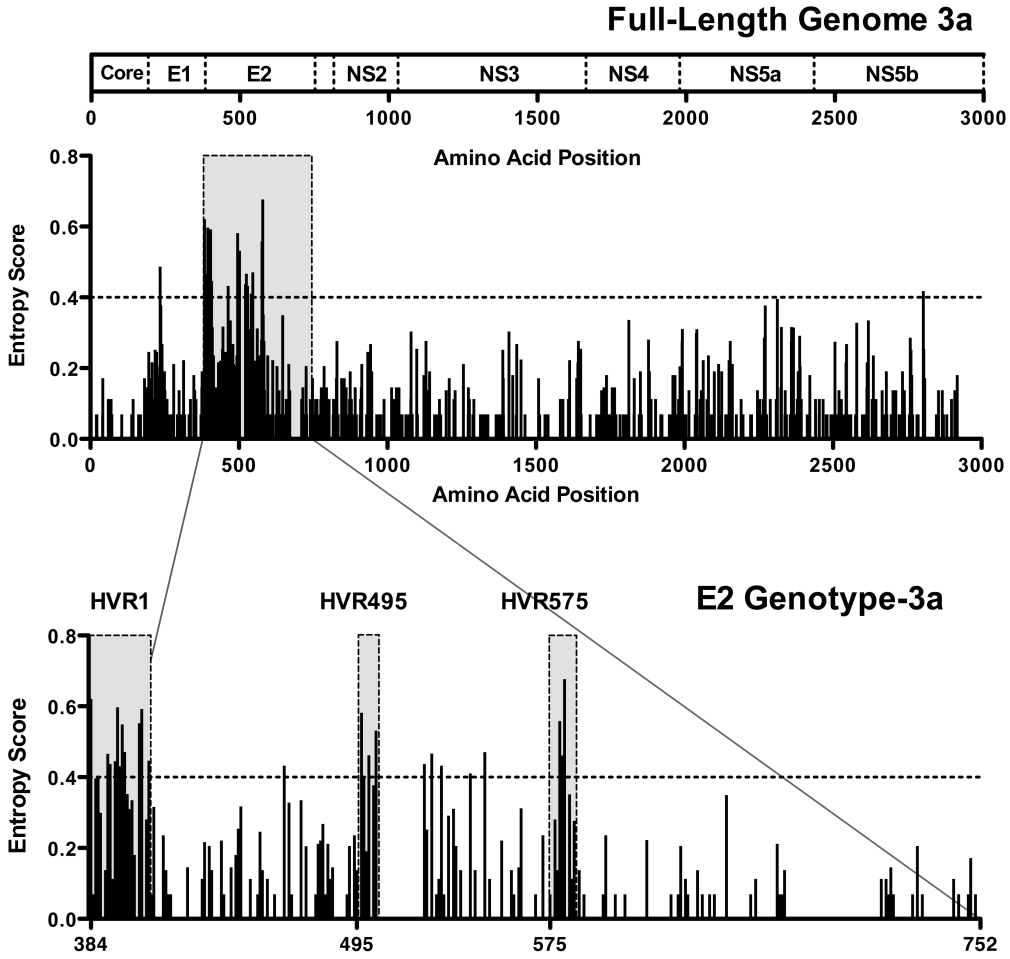


Figure 3.1 Subtype-3a entropy

The entropy score (a mathematical measure of variability) at each amino-acid site following full-length viral genome sequence analysis of 18 patients with chronic HCV subtype-3a infection is shown. Each bar represents variability at a single amino acid site. The corresponding HCV subtype-3a genome map is given above. Analysis of E2 subtype-3a shows the HVR1 at the N-terminal domain of E2, in addition to two novel hypervariable regions (HVR495 and HVR575).

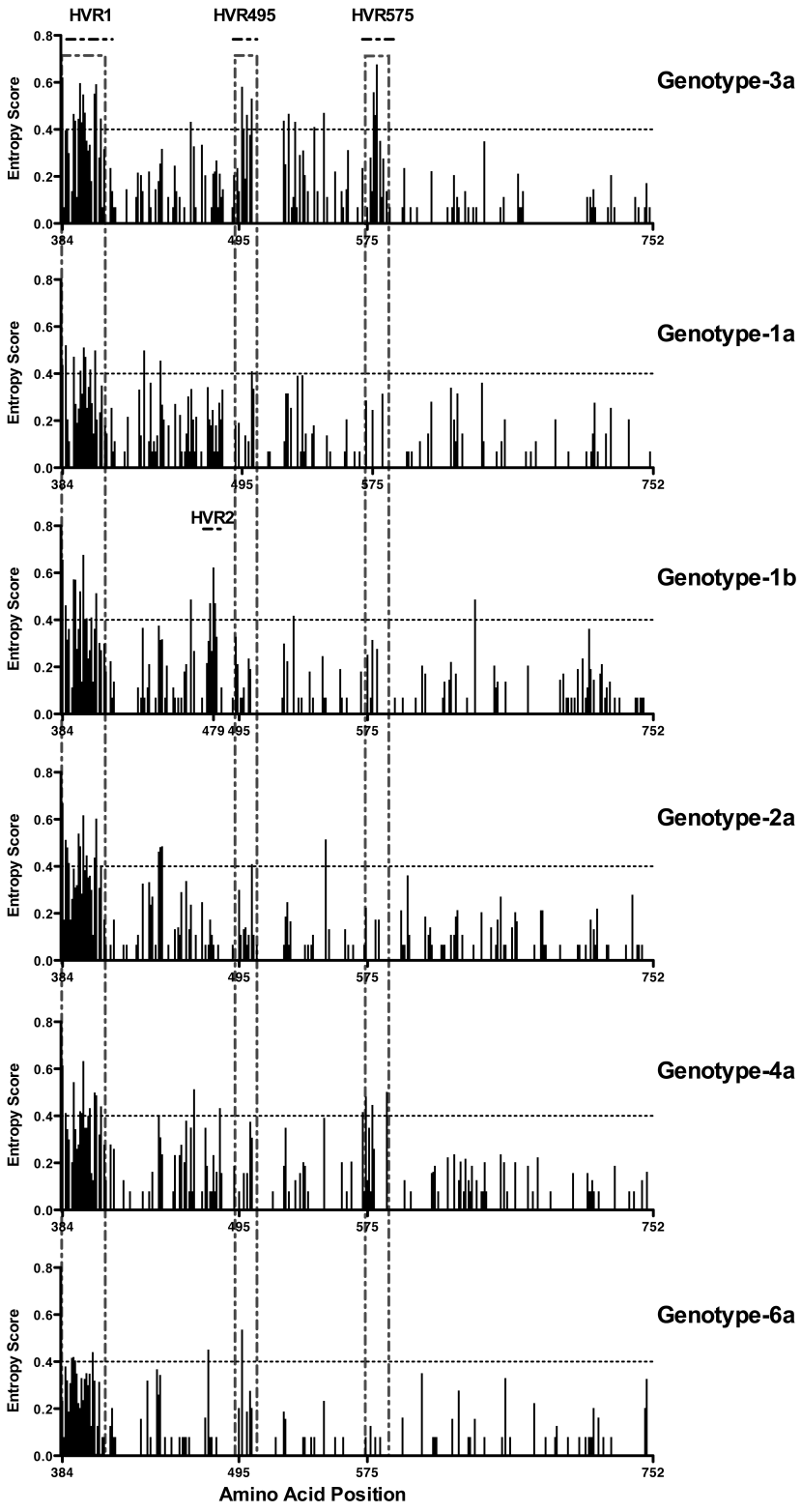


Figure 3.2 HCV entropy

For comparison, E2 entropy scores from 31 patients with HCV subtype-1a, 27 patients with subtype-1b, 20 patients with subtype-2a, 15 patients with subtype-4a and 15 patients with subtype-6a infection (sequences were determined in house with additional sequences randomly selected from the Los Alamos and euHCVdb databases.

the total entropy score for E2 subtype-3a (30.15) was higher than that for subtypes-1a (24.98), 1b (26.15), 2a (25.23), 4a (26.86) and 6a (17.33). HVR495 and HVR575 were not observed in the analysis of the 1a, 1b, 2a or 6a subtypes. There was a single polymorphic amino acid at position 495 in subtype-6a, and variability (less marked than that observed in subtype-3a infection) was observed within HVR575 in subtype-4a infection with an entropy score of >0.4 at position 575b (Figure 3.2).

3.3.3. Evolutionary analysis of positive selection within E2 of HCV subtype-3a

In theory, regions of high variability may arise as some viral genomic regions are simply functionally unconstrained, or may be induced by selective forces. Therefore a selection analysis was performed using the program CODEML to ascertain whether HVR495 and HVR575 were also subject to positive selection. Evolutionary analysis of E2 by CODEML revealed 21 positively selected sites where $\omega > 1$ and with a posterior probability of >90% (Figure 3.3, highlighted in red; Table 3.1) which were concentrated predominantly within HVR1, HVR495 and HVR575. Positively selected sites included amino-acids 495, 496, 498, 500 and 501 within HVR495, and 577, 578a and 578c within HVR575. Only 1 out of 21 selected sites detected were located outside of these three regions. Neither the polymorphic site at position 495 in subtype-6a nor HVR575 in subtype-4a was subject to positive selection by CODEML analysis.

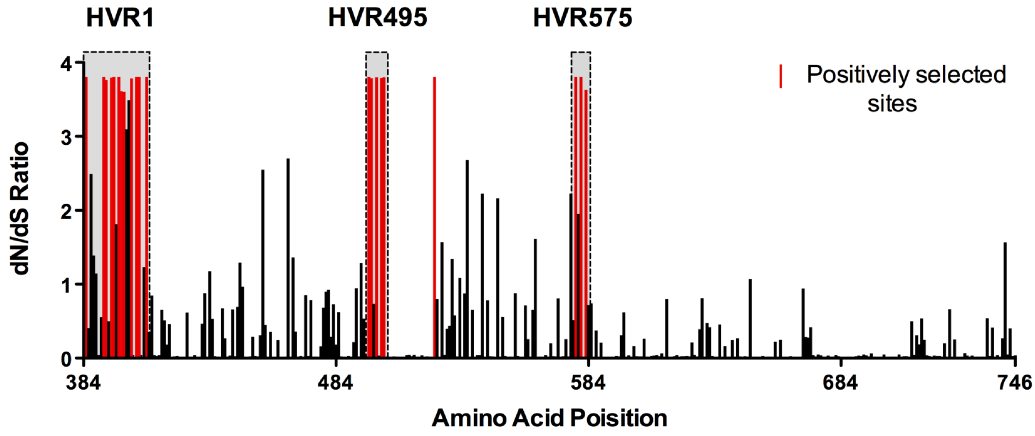


Figure 3.3 Positively selected sites in subtype-3a E2
Assessment of positive selection was performed using CODEML analysis in 18 patients with chronic subtype-3a infection. *dN/dS* ratios within E2 are shown; positively selected sites, with a posterior probability >90% are highlighted in red.

Table 3.1 Positively selected residues
HVR1 spans amino acid positions 384 to 408; HVR495 spans amino acid positions 495 to 501; and HVR575 spans amino acid positions 577 to 578c.

HVR	Amino Acid Position	Amino acid Residue
HVR1	384	E
	391	S
	392	A
	394	H
	395	S
	397	S
	398	G
	399	I
	402	L
	404	S
	405	P
	408	R
HVR495	495	D
	496	T
	498	P
	500	L
	501	N
	521	T
HVR575	577	D
	578a	N
	578c	G

3.3.4 HVR575 contains a genomic insertion unique to HCV subtype-3a

HVR575 contains a 5 amino-acid insertion that is found only in subtype-3a infection. The insertion within HVR575 is represented by lower-case letters a-e (see below).

	575	576	577	578			**578**			579	580	581
	|	|	|	|	**a**	**b**	**c**	**d**	**e**	|	|	|
Geno 1a	G	N	N	T	-	-	-	-	-	L	L	C
Geno 3a	E	G	N	P	**K**	**N**	**E**	**S**	**D**	L	F	C

HVR575 Insertion

The viral sequences that constitute HVR1, HVR495 and HVR575 in the 18 patients with chronic infection are shown in Figure 3.4. Within the 5 amino-acid insertion lies a putative N-linked glycosylation site (N-X-S/T-X; where X represents any amino-acid except proline, N represents asparagine, S/T represents serine/threonine). Detailed analysis of this insertion shows that, in all 18 patients, the only conserved sites are those absolutely required for glycosylation - amino-acids 578b and 578d, which are always asparagine and serine/threonine respectively (Figure 3.4).

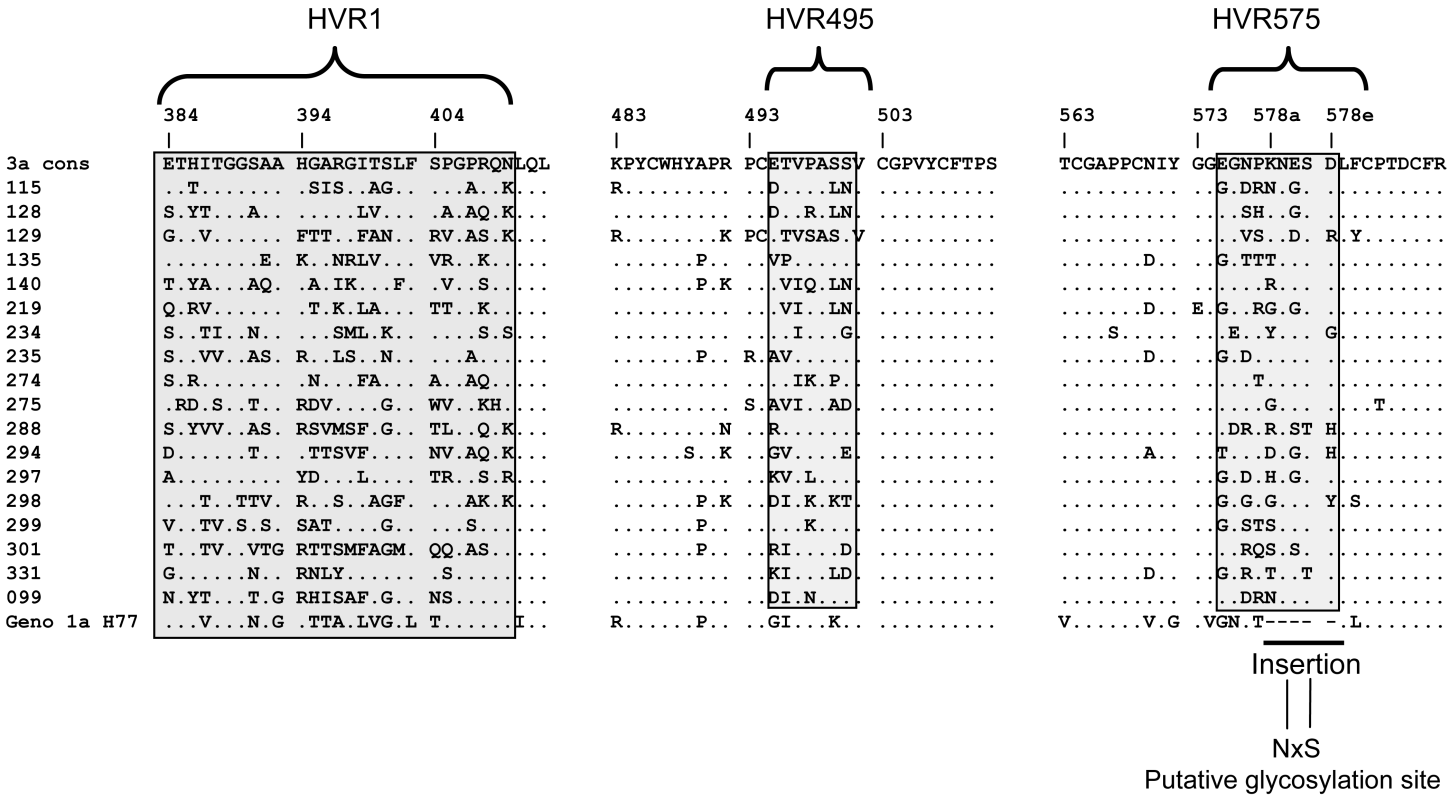


Figure 3.4 Amino acid variation within HVR1, HVR495 and HVR575
Amino-acids within the E2 HVR1, HVR495 and HVR575 are shown. The 5 amino-acid insertion is shown with a bar and is contained within HVR575. The consensus sequence is shown in the top line. A dot indicates that the amino-acid is identical to the consensus amino-acid. The E2 H77-1a sequence is shown for comparison.

3.3.5 HVR495 and HVR575 show clonal variability within a single host that occurs independently of variability observed in HVR1

Having shown that HVR495 and HVR575 are highly variable among individuals infected with the same subtype, next the intra-host variability of these regions was assessed. E2 clonal sequence analysis was performed in 22 patients with chronic HCV infection (9 subtype-3a, 7 with subtype-1a and 6 with subtype-1b). A total of 6 to 26 (mean 18.7) clones were derived from each patient. In subtype-3a patients HVR495 and HVR575 contained multiple variants within a single host; of the 9 patients evaluated, all showed clonal variation within HVR575 and 7 of 9 patients showed clonal variation within HVR495. In contrast, in genotype-1a and -1b these regions were highly conserved except in a single patient (patient 381) that had an aspartic acid to asparagine mutation at position 576 in 5 of the 10 clones sequenced. Clonal analysis of two representative patients of subtype-3a (patients 129 and 299) and one patient with subtype-1a (patient 396) are shown in Figure 3.5.

The structure of HCV E2 is currently not known, therefore in theory, it is possible that HVR495 and HVR575 lie is close proximity to HVR1 to form a common functional unit and may be subject to the same pressures. Therefore I assessed whether sequence variation within HVR1 was connected to variation seen within HVR495 and HVR575. In patient 129, amino-acid variation is seen at multiple sites within HVR575 that is not connected to the variation seen in HVR1 within the same clones. Similarly, patient 299 shows amino-acid variation at multiple sites within HVR495 that is not connected to the variability seen in HVR1 within the same clones.

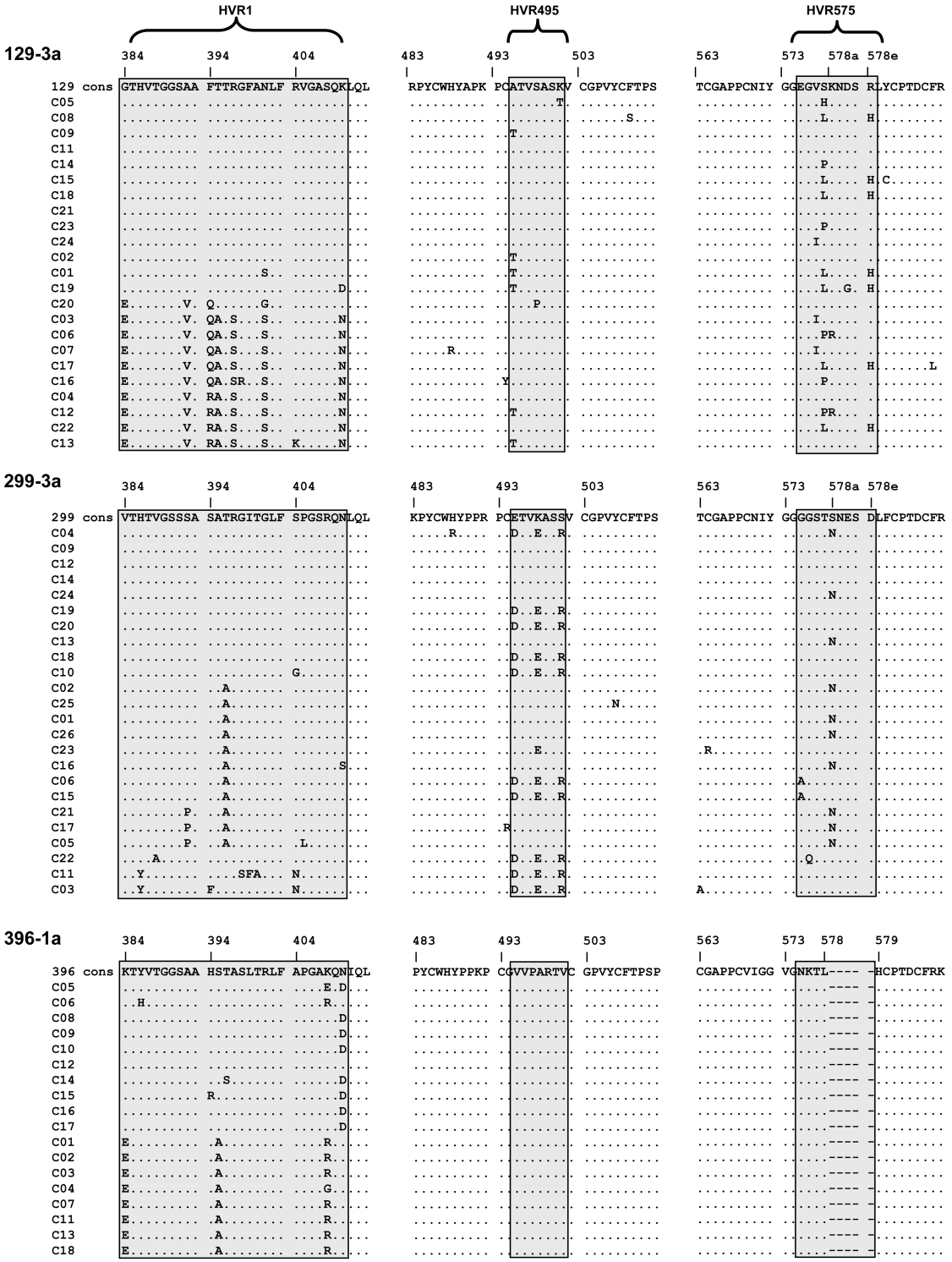


Figure 3.5 Clonal analysis of HVR1, HVR495 and HVR575
Amino-acids within HVR1, HVR495 and HVR575 are derived from 18-25 clones after E2 sequencing in three representative patients: patient 129, subtype-3a; patient 299, subtype-3a; and patient 396, subtype-1a. The consensus sequence is given in the top line. A dot indicates that the amino-acid within a single clone is identical to the consensus amino-acid.

3.3.6 Amino-acid compositions of HVR495 and HVR575

Next I assessed the amino-acid composition of HVR495 and HVR575 in terms of hydropathicity and hydrophilicity. The percentage of individuals expressing a particular amino-acid at each site within HVR495 and HVR575 was determined, and amino-acids were classified into hydrophobic, neutral and hydrophilic categories. HVR495 and HVR575 show highly variable central amino-acids that are largely hydrophilic or neutral, surrounded by highly conserved hydrophobic amino-acids (Figure 3.6). The central, highly variable amino-acids are those that are identified as being under positive selection by the evolutionary analysis performed by CODEML (indicated by a star in Figure 3.6) and include amino-acids 495, 498, 500 and 501 within HVR495, and amino acids 577, 578a and 578c within HVR575.

3.3.7 HVR495 and HVR575 are under positive selection during acute infection

Next, I investigated the evolution of the HVR495 and HVR575 in four individuals with primary HCV subtype-3a infection (It13, It14, It16, and It17) presenting with acute hepatitis. Three individuals acquired acute HCV through intravenous drug use, and one acquired acute HCV 70 days after orthopaedic surgery. The clinical course of infection is described in Figure 3.7 (upper panel graphs). Viral samples were collected at multiple (4-6) time points, and analysis of clonal E2 sequences was performed. Patients It14, It16, and It17 acquired mutations within HVR575 that were detectable in the majority of clones analysed at 39, 309 and 88 days respectively, after acute presentation. Dominant mutations were also observed in HVR495 in patients It13 and It16 at 163 and 309 days respectively, after acute presentation (Figure 3.7 lower panel). Increases in ALT levels observed may be related to sequence changes. However due to a lack of clinical data further assessment of mutations and flares in ALT levels was not possible.

CODEML analysis (of all clones derived from all timepoints) for each acutely infected patient confirmed that mutations within both the HVR495 (amino acid 495 in It16) and the HVR575 (amino-acids 577, 578 and 578b in It17) were under positive selection during acute infection. Although mutations within HVR495 and HVR575 clearly arose early in acute infection, analysis of patient It16 at day 888 after acute infection showed that mutations continued to accumulate within HVR575 after almost a year (day 309) of infection. Due to long intervals during collection of samples, additional sequence changes may have been missed.

Although sporadic mutations outside HVR1, HVR495 or HVR575 (relative to the earliest time point studied) were observed in a low proportion of sequences during acute infection (Figure 3.7), very few dominant mutations (i.e. found within the majority of clones) within E2 were observed outside HVR1 or HVR495 and HVR575 (Figure 3.8) at any time studied.

All four patients progressed to chronicity and as samples from patients that spontaneously resolved infection were not available, the significance of these mutations in terms of progression to chronicity could not be assessed.

Figure 3.6 Amino acid compositions of HVR495 and HVR575
The data is derived from E2 amino-acid sequence in 18 patients with chronic HCV infection. The HVR495 and HVR575 regions are shown. The numbers given are amino-acids relative to the subtype-1a H77 sequence. The percentage of individuals expressing a particular amino-acid at each site is given. Amino-acids are classified as hydrophobic, neutral and hydrophilic. The star graphic highlights sites under positive selection as defined by CODEML analysis.

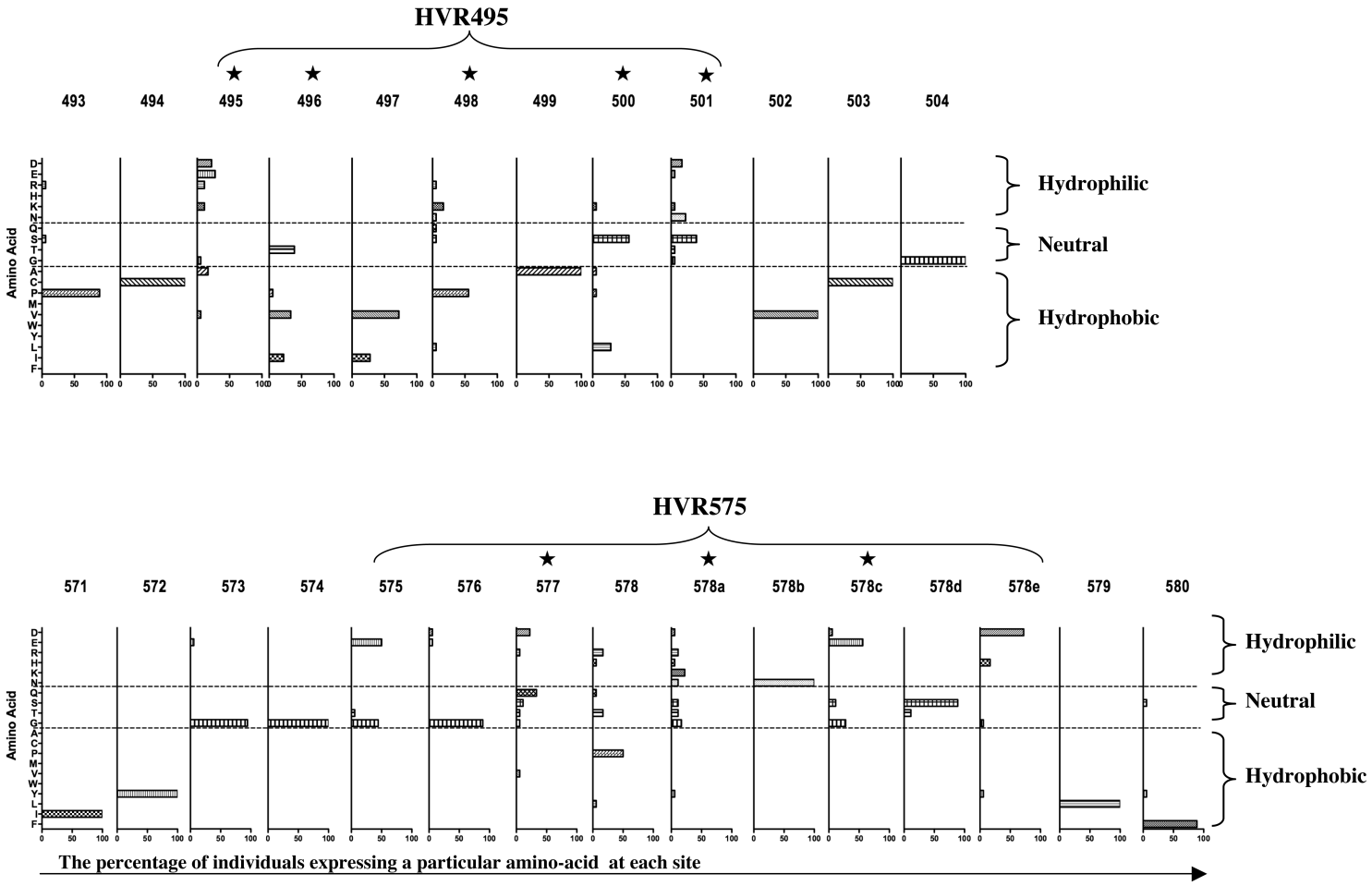
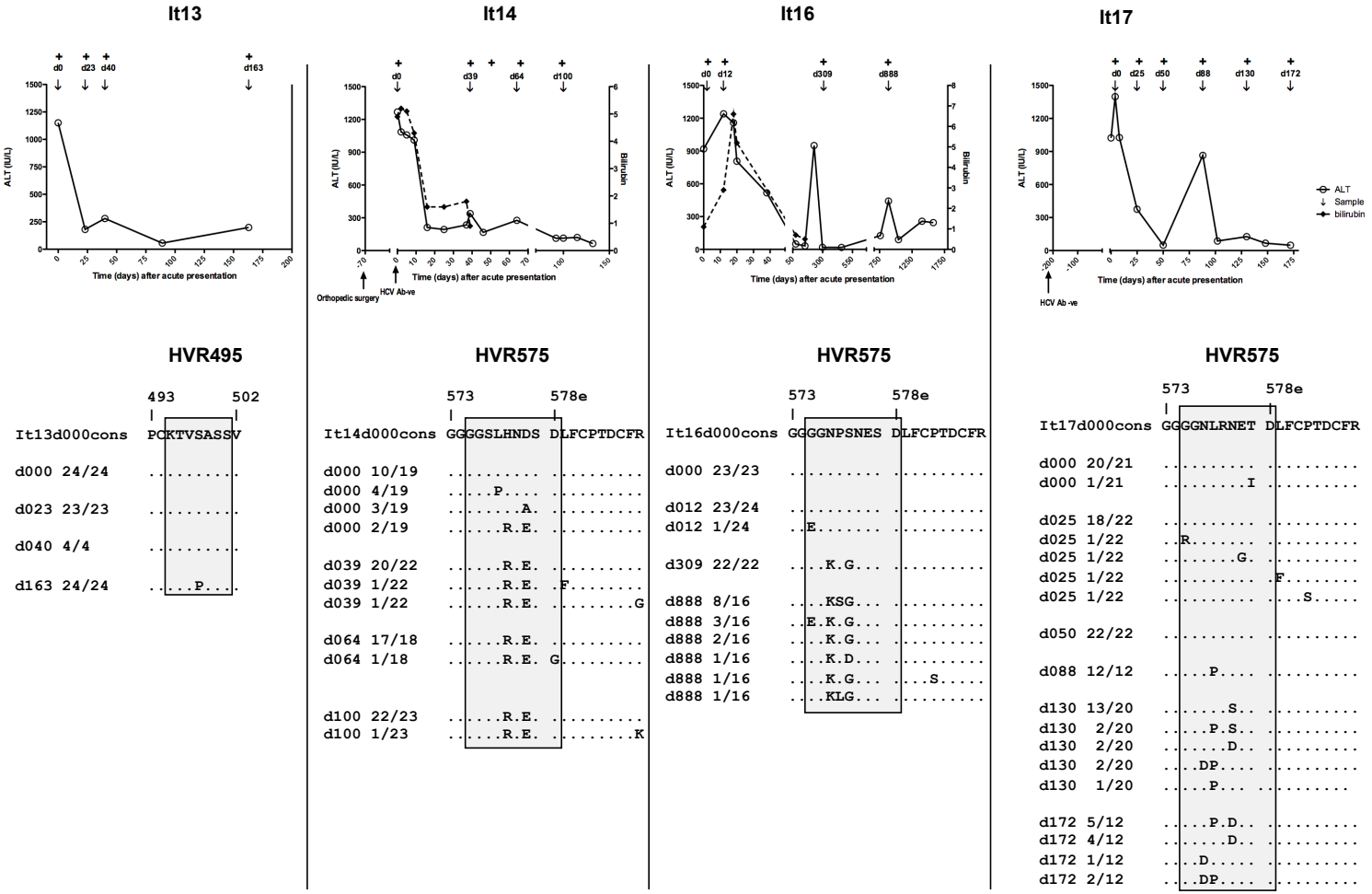


Figure 3.7 HVR495 and HVR575 in acute infection

The clinical courses of 4 patients (It13, It14, It16, and It17) with acute hepatitis of primary HCV infection are shown. HCV RNA was detected by PCR at the timepoints shown (+). Patients were sampled at multiple time points when HCV RNA positive as indicated by ↓ and E2 quasi-species analysis was performed. HVR495 or HVR575 are shaded. The amino-acid sequence of each variant is shown relative to the consensus sequence at the earliest timepoint (day 0). A dot indicates an amino-acid identical to the consensus amino-acid at day 0. The proportion of each variant/total number of variants is given. All patients progressed to chronicity.



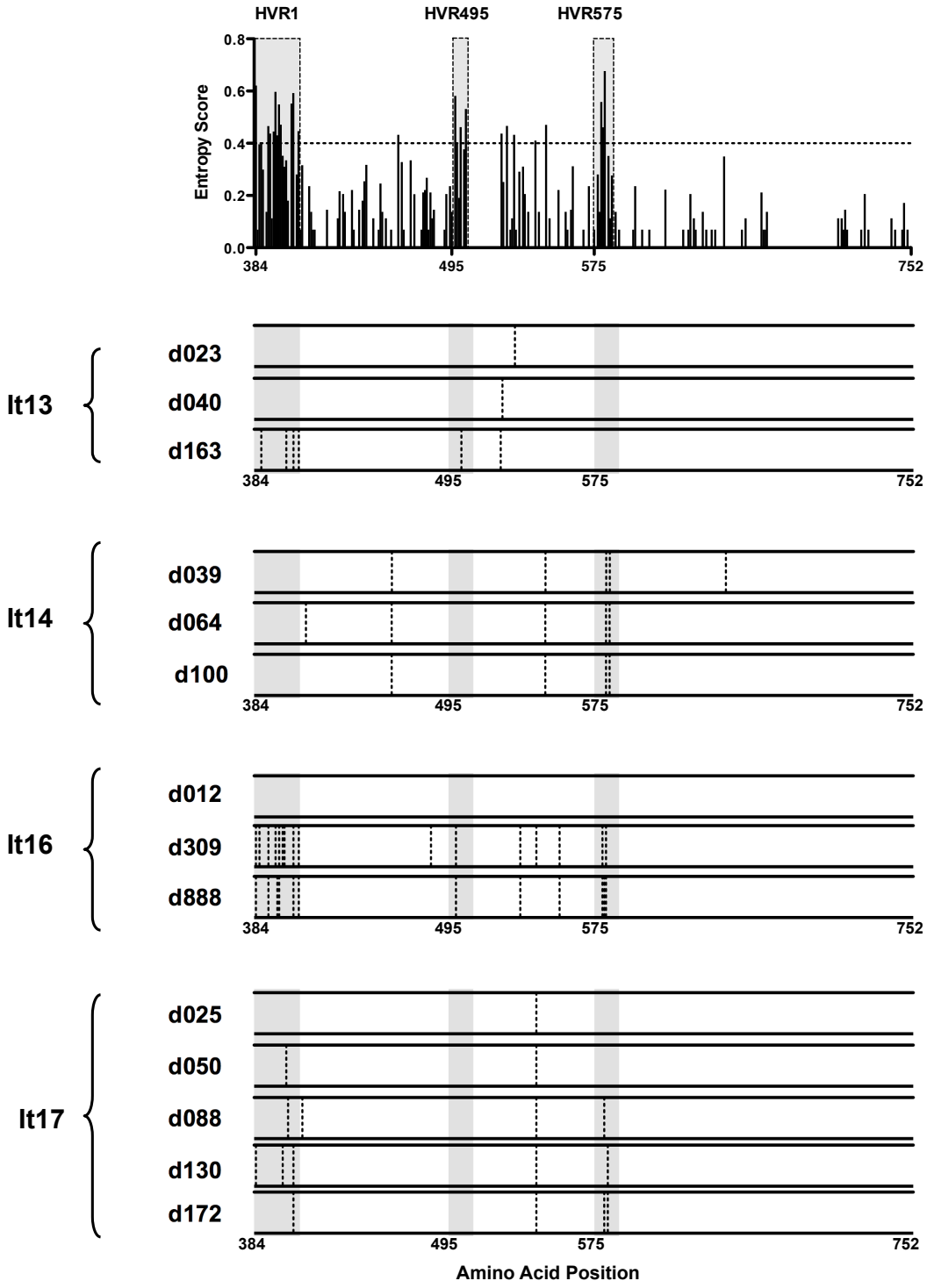


Figure 3.8 Amino acid mutations in acute infection
The upper panel maps the position of the HVRs (shaded regions) derived from 18 patients with chronic HCV subtype-3a infection to the panels below. The data from four patients (It13, 14, 16 and 17) with acute infection are shown in the lower panels. These show mutations (represented by a dotted vertical bar) in the consensus sequence at each time point studied, relative to the consensus sequence at the earliest time point (day 0).

3.4 Discussion

HCV subtype-3a is the predominant infecting strain in newly identified infections in the United Kingdom and is the endemic subtype in parts of Asia. Despite the fact that millions of people are infected with this subtype worldwide, little information is known about the sequence of HCV subtype-3a. This is reflected in the fact that only 5 full-length genomic subtype-3a sequences are currently available in the Los Alamos HCV database (2009), and that a detailed analysis of the entire E2 protein sequence in subtype-3a has not previously been performed. Additionally, lack of sequence data in this common subtype has negative implications for both peptide and protein design, which is essential for assays of adaptive immunity, primer design for sequence analysis, and vaccine design that aims to induce cross-protective immunity between common subtypes.

Following full-length sequencing of 18 patients with chronic HCV subtype-3a, the analysis was focused on the E2 protein. The analysis revealed that HCV subtype-3a contains not only the common HVR1 at the 5' end of E2, but also two additional regions of hypervariability, which I have termed HVR495 and HVR575, that are not present in subtypes-1a, -1b, -2a or -6a. The 5 amino-acid insertion in HCV subtype-3a is contained within HVR575 and this region, in addition to HVR495 appears to be under selection pressure early in acute subtype-3a HCV infection. Analysis of more datasets may be required to confirm or refute the presence of HVR575 in subtype-4a HCV, where some variability was demonstrated but evidence of positive selection was not found. I was unable to assess whether the HVR495 and HVR575 are present in other genotype-3 subtypes as these are rare; only a single subtype-3b and a single subtype-3k E2 sequences are currently available.

The fact that the viral sequence within HVR495 and HVR575 is different in every individual with chronic subtype-3a infection studied suggests that it is highly unlikely

that viral variability in this region is driven by T-cells, where a T cell "footprint" will be directed by human leukocyte antigens, and therefore viral variation will be limited to individuals of a particular human leukocyte antigen type.

Although speculative, the hydrophobicity profile and associated constant/variable amino acid distribution within these sites suggest that the HVR495 and HVR575 sequences lie at the surface of the E2 protein as external loops and as such may act as antigenic determinants or play a role in viral attachment or entry.

HCV envelope proteins are known to be glycosylated with the E2 proteins of subtypes-1a and -3a containing six and seven potential glycosylation sites, respectively (McCaffrey et al., 2007). The potential location of the variable regions at the surface of the protein is further strengthened by the fact that HVR575 contains a putative glycosylation site. N-linked glycosylation has been shown to be required for E2 protein folding (Shaw et al., 2003) and for the formation of E1/E2 complexes (Slater-Handshy et al., 2004). Glycosylation also has an important role in shaping immunogenicity against a protein by shielding epitopes against targeting antibodies, and in maintaining antigenic structure of the protein. A number of studies have shown that N-linked glycosylation can limit the antibody response to HCV envelope proteins, and removal of these glycans increases the ability of antibodies to neutralise their target (Meunier et al., 1999, Fournillier et al., 2001, Helle et al., 2007). In acute human immunodeficiency virus infection, envelope mutations may lead to loss of glycosylation sites and also the development of new glycosylation sites that reduce but do not completely abrogate antibody recognition of antigenic determinants in close proximity (Falkowska et al., 2007). In HCV infection glycosylation sites are thought to be highly conserved (Wei et al., 2003). However changes in glycosylation sites can occur and subsequently affect antibody exposure (McCaffrey et al., 2007). Interestingly I show a dominant mutation developing in the

HVR575 glycosylation site in acute infection (patient It17 - 130 days after presentation). Subtypes-1a, -1b, -2b, -4a and -6a also have a glycosylation site in close proximity to HVR 575 (McCaffrey et al., 2007), suggesting a critical role of the glycosylation site in this region.

Other regions of genotype-specific variability within E2 have been described in chronic HCV infection; this includes a 9 amino-acid region of variability seen in HCV genotype-2 (McCaffrey et al., 2007), and a 7 amino-acid variable sequence 75 amino-acids downstream of HVR1 in HCV subtype-1b infections, termed HVR2 (McCaffrey et al., 2007). A third region (HVR3) of variability has been described between HVR1 and HVR2 (Kato et al., 1992a), although this is confounded by an analysis that grouped patients of mixed genotypes.

The HVR1 region has been intensively studied over recent years and shown to be particularly important as a target for antibody recognition, with further evidence to show that the HVR1, HVR2, (Troesch et al., 2006) and also the intergenotypic variable region (igVR) (Roccasecca et al., 2003) may interplay in a complex fashion to modulate E2 receptor binding and assembly (McCaffrey et al., 2007).

HVR1 has been used as a genetic "marker" to identify and quantify circulating viral genetic diversity (quasispecies) within infected hosts, and to correlate with various clinical outcomes. High quasispecies dynamics based on HVR1 analysis have been linked to interferon treatment responses (McCaffrey et al., 2007, Gaudy et al., 2003, Farci et al., 2002), spontaneous virus resolution after primary infection (Abbate et al., 2004, Farci et al., 2000), and HCV liver pathology (Chen and Wang, 2005). Due to the very real practical difficulties in performing full-length viral genome clonal analysis, it is not clear whether differences in HVR1 evolution and clinical outcome relate directly to this viral

region or whether this region is serving as a marker for sequence differences elsewhere along the viral genome.

In conclusion, I have identified two regions of hypervariability within E2 in HCV subtype-3a chronically infected individuals. Further analysis shows that these regions are subject to strong intra-host selective pressure that arises early during acute infection. Further studies will need to address the functional significance of these specific regions in subtype-3a infection.

Chapter 4 – E2 Sequence Variation and Treatment of Chronic Subtype 3a Infection

Abstract

Successful treatment of chronic HCV infection is dependent on the infecting genotype, such that patients infected with genotype-1 are less likely to respond to therapy compared with genotype-3. The reason(s) for the increased treatment response rates in subtype-3a infection remain unclear. However reasons may be due to viral differences between genotypes. Quasispecies diversity has been assessed in genotype-1 infection with a higher pre-treatment viral complexity and diversity associated with treatment failure. Specific viral sequence motifs such as the E2 PePHD have also been associated with treatment resistance.

Quasispecies diversity and complexity were assessed pre-treatment in 20 subtype-3a chronically infected patients by cloning and sequencing of E2. The affect of treatment on quasispecies was assessed by comparison of clonal sequences at pre-treatment, during and post-treatment in patients that failed treatment. An association between subtype-3a PePHD, HVR495 and HVR575 sequences and treatment outcome was assessed.

In this subtype-3a cohort, pre-treatment quasispecies diversity and complexity was not associated with treatment outcome. In patients that failed treatment, 2 patterns of quasispecies changes were observed, as previously seen in genotype-1 infection, wherein IFN resistant strains were present at pre-treatment in some patients, and were also selected during treatment in others. PePHD, HVR495 and HVR575 sequences were not associated with treatment outcome. Further longitudinal analyses are required in subtype-3a patients that fail treatment to gain a greater understanding of quasispecies population and the affect of treatment.

4.1 Background

Successful treatment of chronic HCV infection is dependent on the infecting genotype, such that patients infected with genotype-1 and -4 are less likely to respond to therapy compared with patients infected with genotypes-2 and -3 (Honda et al., 1994, Fried et al., 2002, Manns et al., 2001). Current treatment consisting of pegylated interferon-α and ribavirin is expensive and patients suffer many side-effects, therefore a method to predict patient's responsiveness to treatment would be invaluable. The reason(s) for the increased treatment response rates in subtype-3a infection compared with genotype-1 remain unclear. However may be due to viral differences between genotypes.

Within a host HCV exists as quasispecies – a population of different but closely related viral strains (Kaplan et al., 2005). The quasispecies nature facilitates the existence of strains within the population with differing sensitivities to IFN, consequently a highly diverse quasispecies population would be more likely to contain strains of resistance (Martell et al., 1992).

A potential mechanism to predict treatment responsiveness would be to assess quasispecies diversity before commencement of treatment, with a highly diverse viral population less likely to respond to treatment. In support of this hypothesis a higher quasispecies complexity and diversity in genotype-1 infection is associated with treatment failure (Marrone and Sallie, 1996, Abbate et al., 2004, Salmeron et al., 2006). In addition, treatment failure in genotype-1 infection is associated with the existence of a resistant pre-treatment strain that dominates during treatment (Torres-Puente et al., 2008, Farci et al., 2002). However, other studies have not found an association between pre-treatment quasispecies diversity and treatment outcome (Pawlotsky et al., 1999, Sookoian et al., 2001).

The majority of previous studies of HCV quasispecies have focused on genotype-1 infection with the inclusion of few, if any subtype-3a infected patients, therefore an analysis of viral complexity in relation to treatment in subtype-3a infection remains largely unexplored. Only a single previous study has assessed an entire subtype-3a cohort and found an association between lower quasispecies complexity and diversity of quasispecies before treatment and treatment success (Farci et al., 2002). To confirm this finding, additional quasispecies studies of subtype-3a populations are required.

Specific viral sequence motifs such as the PePHD within E2, have also been associated with treatment resistance (Chayama et al., 2000, Sarrazin et al., 2000b, Sarrazin et al., 2001). Genotype-1 PePHD is analogous to the PKR phosphorylation site, enabling E2 to inhibit PKR activity and is therefore likely responsible for the higher resistance of genotype-1 to treatment (Moreau et al., 2008, Kato et al., 1990, Taylor et al., 1999, Sarrazin et al., 2000). However, PePHD sequences have been found to predict treatment response in some but not all studies (Polyak et al., 2000, Cochrane et al., 2000, Chayama et al., 2000, Abid et al., 2000, Berg et al., 2000, Gerotto et al., 2000, Puig-Basagoiti et al., 2001, Sarrazin et al., 2001).

In the current study an association between quasispecies diversity and treatment outcome was assessed at multiple timepoints. Clonal E2 sequencing also enabled the study of the PePHD region at pre-treatment and treatment outcome in a larger cohort of patients.

4.2 Chapter Specific Materials and Methods

4.2.1 Patients

Patients with samples for E2 cloning are shown in Table 4.1. An additional 30 patients were analysed for PePHD sequence comparison. These patients were treatment naïve and received combination treatment for 24 weeks (19 SVR; 11 REL).

Table 4.1 Subtype-3a patients

Patient	Treatment Outcome	Treatment Naïve	Treatment Duration	Timepoints Cloned (weeks)	Age	Fibrosis Score (0-6)
110	SVR	Y	24	0	32	2
115	SVR	Y	24	0	41	2
128	SVR	Y	24	0	44	5
235	SVR	Y	24	0	48	nd
275	SVR	Y	24	0	55	nd
278	SVR	Y	24	0	45	4
394	SVR	Y	24	0	49	nd
398	SVR	Y	24	0	56	nd
437	SVR	Y	24	0	50	nd
442	SVR	Y	24	0	40	6
098	REL	Y	24	0, 36	NA	NA
129	REL	Y	24	0, 36, 108	67	1
288	REL	Y	24	0, 36	57	nd
428	REL	Y	24	0, 36	60	6
450	REL	Y	24	0, 36	59	0
301	NR	Y	24	0, 4, 25	45	2
410	NR	Y	16*	0, 3, 12, 20, 36, 56	39	3
096	REL	N	24	0, 25	56	NA
097	REL	N	24	0, 30	48	NA
299	NR	N	16*	0, 4, 16, 56, 80	54	3
411	NT	-	-	0, 23	39	nd
420	NT	-	-	0, 13	59	3

SVR – Sustained virological response; REL – virological relapse; NR – nonresponder; NT – not treated. * Stopped treatment at treatment week 16 due to non-response. nd – not determined; NA – not available. Fibrosis score based on Ishak score, 0 - no fibrosis, 6 – cirrhosis (Chayama et al., 2000).

4.2.2 Sequence Alignment

Sequences were aligned using ClustalW (Ishak et al., 1995, Larkin et al., 2007) and Se-AL (v2.0 a11: http://tree.bio.ed.ac.uk).

4.2.3 Phylogenetic Analysis

Tree models of evolution were assessed using JModelTest (Goujon et al., 2010). The best-fit tree General Time Reversible with gamma distribution was selected based on lowest likelihood score and Akaike Information criterion (AIC) correction analysis. Trees were constructed using MEGA5 program (Posada, 2008) and bootstrap scores calculated based on 1000 replicates.

4.2.4 Viral Loads

Quantification of HCV viral load was performed using real-time PCR with SYBR Green I detection (Roche) based on the method by Komurian-Pradel *et al.* (Tamura et al., 2011). Initially viral RNA was transcribed into cDNA (see Methods 2.2.2) using RC1 (5'-GTCTAGCCATGGCGT-3') and RC21 (5'-CTCCCGGGGCACTCGCAAGC-3') primers to amplify 5'UTR region (Komurian-Pradel et al., 2001). cDNA (1μl) was amplified in triplicate by real-time PCR with RC1 and RC21 primers (10pmol), performed in a Lightcycler 480 (Roche). PCR conditions were an initial denaturation step 95°C for 15min, followed by 45 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 15 sec. Fluorescence measurements were taken after each elongation step. Viral loads were determined as copies/ml (not standardised WHO units of IU/ml) as the viral standard contained viral loads too low for accurate comparison.

4.3 Results

4.3.1 Pre-treatment quasispecies in treatment naïve patients

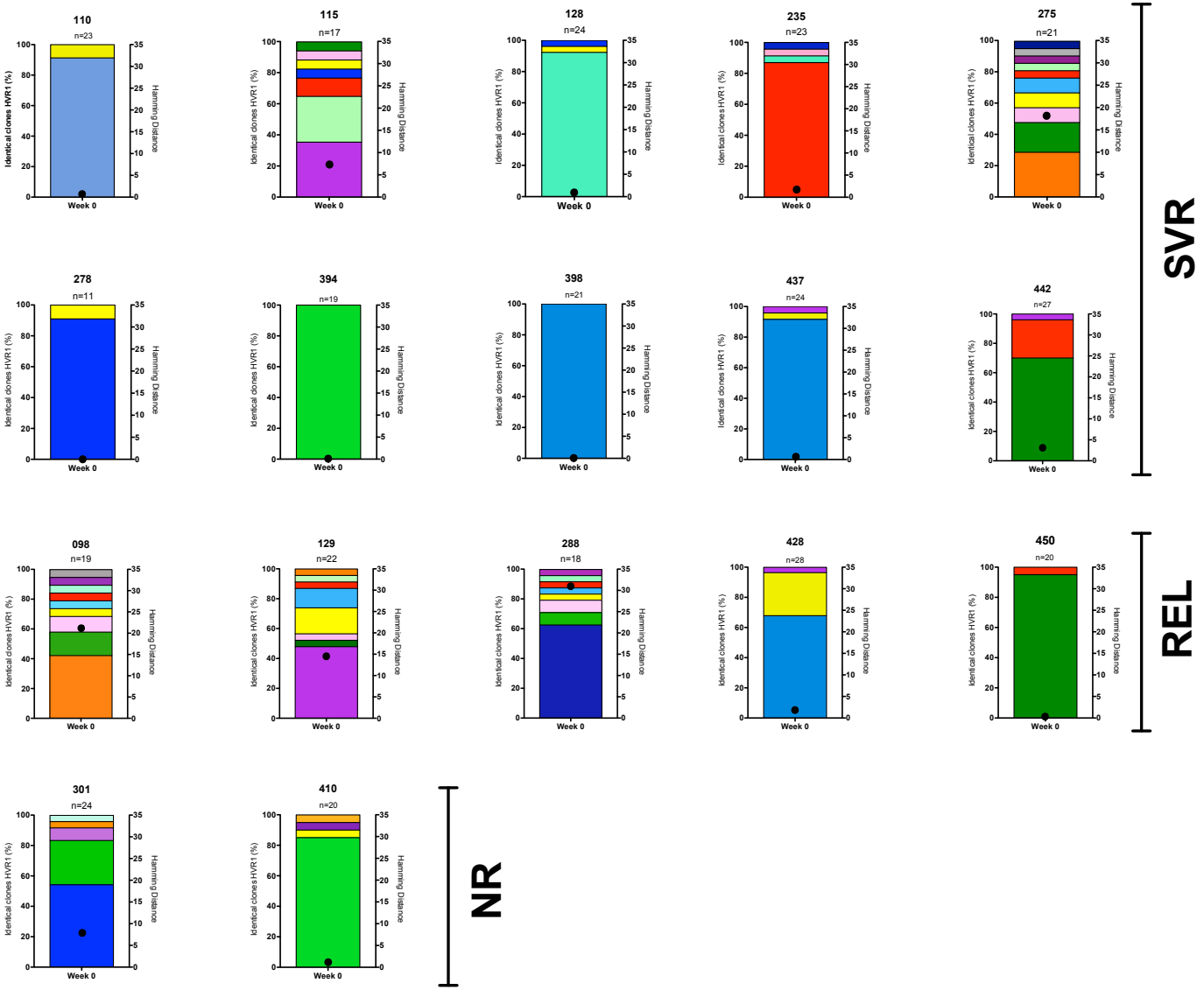
Pre-treatment E2 clones (total 361, mean 21.23 clones, range 11-28) were successfully obtained for 17 treatment-naïve patients chronically infected with HCV subtype-3a. Ten patients achieved SVR (59%), 5 patients experienced virological relapse within 6 months post-treatment (REL) (29%) and 2 patients did not exhibit a virological response to treatment (non-responders) (12%) (Table 4.1).

Cirrhosis and older age (>40 years) is associated with non-response to treatment (Besnard and Andre, 1994, Nousbaum et al., 1995, Le Guen et al., 1997, Valla et al., 1999) as cirrhosis results in abnormal liver architecture (Dusheiko, 1998, Liang et al., 2009), and older age may represent longer duration of infection. Unfortunately histology data was not available for all patients, nevertheless a range of fibrosis scores were seen in all treatment groups. Although patients that failed treatment were older than those achieving sustained virological response, this was not statistically significant (54.50 ± 4.256 vs. 46.00 ± 2.290, two-tailed t-test, p=ns). However the age of patients with virological relapse was significantly higher than SVR patients (60.75 ± 2.175 vs. 46.00 ± 2.290, p=0.0027).

An association between quasispecies diversity and treatment outcome was sought in treatment naïve patients by assessing the number of viral strains, Hamming distance (HD) and Shannon entropy (SE) scores at pre-treatment. Of the 10 SVR patients, eight patients had 4 or fewer viral strains at pre-treatment (mean 3.6, range 1-10) (Figure 4.1) and had very low diversity and SE (HD amino acids mean 0.8596, range 0.0-3.1233; SE aa mean 1.794, range 0.0-0.4926) with a clear dominant strain that represented over 70% of the population (Figure 4.1 and Figure 4.2). The two remaining SVR patients had 7 or more viral strains, higher quasispecies diversity and complexity (e.g. patient 275, aa HD

Figure 4.1 HVR1 quasispecies at pre-treatment in treatment naïve patients

The percentage of viral strains at pre-treatment are shown on the left y-axis, and viral complexity measured by hamming distance is shown on the right y-axis. Viral strains are indicated by colour. Colours do not represent the same strains between patients. Number of viral strains sequenced are indicated (n). Patients are grouped according to treatment outcome. SVR-sustained virological response; REL-relapse patients; NR-nonresponders.



18.1834; SE 2.3660). Of the five REL patients, three patients had 8 or more viral strains at pre-treatment, with a higher diversity (aa HD 14.5582-30.9764) and complexity (aa SE 1.8174-4.3378), whilst the remaining two patients had 3 or fewer strains and lower quasispecies diversity and complexity with the dominant strain representing over 65% of the viral population. The two non-responders (301-NR, 410-NR) had 5 and 4 viral strains respectively, however the dominant strains varied representing 40-85% of the population. Diversity and complexity also differed between the non-responders (aa HD range 1.1111-7.9844; SE 0.1926-0.2433) (Figure 4.1 and Figure 4.2).

No association was found between treatment outcome and quasispecies diversity or complexity (p-test non-significant) (Figure 4.2). However SVR patients were more likely to have lower quasispecies complexity, with a trend towards significantly lower SE at the amino acid level (p=0.0509) (Figure 4.2).

To determine whether the viral E2 sequences were genetically related in terms of treatment outcome, the pre-treatment E2 quasispecies sequences from 17 patients were analysed by phylogenetic tree. However the phylogenetic tree revealed that the E2 sequences did not cluster according to treatment outcome (Figure 4.3).

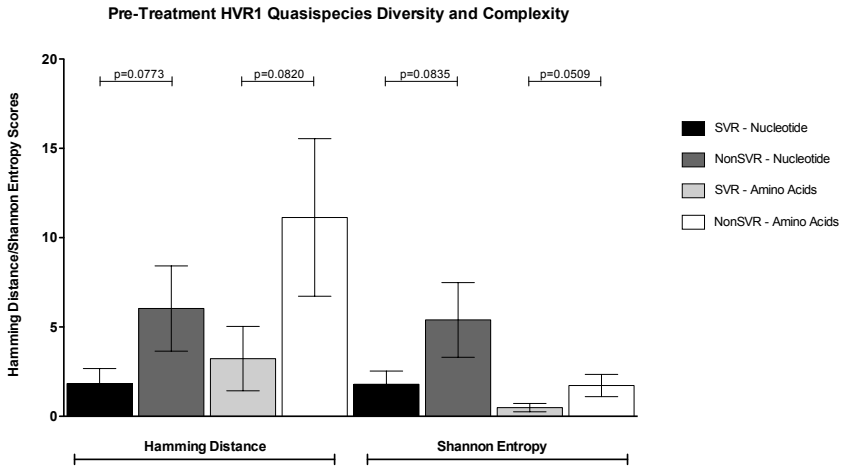
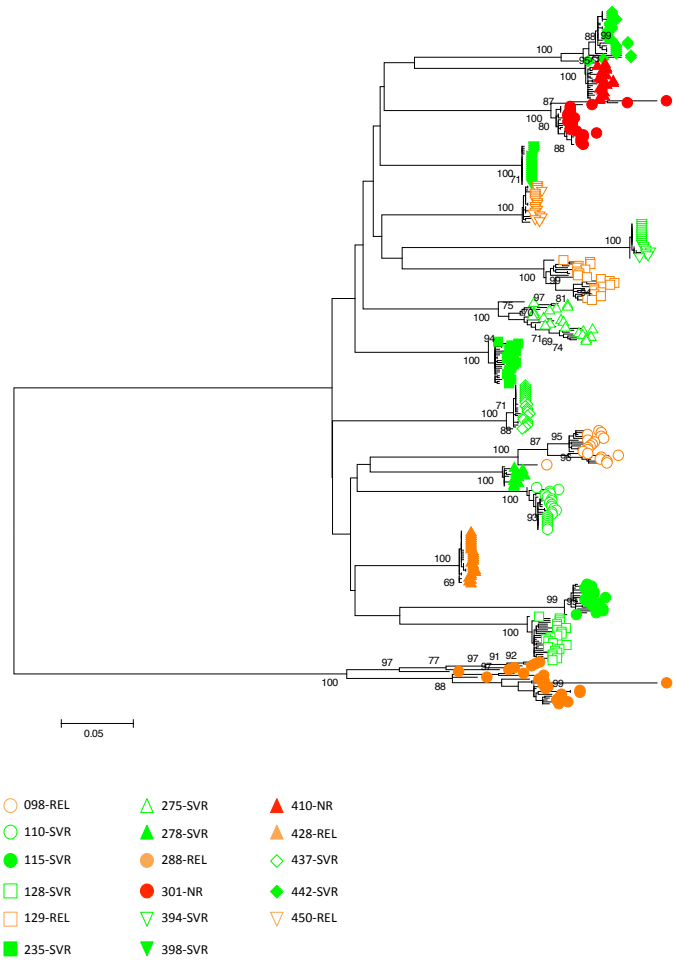


Figure 4.2 Pre-treatment quasispecies diversity and complexity in subtype-3a patients

HVR1 pre-treatment diversity and complexity measured by hamming distance and Shannon entropy, respectively in subtype-3a patients.
SVR – sustained virological response, NonSVR – patients that failed treatment (relapse patients and non-responders). Significance unpaired t-test $p<0.05$.

Figure 4.3 E2 quasispecies at pre-treatment
General time reversible plus Gamma distribution tree of entire E2 clonal sequences at pre-treatment. Bootstrap scores >70% are shown based on 1000 replicates.
SVR patients are shown in green, Relapse patients in orange and Nonresponders in red.



4.3.2 Therapy changes Quasispecies populations in Non-responder Patients

To determine whether changes in viral quasispecies population occurred during treatment, E2 clonal sequences were assessed during and post-treatment in treatment naïve non-responder patients 301-NR and 410-NR.

The HVR1 E2 quasispecies of patient 301-NR were assessed at treatment week (TW) 4 and 7 days after cessation of treatment (week 25) (Figure 4.4). The pre-treatment quasispecies were replaced by a new strain by TW4 indicating that it was not sensitive to IFN. At 7 days post-treatment, the IFN insensitive strain at TW4 was only detected at low levels, whilst the pre-treatment dominant strain re-emerged representing 70% of the population. However not all pre-treatment strains were detected post-treatment

suggesting that they had been eliminated or had not yet returned to a detectable level. New minor variants were also detected at post-treatment. A higher diversity was detected at post-treatment compared with pre-treatment (HD 12.64 and 7.98, respectively).

For patient 410-NR, viral quasispecies were assessed at treatment weeks 3 and 12 and also at 4 weeks post-treatment (week 20) (Figure 4.4). During treatment, the dominant pre-treatment strain remained dominant but decreased in proportion (Pre 85% vs. TW12 61%), whilst a minor pre-treatment strain increased (Pre 5% vs. TW12 33%). Treatment was stopped at week 16 due to lack of response. By 4 weeks post-treatment (week 20), a single dominant strain was detected that had been detected at low levels at pre-treatment and TW3. Despite the continuing change in quasispecies population, this was not reflected in diversity (range 0.00 – 2.2755).

Viral loads were also assessed to determine whether the change in quasispecies in 301-NR, was due to a fall in viral load and hence a "bottleneck" effect. The viral loads (RNA copies/ml) revealed in a slight decrease by TW4 of patient 301-NR (Figure 4.4), but overall the viral load was low compared to that of 410-NR. Patient 410-NR had an increase in viral load by TW3 that returned by TW12 to levels seen at pre-treatment. However at 4 weeks post-treatment (week 20), the viral load increased once more (Figure 4.4). The change in viral load during treatment in 301-NR and post-treatment in 410-NR, may be responsible for a change in quasispecies. A very low viral load was detected for patient 301-NR that is unusual for viral loads detected in chronic disease and therefore the decrease in viral load may not be related to the change in quasispecies.

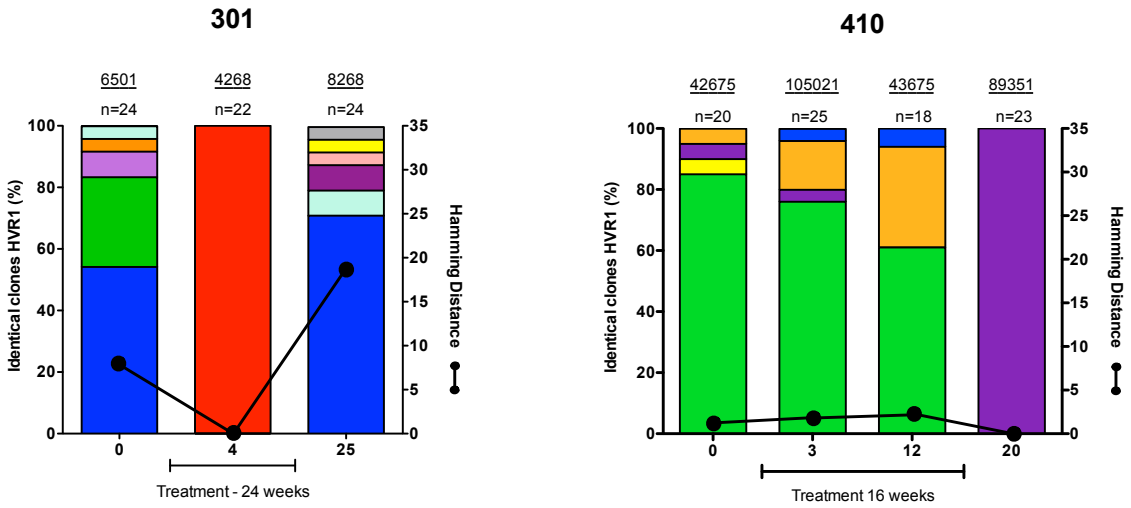


Figure 4.4 HVR1 quasispecies in non-responder patients
Viral strains are shown at pre-treatment (week 0), during treatment and post-treatment. Treatment duration is indicated by horizontal bar. Each viral strain is represented by different colours. The same colour does not represent the same strain between patients. Percentage of viral strains detected are indicated on the left y-axis. Viral complexity measured by hamming distance is shown on the right y-axis. Number of viral strains sequences are indicated (n). Viral load (RNA copies/ml) is indicated above each bar and underlined.

4.3.3 Quasispecies Complexity and Diversity in Relapse Patients

In five treatment naïve patients that experienced virological relapse, E2 quasispecies were assessed at pre-treatment and point of relapse (Figure 4.5). In three patients a new resistant viral strain was detected at post-treatment whilst in the remaining two patients, the post-treatment dominant strain was present at pre-treatment albeit at varying proportions (428-REL and 450-REL, 95% and 28%, respectively), indicating insensitivity to IFN. In all cases a dominant viral strain representing more than 90% of the population was detected at the point of relapse, resulting in a decrease in amino acid viral diversity (HD mean 0.4363, range 0-1.4815) and complexity (SE mean 0.0758, range 0-0.2569) (Figure 4.5). Overall two patterns of quasispecies change were seen in relapse patients. The presence of IFN insensitive strains before commencement of therapy suggests that treatment does not give rise to insensitive strains in all patients that experience virological relapse.

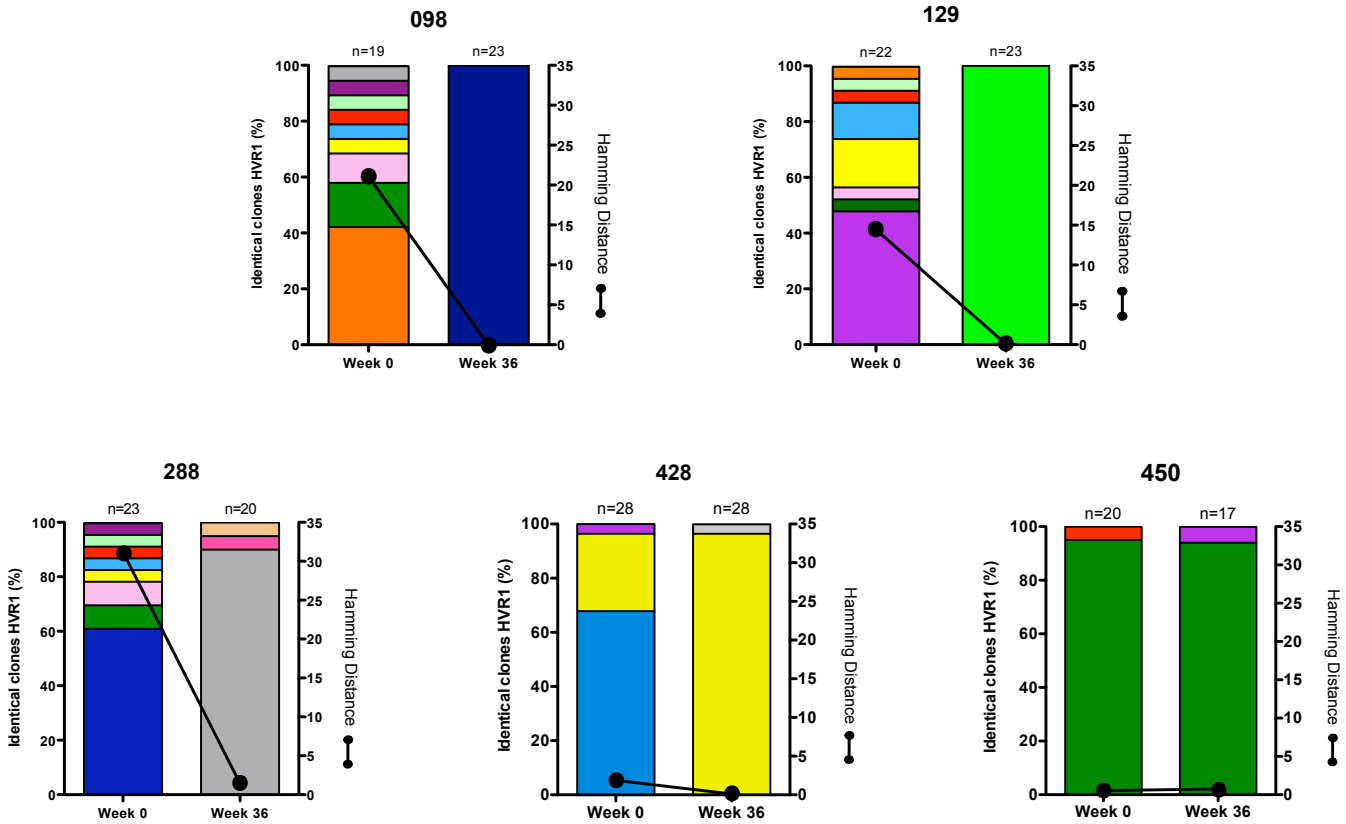


Figure 4.5 HVR1 quasispecies in relapse patients
Quasispecies sequenced at pre-treatment and point of relapse in relapse patients. All patients were treatment naïve. The percentage of viral strains at pre-treatment are shown on the left y-axis, and viral complexity measured by hamming distance is shown on the right y-axis. Viral strains are indicated by colour, however colours do not represent the same strains between patients. Number of viral strains sequenced are indicated (n).

4.3.4 Quasispecies of previously treated patients

Samples were available from three patients that had previously failed IFN treatment prior to inclusion in this study, and were assessed during the second course of treatment to determine whether previous treatment affected quasispecies populations - 096-REL, 097-REL and 299-NR.

In the previously treated relapse patients, high viral diversity at pre-treatment was detected in 096-REL (8 viral strains, HD 16.1946) whilst in 097-REL very low diversity was detected with a dominant strain representing 88% (2 viral strains, HD 0.7746). At the point of relapse a single IFN insensitive dominant strain was detected in both patients, which had been detected at pre-treatment (Figure 4.6).

Patient 299-NR at pre-treatment had 2 dominant and 7 minor strains; however a low diversity reflected few amino acid differences between the strains (HD 6.3875). A pre-treatment minor variant increased in proportion during treatment and represented over 90% population by TW16. New minor variants were detected transiently during treatment, with an initial increase in diversity by TW4 that decreased by TW16 (HD 11.32 and 0.22, respectively). Due to the presence of viral RNA at week 16, treatment was stopped. At week 56, the dominant strain during treatment declined to minor levels, with the re-appearance of the dominant pre-treatment strains in addition to new variants, increasing the quasispecies diversity (HD 5.84). Viral loads revealed that a decrease in viral load levels had occurred by TW16, which increased greatly by 56 weeks, possibly responsible for the change in quasispecies detected during treatment.

In all three patients, an insensitive IFN strain was present before treatment. However it is unclear whether these insensitive strains were selected during the first course of treatment rendering any further treatment ineffective or present before the initial course of treatment.

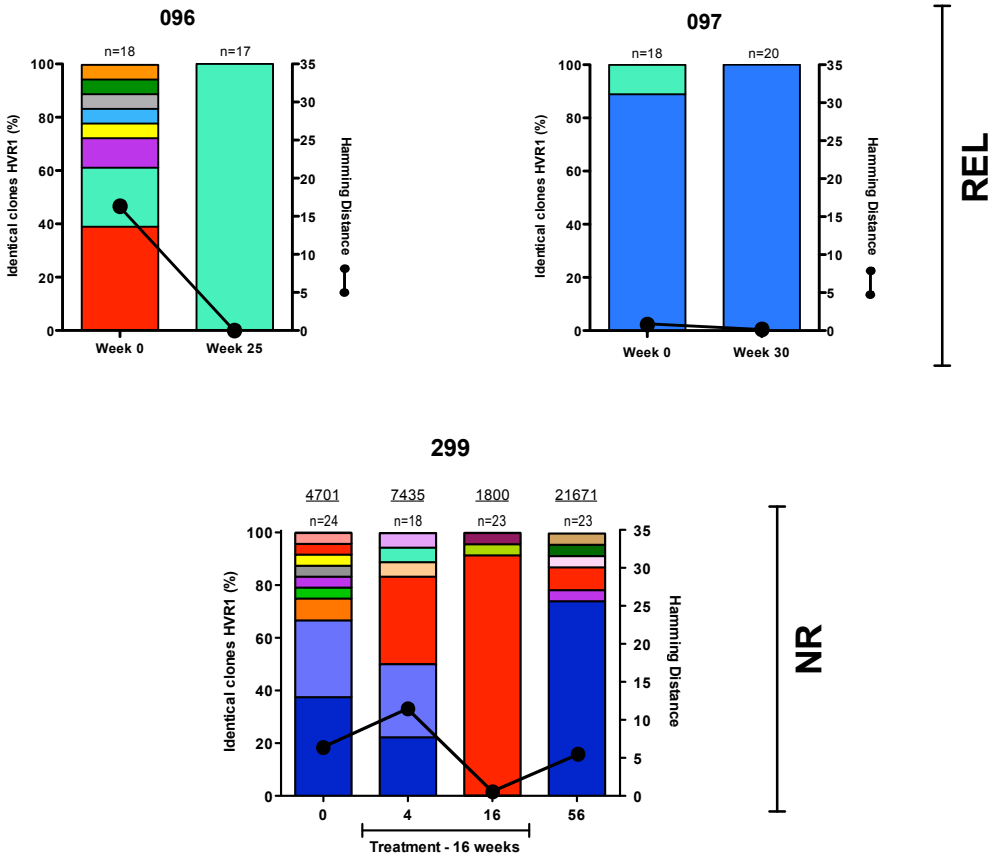


Figure 4.6 HVR1 quasispecies in previously treated patients
Quasispecies were measured at pre-treatment and at point of relapse in patients 096 and 097, and quasispecies were measured at pre-treatment, 2 timepoints during treatment and post-treatment in patient 299. The percentage of viral strains are shown on the left y-axis, and viral complexity measured by hamming distance is shown on the right y-axis. Viral strains are indicated by colour, however colours do not represent the same strains between patients. The number of viral strains sequenced are indicated (n). REL-relapse patients; NR-nonresponders. Viral load for patient 299 (RNA copies/ml) is indicated above each bar and underlined.

4.3.5 Quasispecies in Treatment Naïve Untreated Patients

Quasispecies populations were also assessed in two patients not receiving treatment, to determine whether quasispecies populations vary during natural infection, without pressure from treatment. Plasma samples were available from 2 timepoints in two chronically infected patients - patient 411 (samples 5 months apart) and patient 420 (samples 3 months apart).

E2 clones were obtained (total 84, mean 21, range 18-24), and quasispecies assessment revealed that all timepoints contained a dominant variant representing over 70% of the population (Figure 4.7). Patient 411 had a new single dominant variant by the second timepoint resulting in the decrease in diversity (HD 8.0126 and 0.00, respectively). Patient 420 had the same dominant variant at both timepoints with very few minor variants (HD 0.00 and 3.5427, respectively). Therefore during natural chronic HCV infection the quasispecies population may vary.

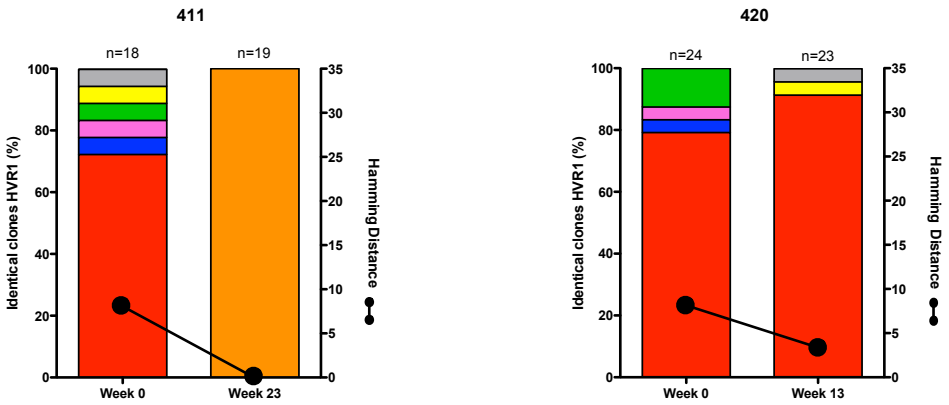


Figure 4.7 HVR1 quasispecies in untreated patients
Quasispecies were measured at 2 timepoints of chronic infection. The percentage of viral strains are shown on the left y-axis, and viral complexity measured by hamming distance is shown on the right y-axis. Viral strains are indicated by colour, however colours do not represent the same strains between patients. The number of viral strains sequenced are indicated (n).

4.3.6 Sequence Diversity across the entire E2 protein

The viral diversity and complexity of the entire E2 protein and the E2 sequence outside the HVR1 region was also assessed to determine whether diversity and complexity of E2 was associated with treatment outcome (Figure 4.8). No difference was found in the diversity or complexity of the entire E2 protein of SVR compared with nonSVR patients (t-test $p>0.05$). The diversity and complexity of the E2 protein without HVR1 sequence was not associated with treatment response (Figure 4.8).

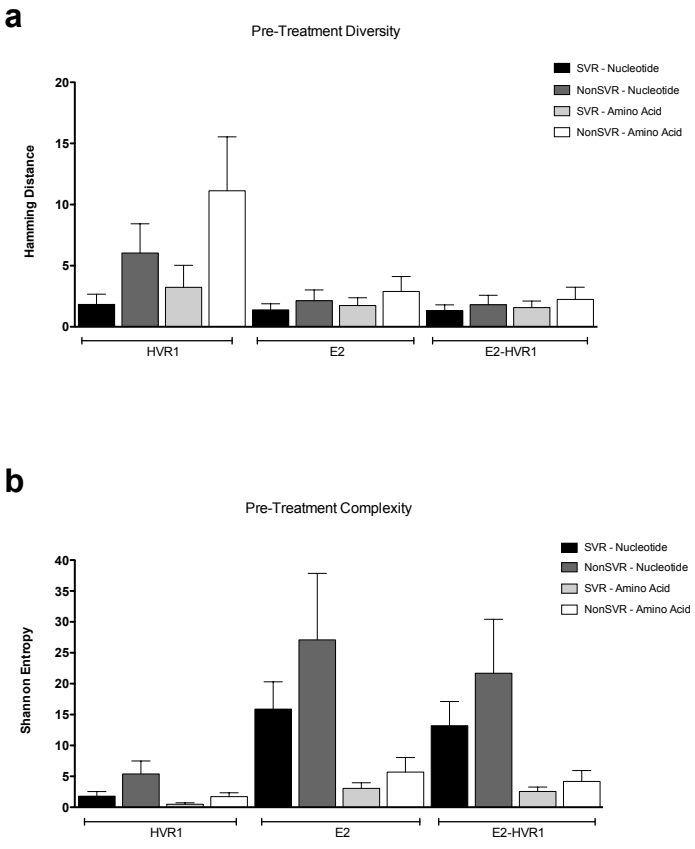


Figure 4.8 Pre-treatment diversity and complexity across the E2 protein
(a) Diversity at pre-treatment measured by hamming distance, (b) complexity at pre-treatment measured by Shannon entropy. Scores are shown for the HVR1, the entire E2 protein and also the E2 protein excluding HVR1 (HVR1-E2). SVR – sustained virological response, NonSVR – patients that failed treatment (relapse patients and non-responders).

4.3.7 Quasispecies Populations After Treatment Failure

To determine whether the quasispecies detected post-treatment remained within the population in the absence of therapy, additional samples at post-treatment were available in three patients that had failed treatment - two treatment naïve (129-REL and 410-NR) and one previously treated patient (299-NR).

An additional sample for patient 129-REL, at 108 weeks (Figure 4.9, indicated by an asterisk *) was assessed and revealed a population of new variants and a complete change in quasispecies since the point of relapse. Phylogenetic analysis revealed that the viral strains at 108 weeks were more closely related to strains at point of relapse (36 weeks) than those at pre-treatment (see Appendix II).

Two additional samples were available for 410-NR at 36 and 56 weeks after the start of treatment. The dominant strain at week 20 had decreased in proportion and was not detected by week 56, whilst the dominant pre-treatment strain re-emerged and was dominant again by week 56. A second pre-treatment strain was also detected with the emergence of new minor strains.

Previously treated patient 299-NR had an additional sample at 80 weeks since start of treatment (Figure 4.9). Little change was observed at week 80 as all the strains detected at the previous timepoint (week 56) remained, with the appearance of 2 new minor variants. However as a sample immediately after treatment was not available, additional changes may have been missed.

Therefore changes detected in quasispecies populations due to treatment are not maintained within the host after treatment.

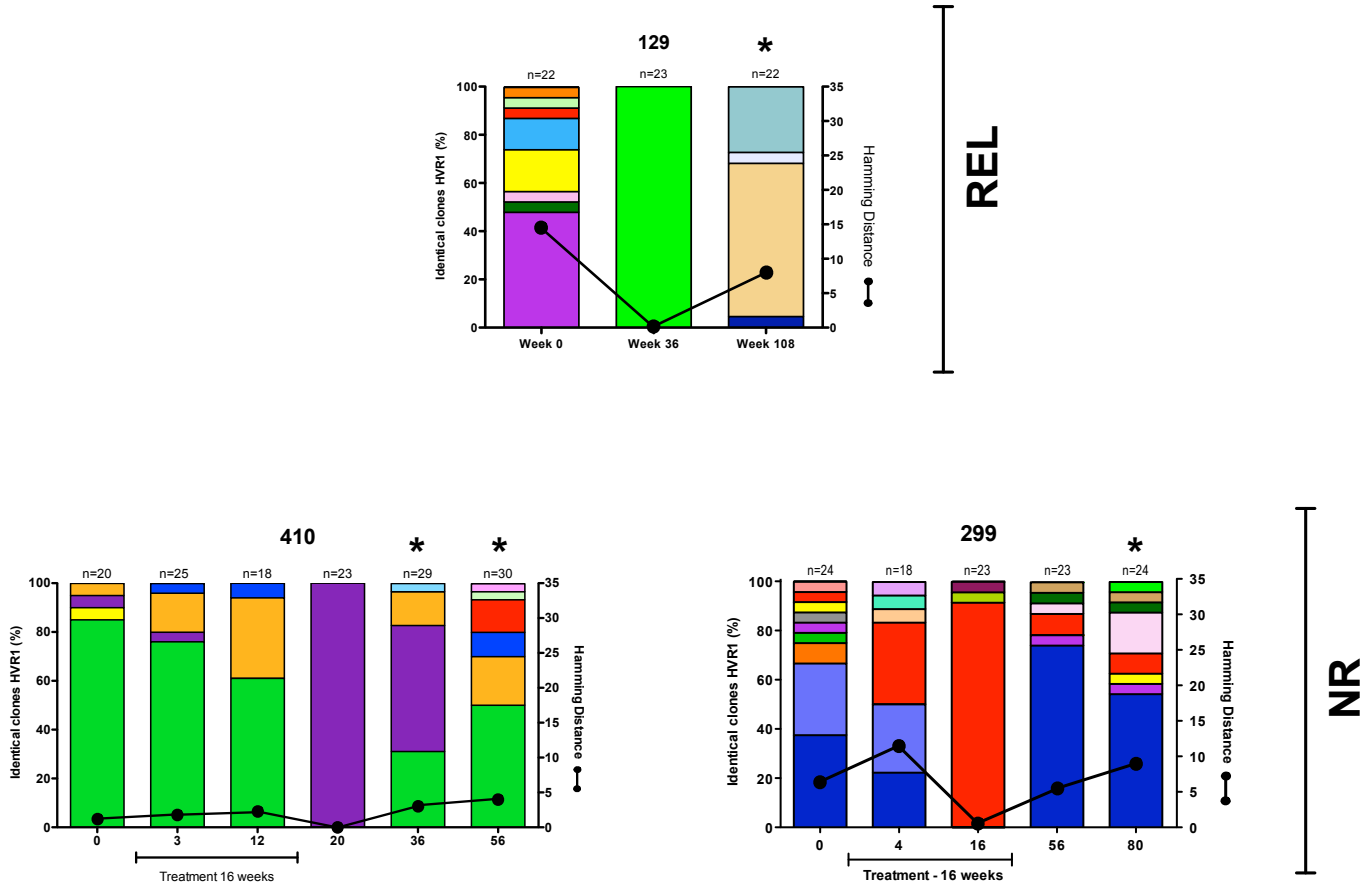


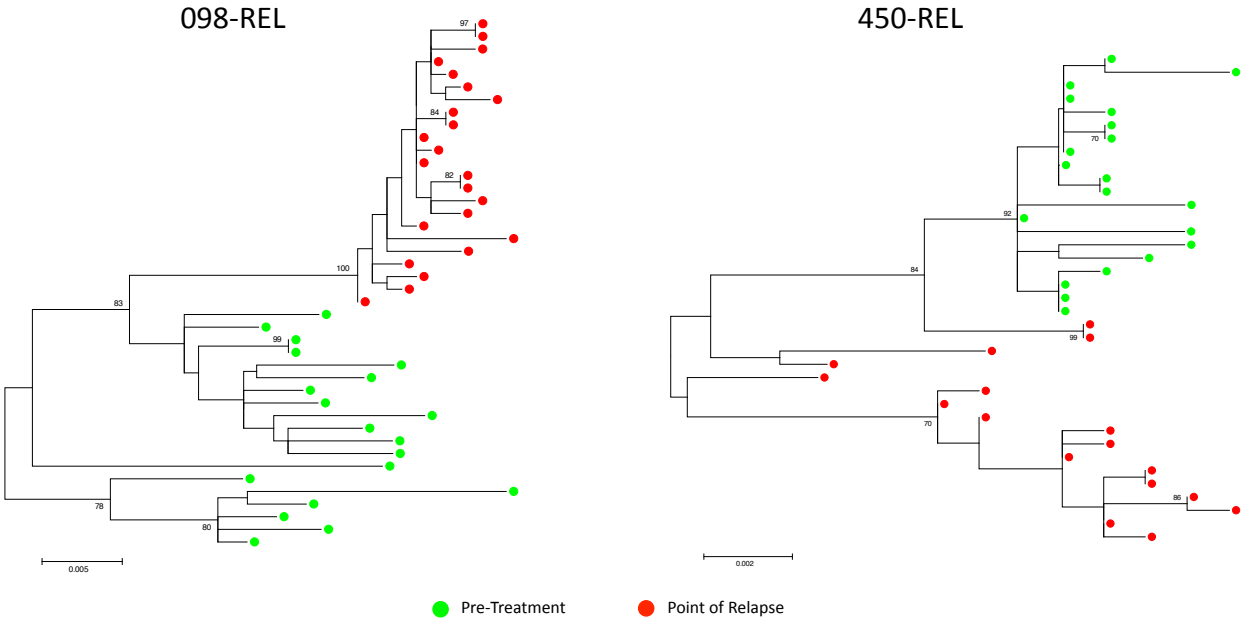
Figure 4.9 Quasispecies at post-treatment timepoints
Additional timepoints post-treatment are indicated with an asterisk (*).The percentage of viral strains at pre-treatment are shown on the left y-axis, and viral complexity measured by hamming distance is shown on the right y-axis. Viral strains are indicated by colour, however colours do not represent the same strains between patients. The number of viral strains sequenced are indicated (n). REL-relapse patient; NR-nonresponders.

4.3.8 Phylogenetic Analysis of E2 sequences

Phylogenetic analysis was performed to assess E2 quasispecies at pre-treatment, during and post-treatment in treatment naïve and previously treated patients. The treatment naïve relapse patients showed 2 patterns and representative trees are shown in Figure 4.9a. The pre-treatment and point of relapse quasispecies in three of five relapse patients formed distinct clusters. The two remaining patients also formed clusters according to time of sampling, however at least 2 point of relapse strains clustered with the pre-treatment strains, indicating they were closely related and IFN insensitive (Figure 4.10a; Patient 450-REL). This pattern was also observed in the two previously treated relapse patients (Figure 4.10b).

Two patterns were also seen in treatment naïve non-responders. Phylogenetic tree of 301-NR E2 sequences showed that the viral sequences during treatment (treatment week 4) formed a distinct cluster and are distantly related to pre-treatment sequences. The majority of post-treatment sequences are directly related to the pre-treatment sequences indicating the re-emergence of pre-treatment strains (Figure 4.10c). This pattern was also observed in the previously treated non-responder 299-NR (Figure 4.10d). The phylogenetic tree of 410-NR sequences showed that the sequences were closely related, with no clustering according to timepoint, except for the sequences detected at week 20 (4 weeks post-treatment) that formed a distinct cluster.

a) Relapse Patients



b) Previously Treated Relapse patients

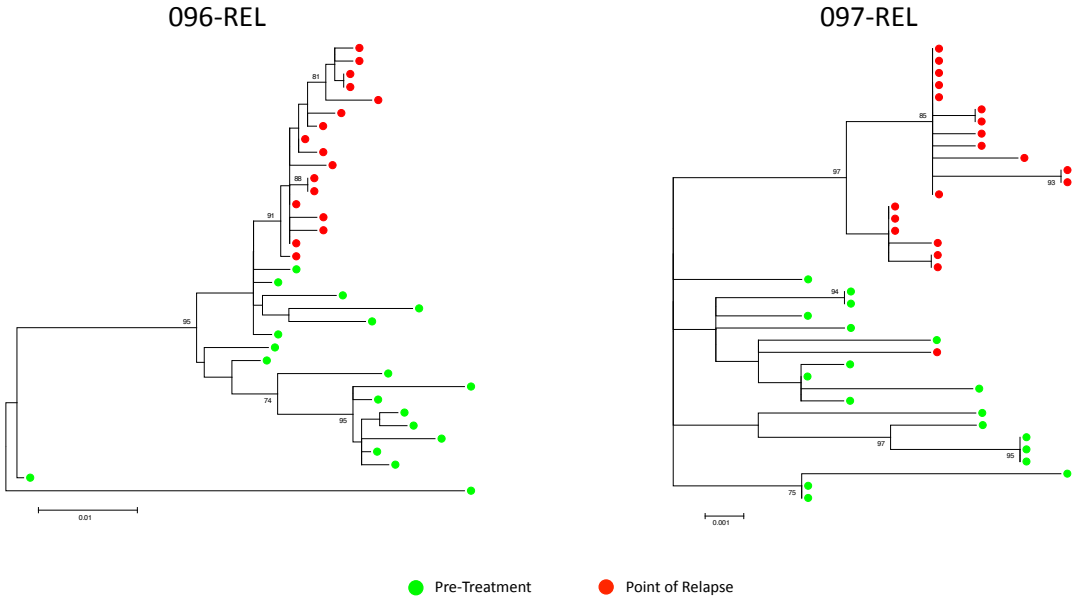
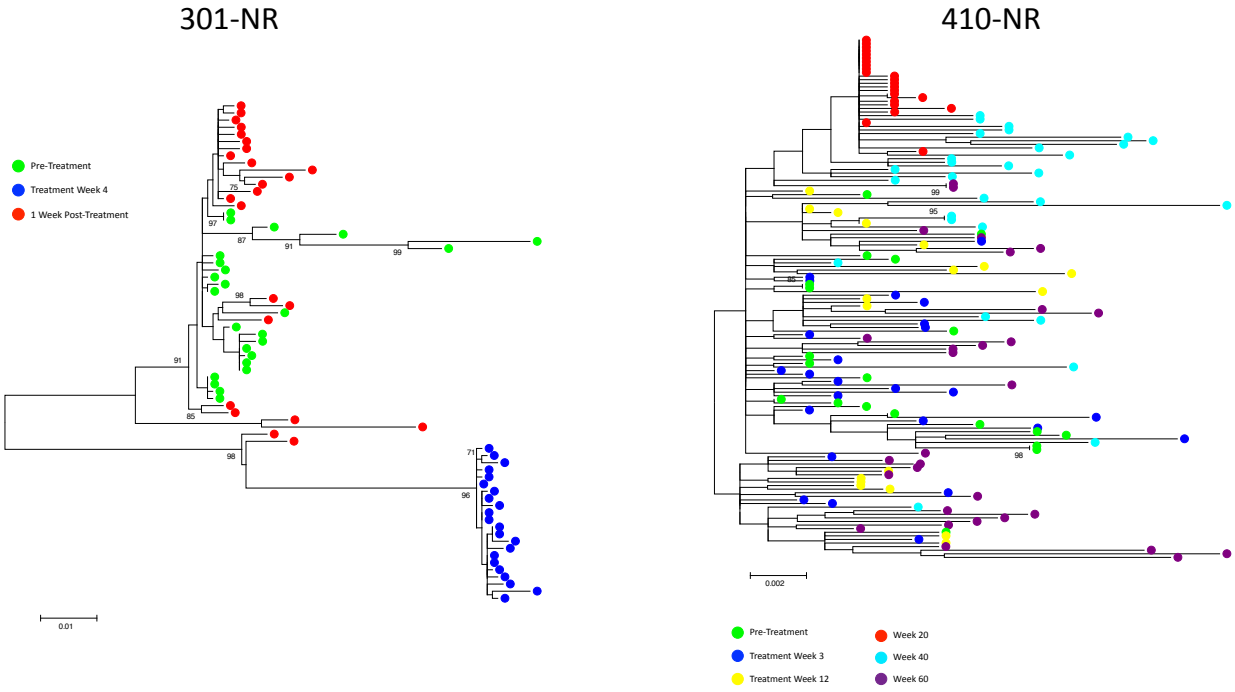


Figure 4.10 Phylogenetic analysis of E2 sequences
Phylogenetic trees using general time reversible with gamma distribution of E2 clonal sequences from all timepoints of a) representative relapse (REL) patients and b) previously treated relapse patients. Timepoints are indicated by different colours. Bootstrap values over 70% shown determined by 1000 replicates.

c) Non-responder Patients



d) Previously Treated Non-responder

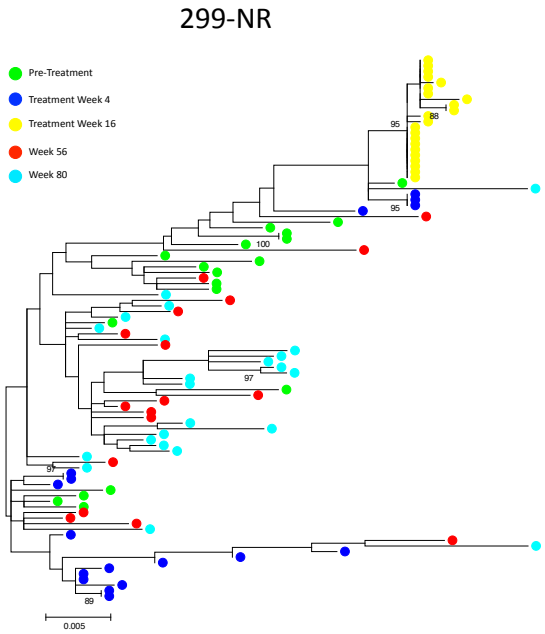


Figure 4.10 cont. Phylogenetic analysis of E2 sequences
Phylogenetic trees using general time reversible with gamma distribution of E2 clonal sequences from all timepoints of c) nonresponder (NR) patients and d) previously treated non-responder (NR) patient. Timepoints are indicated by different colours. Bootstrap values over 70% shown determined by 1000 replicates.

4.3.9 Positively Selected Sites in E2 Quasispecies

The E2 sequences of all subtype-3a patients were analysed using CODEML to identify sites throughout the entire E2 protein that were under positive selection (Table 4.2). Positively selected sites were identified by CODEML with $\omega>1.0$ and a posterior probability >95% (see Methods 2.3.3).

Positively selected sites were identified in seven subtype-3a patients - three SVR patients, two relapse patients, one non-responder and one previously treated patient (Table 4.2). There was no clear pattern of positive selection corresponding to treatment outcome (total 49, mean 7, range 1-11). Positively selected sites were identified within HVR1 and HVR495 in the majority of patients. Positively selected sites were also found within HVR575, a CD81 binding region (CD81-II), and within the remaining E2 protein, although the function of these regions in not known. Ten sites were identified in more than 1 patient (Table 4.2; indicated in italics). However these did not correspond to treatment outcome, and suggest that regions within E2 are under selective pressure regardless of treatment outcome.

Table 4.2 Amino acids under positive selection in subtype-3a patients
Table shows positively selected sites and residue selected. HVR1 amino acids 384-410; HVR495 aa 495-501; HVR575 aa 575-578e; CD81-II binding site aa 522-551.[a] Previously treated patients. Residues selected in more than one patient are shown in *italics*.

Patient	Treatment Outcome	Positively Identified Sites	Amino Acids Positively Selected				
			HVR1	HVR495	HVR575	CD81-II	Other
110	SVR	8	408 R	*495 Q* *498 K*		*546 S*	412 Q 442 M 640 F 699 V
275	SVR	7	397 R *399 L*	*501 D*		*528 N* *546 A*	414 V 622 I
442	SVR	5		*501 R*	*578a T*	*528 S*	*464 S* 474 Y
288	REL	10	384 S 394 R	*495 R*	*578 P* *578a R* *578c S* 578d T 578e H		*464 F* 741 V
428	REL	1	395 V				
301	NR	11		497 V *498 N*	577 E *578 P* *578a E* *578c G*	524 A 533 V	454 K 521 H 608 M
299	NR[a]	7	396 T *399 I* 404 S	*495 D* *498 E* *501 R*			641 T

4.3.10 HVR495, HVR575 and treatment of Chronic Infection

Treatment responsiveness has been linked to HVR1 quasispecies diversity in genotype-1 infection, and this may also be the case for HVR495 and HVR575 in subtype-3a infection. Pre-treatment E2 sequences were available from 17 chronically infected subtype-3a patients - ten SVR patients, five relapse patients and two non-responders. The consensus sequence from each patient was compared and variation within HVR495 and HVR575 was found between patients. However no specific mutations were identified in association with treatment response (Figure 4.11).

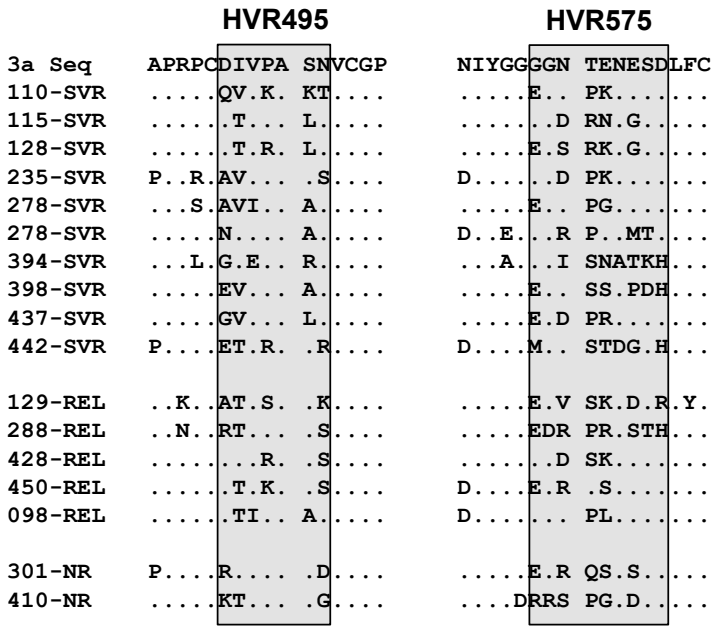


Figure 4.11 Variation within HVR495 and HVR575
Bulk or consensus sequences of HVR495 and HVR575 from pre-treatment in chronic patients. Subtype-3a consensus is shown at the top line. A dot represents a residue identical to the consensus sequence. HVR495 and HVR575 are indicated within boxes. Treatment outcome is also indicated – SVR-sustained virological response; REL- relapse patients; NR- non-responders.

Analysis of the E2 clonal sequences at pre-treatment revealed that of the 10 SVR patients (Figure 4.12a), HVR495 and HVR575 had very little or no variation in the majority of clones of eight patients. Three of five relapse patients (Figure 4.12b) had either none or very little variation. Of the remaining two patients, 129-REL had 1-2 amino acid changes within HVR575 (68% of clones), whilst patient 288-REL had 2-11 amino acid changes within both HVR495 and HVR575. At pre-treatment in non-responder patient 301-NR (Figure 4.12c), 1-2 amino acid changes were detected in HVR495 in 50% of clones, with 5-6 amino acid changes in HVR575 in only 12% of clones. Very little variation was detected in patient 410-NR. Therefore the amount of amino acid variation at pre-treatment within the clonal sequences of HVR495 and HVR575 were not associated with treatment outcome.

The effect of treatment on HVR495 and HVR575 quasispecies was also assessed in patients that failed treatment (Figure 4.13). Three of five relapse patients showed very little or no variation in HVR495 and HVR575 at the point of relapse. However 129-REL and 288-REL had dominant amino acid mutations at the point of relapse in HVR495, and both HVR495 and HVR575, respectively (Figure 4.13a). Non-responder 301-NR had dominant mutations in both HVR495 and HVR575 during treatment which were seen at post-treatment in HVR495, whilst little variation was observed in HVR575 post-treatment. Patient 410-NR had very little variation during and 1-month post treatment (Figure 4.13b). Therefore variation within HVR495 or HVR575 was not associated with treatment outcome at the clonal level.

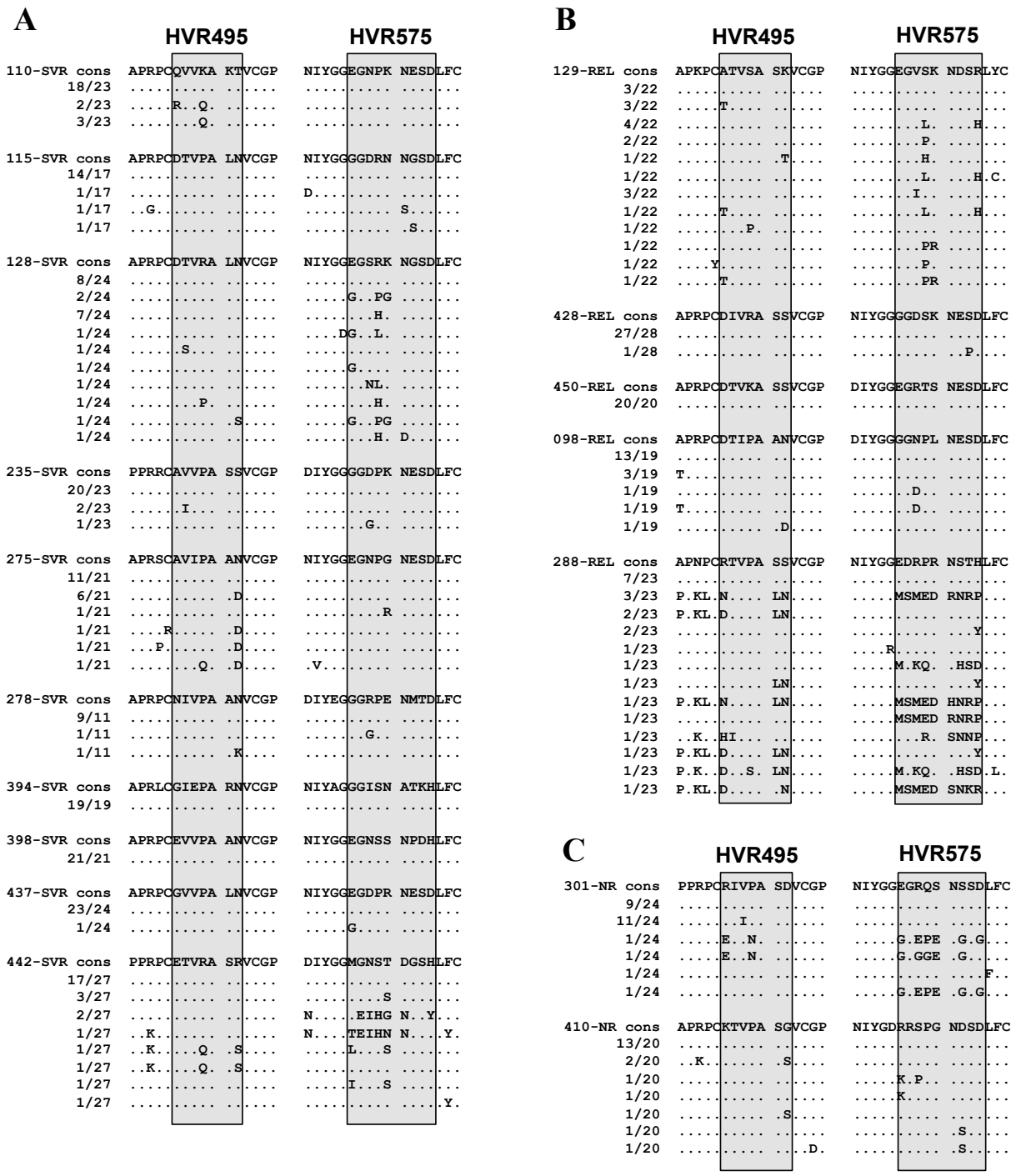


Figure 4.12 HVR495 and HVR575 quasispecies at pre-treatment
Sequence of Novel Regions HVR495 and HVR575 at pre-treatment in a) SVR patients, b) Relapse patients and c) Non-responder patients. Patient consensus sequence is shown at the top line. A dot represents a residue identical to the consensus sequence. Proportion of clonal sequences are indicated. HVR495 and HVR575 are indicated within shaded boxes.

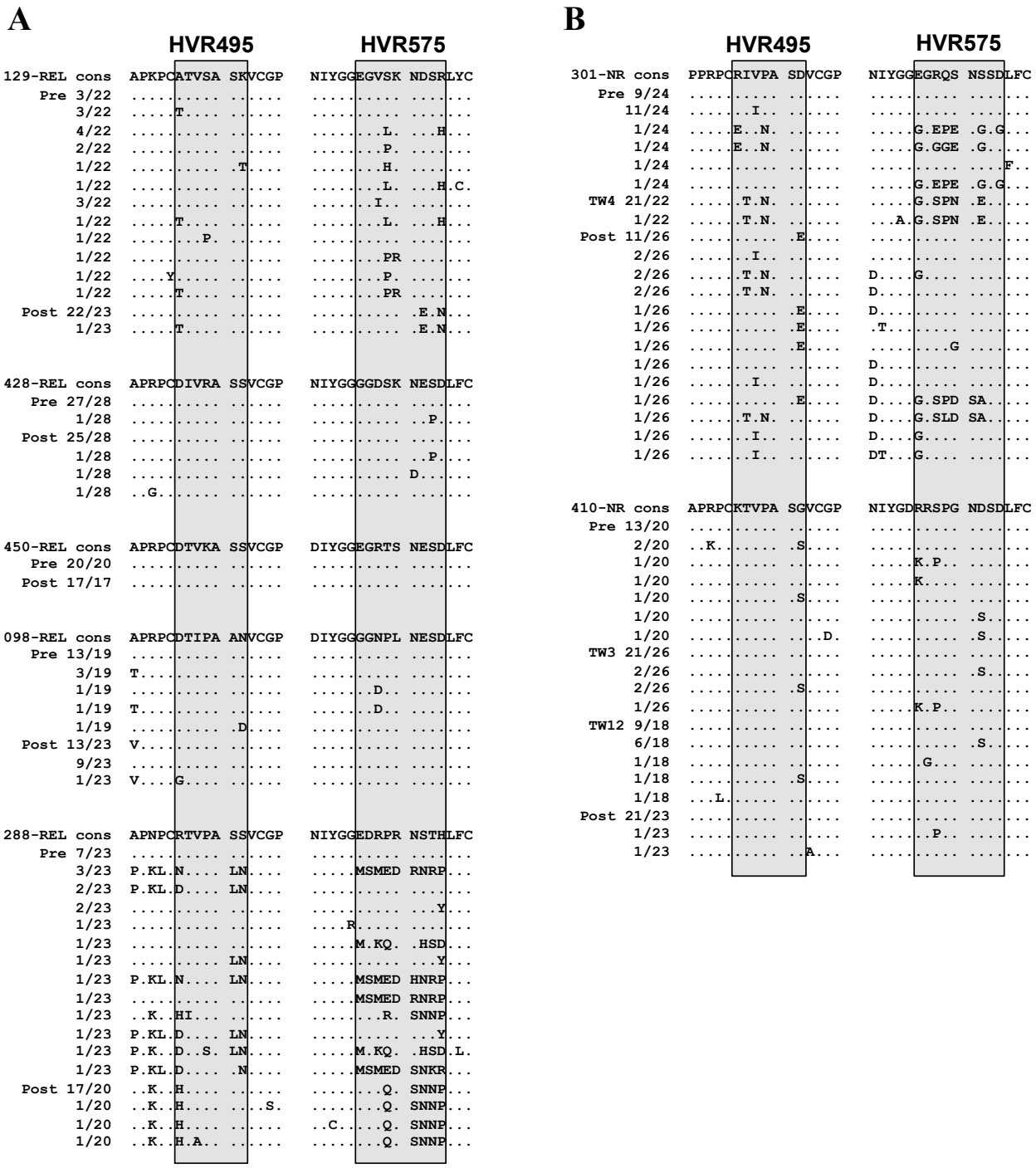


Figure 4.13 HVR495 and HVR575 quasispecies and effect of treatment
Sequence of novel Regions HVR495 and HVR575 at a) pre-treatment (Pre) and point of relapse (Post) in patients that experienced virological relapse and at b) pre-treatment, during (TW) and post-treatment (Post) in non-responder patients. Pre-treatment consensus for each patient is shown at the top line. A dot represents a residue identical to the consensus sequence. Number of clonal sequences are indicated. Novel regions are indicated within shaded boxes.

4.3.11 PePHD Sequence and Treatment Outcome

As an association was not found between HVR1, HVR495 and HVR575 quasispecies and treatment outcome, the PePHD sequence within E2 was analysed. The bulk or consensus E2 sequence of 45 subtype-3a patients was sequenced before the initiation of treatment. Of the 45 treatment naïve patients, 32 achieved SVR (71%), 11 experienced virological relapse within 6 months of cessation of treatment (REL) (24%), and two were non-responders (5%) (Figure 4.14).

Amino acid mutations were found in the viral sequences of ten SVR patients, predominately a single amino acid change at residue 662 (position 4) of the PePHD sequence. The dominant mutation was glutamine (Q) to leucine (L). Only 1 mutation was detected in the relapse patients - histidine (H) to glutamine (Q) at position 663 (position 5). This H to Q mutation at 663 was also seen in one nonresponder and two SVR patients (Figure 4.14). Amino acid mutations resulting in hydrophobic residues were seen only in SVR patients. No association between number of mutations within PePHD and treatment outcome was observed ($p=0.4956$, Fisher's exact test).

Clonal sequencing was also available for 16 of 45 patients (Figure 4.15a). None or little variation was seen in the clonal sequencing for eight of ten SVR patients. In patients 275-SVR and 394-SVR, a single mutation at residue 662 (position 4) was detected in the majority of clones (Q to L). Of the four relapse patients, three had none or very little variation within PePHD, whilst 428-REL had H to Q mutation at position 663 (position 5) in all clones (Figure 4.15). Of two nonresponder patients, only patient 301-NR had a substitution detected at position 663 (position 5) in all clones (histidine (H) to Q). The majority of substitutions detected in SVR patients occurred at amino acid 662 (position 4), Q to L, whilst in relapse and non-responder patients the majority of substitutions were seen at amino acid 663 (position 5), with H to Q mutation.

	659	670
Seq 3a PePHD	RSEQHPLLHSTT	
110-SVR	.....	
115-SVR	.....SV.....	
118-SVR	...L.....	
128-SVR	.....	
135-SVR	.....	
140-SVR	.....	
218-SVR	.....	
219-SVR	...L.....	
227-SVR	.....	
231-SVR	.....	
233-SVR	.....	
235-SVR	.....	
259-SVR	.....	
275-SVR	...L.....	
278-SVR	.....	
285-SVR	...L.....	
331-SVR	...I.....	
335-SVR	.....	
362-SVR	.....	
373-SVR	.....	
374-SVR	.....	
377-SVR	...L.....	
389-SVR	.....	
394-SVR	...L.....	
423-SVR	.....	
437-SVR	.....	
442-SVR	.....	
447-SVR	.....	
457-SVR	.....	
540-SVR	.....	
570-SVR	....Q.....	
589-SVR	....Q.....	
102-REL	.....	
129-REL	.....	
154-REL	.....	
274-REL	.....	
288-REL	.....	
294-REL	.....	
332-REL	.....	
428-REL	....Q.....	
450-REL	.....	
579-REL	.....	
582-REL	.....	
410-NR	.....	
301-NR	....Q.....	
Geno1 PePHD	...LS...LT..	

Figure 4.14 Pre-treatment PePHD sequences
Bulk or consensus sequences of PePHD patients at pre-treatment compared with subtype-3a PePHD sequence. The consensus sequence is given in the top line. A dot indicates that the residue is identical to the consensus amino-acid. Treatment outcome is indicated – SVR-sustained virological responder; REL-experienced virological relapse; NR-nonresponder. Genotype-1 sequence is also shown.

The effect of treatment on the PePHD sequence was also assessed by clonal sequencing of E2, during and post-treatment in those that failed to clear the virus (Figure 4.15b). For the majority of relapse and nonresponder patients, the PePHD sequence did not alter from pre- to post-treatment, which is consistent with that found by Sarrazin *et al*. (Aghemo et al., 2009). However 288-REL had a dominant Q to L mutation at position 662 (position 4) at post-treatment that was not detected at pre-treatment. This mutation is the most common mutation detected in SVR patients.

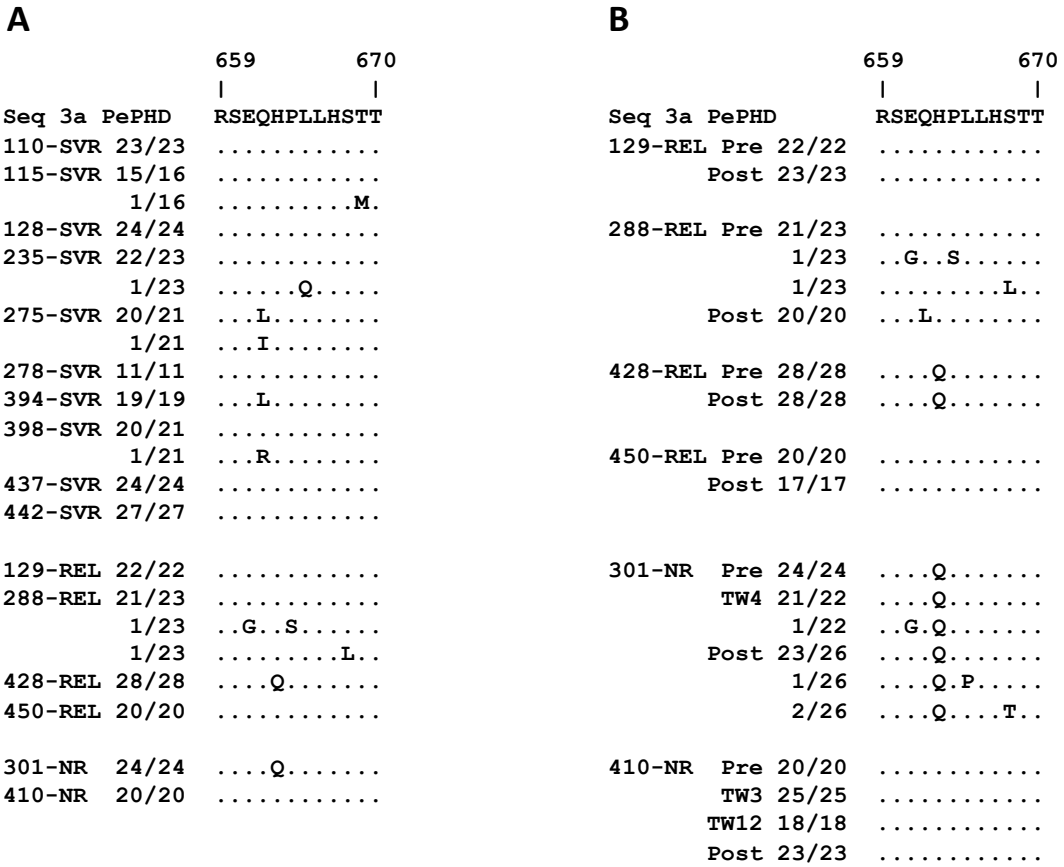


Figure 4.15 PePHD quasispecies
Clonal PePHD sequences at pre-treatment (A), and at pre-, during and post-treatment in relapse and nonresponder patients (B). Sequences are compared to subtype-3a PePHD sequence. A dot indicates that an amino-acid residue identical to the consensus residue. Treatment outcome is indicated – SVR-sustained virological responder; REL-virological relapse patient; NR-nonresponder.

4.3.12 Insertion of amino acids in within E2 sequence

E2 sequencing revealed amino acid insertions and deletions in four subtype-3a patients (Figure 4.16). Two relapse patients, 129-REL and 288-REL, and an untreated patient 420 had amino acid insertions within the E2 sequence. Patient 129-REL had a 4 amino acid insertion at the C-terminal of HVR1 at the point of relapse (Post1) that was also detected at 108 weeks (Post2) (Figure 4.16a). The sequence of the insertion was conserved only at the point of relapse. CODEML analysis did not identify any of the inserted amino acids as under positive selection. Untreated patient 420 had a 2 amino acid insertion at the N'terminal of HVR1 that was detected in all clones and at both timepoints. Neither amino acid was under positive selection. Relapse patient 288-REL had 2 amino acid insertions within HVR575 in all clones and at both timepoints, and conserved at point of relapse only (Figure 4.16b). CODEML analysis revealed that both amino acids of the insertion were under positive selection. An amino acid deletion was also observed in untreated patient 411. The deletion of residue 480a (an insertion relative to genotype-1 sequence) was observed in all clones at both timepoints.

Insertions and deletions are uncommon in HCV infection. However the significance of insertions and deletions within E2 with regards to protein structure, antigenicity and treatment response are unknown.

a) Patient 129-REL

```
                     384        394        404        410
                     |          |          |          |
Geno 3a HVR1 Seq     TTYITGGSAA RSASGFTSLF S----VGAKQ N
129-REL  Pre 11/22   G.HV...... FTTR..AN.. R----...S. K
              4/22   E.HV....V. QAT...A... R----...S. .
              3/22   E.HV....V. .AT...A... R----...S. .
              1/22   G.HV...... FTTR..A... R----...S. K
              1/22   E.HV....V. QTTR..AG.. R----...S. K
              1/22   E.HV....V. QAT.R.A... R----...S. .
              1/22   E.HV....V. .AT...A... K----...S. .

129-REL Post1 23/23 E.RV...TE. .A.Q.L..F. DRALFR.... .

129-REL Post2 14/22 E.RV...TE. .A.Q.L..V. GRAFSR.... .
               6/22 E.RV...TE. .A.Q.L..FL DIAFVR.... .
               1/22 E.RV...TE. .A.Q.L.GF. GRALSI.... .
               1/22 E.R-...TE. .A.Q.L..V. GRAFSR.... .
```

b) Patient 288-REL

```
                     570      578a   579
                     |        |       |
  Geno3a E2 Seq      NIYGGGGNTE NES--DLFCP TDC
  288-REL Pre  9/23  .....EDRPR .STSPH.... ...
               4/23  .....MSMED RNRPKP.... ...
               2/23  .....EDRPR .STNPY.... ...
               2/23  .....EDRPR .STRPY.... ...
               1/23  ....REDRPR .STSPH.... ...
               1/23  .....EDRRR SNNSSP.... ...
               1/23  .....MDKQR .H.ER..... A..
               1/23  .....MDKQR .H.AQ..L.. ...
               1/23  .....MSMED HNRPKP.... ...
               1/23  .....MSMED SNKSKR.... ...

  288-REL Post 19/20 .....EDRQR SNNSSP.... ...
                1/20 ..C..EDRQR SNNSSP.... ...
```

Figure 4.16 Amino acid insertion in E2 clonal sequences
Amino acid sequence from each timepoint are shown and compared with subtype-3a consensus sequence. Amino acid residue identical to consensus is shown as a dot. Inserted amino acids are shown in red. Number of clones are indicated.

4.4 Discussion

Treatment of HCV infection is effective in up to two thirds of subtype-3a and only half of genotype-1 infected patients (Sarrazin et al., 2000). The reason(s) for the better treatment response of subtype-3a infection is not known. However an ability to predict patients that would respond to treatment would be invaluable.

The quasispecies nature of HCV enables the virus to establish chronic infection by evolving within the host to obtain its optimal fitness, and facilitating the fast adjustment to any variation in environmental conditions (Fried et al., 2002, Martell et al., 1992). A low pre-treatment HCV complexity is associated with a better response to treatment in genotype-1 infection (Marrone and Sallie, 1996, Salmeron et al., 2006, Torres-Puente et al., 2008). Previous studies of viral diversity and complexity with treatment have focused on genotype-1 infection, with only a single study exclusively assessing subtype-3a patients which found an association between low pre-treatment viral complexity and successful treatment outcome (Abbate et al., 2004).

Subtype-3a Quasispecies Analysis

Quasispecies diversity and complexity were assessed in subtype-3a patients, to address the hypothesis that in subtype-3a infection a lower quasispecies diversity and complexity is found at pre-treatment in patients that successfully clear virus, as seen by Moreau *et al.* (Moreau et al., 2008). However I did not such an association between pre-treatment viral diversity and complexity, and treatment outcome. Low viral diversity and complexity were observed in patients that successfully cleared and those that failed therapy, in contrast to that found by Moreau *et al.* and in genotype-1 infection (Moreau et al., 2008, Torres-Puente et al., 2008, Abbate et al., 2004, Salmeron et al., 2006). The lack of an association in my subtype-3a cohort was also supported by phylogenetic analysis of the

E2 viral sequences at pre-treatment that did not show clustering according to a specific treatment outcome, as previously observed in genotype-1 infection (Moreau et al., 2008).

To address the hypothesis that subtype-3a quasispecies behave similarly to quasispecies in genotype-1 infection, where a pre-treatment IFN resistant strain persists during treatment in non-responder patients (Farci et al., 2002, Sookoian et al., 2001, Pawlotsky et al., 1999, Abbate et al., 2004), I assessed quasispecies diversity during and post-treatment in two non-responder subtype-3a patients. I observed different patterns of quasispecies changes.

The first pattern observed was the emergence of an IFN insensitive strain during treatment, with the re-emergence of pre-treatment strains after cessation of treatment. This is in contrast to previous studies (Moreau et al., 2008, Pawlotsky et al., 1999, Abbate et al., 2004, Farci et al., 2002). This suggests that either an insensitive strain was selected during treatment or was previously present within the population at undetectable levels. The second pattern I observed was the presence of an insensitive pre-treatment strain that remained during treatment, consistent with genotype-1 infection and subtype-3a study by Moreau *et al.* (Moreau et al., 2008, Pawlotsky et al., 1999, Abbate et al., 2004, Farci et al., 2002).

In both patterns, strains detected at pre-treatment re-emerged after cessation of treatment, suggesting their suppression to undetectable levels during treatment, possibly due to lower inherent IFN sensitivity. Alternatively these strains may have been retained within other sites such as the liver, where IFN was less effective. In addition, their re-emergence within the population suggests an inherent greater replicative capacity enabling them to dominate within the quasispecies.

The effect of treatment on virus levels was assessed through viral loads. Viral load was expressed in RNA copies/ml and are unfortunately not directly comparable with international units that are usually expressed for viral loads (Moreau et al., 2008). Therefore viral load data in this study provides additional information to obtain a greater understanding of the changing quasispecies population during treatment. The viral load levels for two non-responder patients were lower than that in the third non-responder patient. This may have been due to an error of the assay used, which involved many dilution steps, and relies on the integrity of the RNA samples (Strader et al., 2004).

No specific quasispecies pattern was observed in subtype-3a non-responder patients. Further assessment in non-responder patients is required to elucidate the behaviour of subtype-3a quasispecies and treatment non-response.

The effect of treatment was also assessed in subtype-3a patients that experienced virological relapse, to address the hypothesis that IFN sensitive strains are present before treatment with the emergence of an IFN insensitive strain at the point of relapse.

I observed two quasispecies patterns in relapse patients. Interferon insensitive strains were detected at both pre-treatment and point of relapse, in contrast to a previous study by Abbate *et al.* in genotype-1 infection (Lauer and Walker, 2001). This suggests that during treatment, IFN insensitive strains were suppressed to undetectable levels, or that viral suppression by treatment occurred only transiently (Abbate et al., 2004). The second pattern observed was that IFN insensitive strains were detected only at point of relapse, possibly due to their selection during treatment, as previously seen in genotype-1 infection (Farci et al., 2002). Alternatively these strains may have been present before treatment at undetectable levels or contained within sites that were less accessible to IFN. As a clear dominant viral strain was observed at the point of relapse it suggests that these

strains were the first to emerge possibly due to higher replicative fitness or simply by chance.

Previous courses of treatment may alter the quasispecies population, possibly selecting an IFN insensitive strain that may render further treatment ineffective. In previously treated patients an IFN insensitive viral strain was present before commencement of second course of treatment. It is unclear whether IFN insensitive strains were selected during the first treatment regimen and hence further treatment was ineffective, or if these strains were present before the first course of treatment rendering all courses of treatment ineffective.

Assessing viral quasispecies provides information on the fluctuating quasispecies population during treatment. However detection of viral strains during treatment is not a definitive measure of resistance and therefore can only indicate whether viral strains are sensitive or not to interferon. The lack of correlation between quasispecies diversity within plasma and treatment outcome, may be due differences between quasispecies contained within the liver and plasma. This raises an important question regarding the significance of quasispecies analysis within plasma, as it has been demonstrated that quasispecies detected within the blood do not correlate with the quasispecies population within the liver, as found by Cabot *et al.* and Maggi *et al.* (Abbate et al., 2004, Cabot et al., 1997). Nevertheless assessing quasispecies within the plasma are vital, and may also represent the changes in viral quasispecies within the liver. The requirement for a liver biopsy is specific for each individual, multiple biopsies are not standard and are not required before treatment in the majority of HCV infected patients due to potential risks involved with the procedure (Maggi et al., 1997, Strader et al., 2004). Therefore studies

of viral quasispecies in the blood are valuable in obtaining a greater understanding of the quasispecies population and treatment effect in subtype-3a infection.

The continuous change in viral quasispecies in the absence of therapy suggests that treatment-induced changes in the quasispecies population are not maintained in all patients and that variation may represent continuous replication and varying strains within the liver (Lauer and Walker, 2001, McKechnie et al., 2000). Alternatively, variation may reflect host immune pressure. Therefore changes in the HVR1 sequence may not only reflect specific characteristics of the viral strain such as resistance but may also reflect pressure from the immune response, as HVR1 has been shown to continuously evolve to escape the antibody response in chronic HCV infection (Moreau et al., 2008). It is also possible that changes within quasispecies during natural infection occur at varying rates within different hosts, possibly due to the host immune response. Therefore longitudinal analyses, consisting of samples obtained at regular intervals are needed to understand the nature of quasispecies during natural chronic infection.

The HCV E2 protein is targeted by both adaptive and humoral immune responses, therefore subtype-3a clonal E2 sequences were assessed to determine whether the host immune response exerts selection pressure on the entire E2 protein (von Hahn et al., 2007, Tsai et al., 1998, Farci et al., 1994, Shimizu et al., 1994). Positive selection was detected throughout the E2 protein indicating that it is targeted by the immune system in chronic subtype-3a infection. However the number and location of the selected sites within subtype-3a patients undergoing treatment did not correspond with treatment outcome, indicating that treatment does not exert specific selective pressure on the E2 protein nor induce a host immune response targeting a specific E2 region.

HVR495, HVR575 and Treatment Outcome

Residues within HVR495 and HVR575 were identified as being under positive selection. However sequence variation within these sites did not correspond to treatment outcome, suggesting that HVR495 and HVR575 are not involved in the HCV response to treatment. The function of these regions remains unknown and further studies are needed to determine the significance of these unique subtype-3a regions.

PePHD in Subtype-3a Infection

As a correlation was not found between quasispecies diversity and treatment outcome of subtype-3a patients, the PePHD sequence of the E2 protein was assessed.

PKR is an important protein involved in host signalling pathways in response to viral infections. Genotype-1 E2 protein inhibits host PKR protein through the PePHD sequence. However mutations within genotype-2 and -3 PePHD sequences have only a weak inhibitory effect against PKR activity (Del Porto et al., 2000). However an association between PePHD sequence and treatment response remains controversial (Polyak et al., 2000, Berg et al., 2000, Sarrazin et al., 2001, Gerotto et al., 2000, Abid et al., 2000, Puig-Basagoiti et al., 2001). I did not find an association between PePHD sequences and response to treatment, as very few mutations were observed within the PePHD sequence at both consensus and quasispecies level, and before or during treatment as previously observed (Taylor et al., 1999, Squadrito et al., 1997, Cochrane et al., 2000, Sarrazin et al., 2000, Puig-Basagoiti et al., 2001). The lack of sequence mutations may indicate possible functional or structural limitations of this region (Afzal et al., 2010, Polyak et al., 2000, Chayama et al., 2000).

Hydrophobic mutations were only in SVR patients - leucine at position 4, as previously found (Sarrazin et al., 2000). However genotype-1 PePHD also contains leucine at

position 4. The significance of a subtype-3a mutation to generate a sequence more similar to genotype-1 PePHD is unclear, although may suggest that residues at this position do not play a role in PePHD binding. The majority of substitutions in SVR patients occurred at position 4, compared with position 5 in nonSVR patients. And may suggest different pressures and functional restraints within different treatment groups, or that neither positions are essential for PePHD binding, as found for positions 2 and 3 of genotype-1 PePHD (Sarrazin et al., 2000). Despite the lack of correlation between PePHD and treatment outcome in both genotype-1 and subtype-3a infection, the *in vitro* inhibitory effect of E2 PePHD of different genotypes suggests that this region is involved in HCV resistance but may not be the exclusive mechanism (Chayama et al., 2000).

Amino Acid Insertions Within Subtype-3a E2

E2 sequencing of subtype-3a patients identified amino acid insertions and deletions in four patients. Two and 4-amino acid insertions were observed at the N- and C-terminal of HVR1, respectively. The majority of previous studies have detected amino acid insertions at the N-terminal of HVR1, varying in length from 1-4 amino acids (Berg et al., 2000, Torres-Puente et al., 2007, Gerotto et al., 2001), with insertions also detected at other regions of E2 such as HVR2 of genotype-1 (Aizaki et al., 1996). A 2-amino acid insertion was also observed within HVR575. The insertions and deletions did not differ in frequency within the quasispecies population, and were detected at subsequent timepoints, suggesting they were not deleterious to the virus, in contrast to those detected in genotype-1 infection (Torres-Puente et al., 2007). Insertions were only detected in patients that failed treatment, in contrast to genotype-1 infection where insertions were detected in both SVR and nonSVR patients (Torres-Puente et al., 2007). The size of the HVR1 is conserved across genotypes 1-5, suggesting functional restrictions of this region

(Torres-Puente et al., 2007). Insertions are rare, as they may affect HVR1 binding ability to cellular receptors, tissue tropism and antigenicity (Chamberlain et al., 1997, Torres-Puente et al., 2007). As the function of HVR575 is not known, it is unclear whether insertions within this region affect its potential function and also the overall structure of the E2 protein.

IL-28B Status

At the time of this study, the importance of IL-28B status was not known, and therefore not available for this group of patients. IL-28B status is associated with treatment outcome in genotype-1 infection (Sobolev, 2000, Ge et al., 2009). However as the association of IL28B status in genotype-3 infection is not clear, and other factors such as quasispecies complexity should continue to be assessed.

In Summary

The treatment outcome of subtype-3a infection cannot be predicted by assessing the quasispecies diversity and complexity of HVR1, HVR495 nor HVR575 regions before initiation of treatment. Various patterns of quasispecies diversity were observed during treatment, which were not specific to a particular treatment outcome. IFN insensitive strains were detected in some but not all patients that failed treatment indicating that IFN treatment does not select an insensitive or resistant strain in all cases. The PePHD region was not associated with treatment outcome and did not vary during treatment. Further analyses are required to determine additional viral factors that are responsible for the favourable treatment outcome of subtype-3a infection.

Chapter 5 - Distinct Specificity and effects of therapy on HCV subtype-3a specific T-cell immunity

Abstract

HCV subtype-3a infection is now dominant in newly diagnosed infections within the UK. A characteristic feature is a favourable response to therapy; the reasons for this are unknown but may include distinct subtype-3a specific T-cell immunity. However, T-cell immunity to this subtype is poorly defined. In contrast immune responses to genotype-1 have been comprehensively assessed. My aim is to a) define frequency, specificity and cross reactivity of T-cell immunity across the whole viral genome in subtype-3a infection and b) assess the impact of IFN-α/ribavirin on T-cell immunity. I analysed T-cell responses by IFN-γ ELISpot in treatment-naïve, chronically infected subtype-3a and genotype-1 patients using specific peptide panels. T-cell responses were followed longitudinally in a subset of 21 subtype-3a infected patients receiving therapy. CD8+ T-cell responses targeted non-structural proteins in subtype-3a infection, whereas in genotype-1 infection CD4+ responses predominantly targeted HCV core with CD8+ T-cell responses rarely detected ($p=0.019$). Paradoxically, a sustained response to therapy was associated with a brisk decline in HCV-specific and non-specific T-cell responses and total lymphocyte counts that recovered after treatment, and therefore the data do not support the theory that subtype-3a viral clearance is associated with a further enhanced antiviral T-cell response. In contrast, a reduction of these responses may serve as a biomarker of interferon responsiveness.

5.1 Background

HCV is a globally distributed pathogen that currently infects 3% of the worlds population (Suppiah et al., 2009). Over the last one hundred years a small number of distinct strains, in particular HCV subtypes-1a, -1b and -3a, have become more globally distributed in an epidemic that is associated with medical practice and IVDU (World Health Organisation, 1999).

Within the UK, approximately 0.5% of the population are currently infected with HCV and the predominant strain in newly diagnosed infections is now subtype-3a (Pybus et al., 2001). This subtype is also endemic in parts of Asia, Western Europe and common (5-10%) in the United States of America (US). However in the US genotype-1 remains the dominant strain (HPA, 2009).

The classification of HCV by viral genotype has proven to be highly informative, not only in terms of the assessment of global viral evolution and epidemiology, but also in predicting the response to IFN based treatment regimens. Large randomised clinical studies have consistently shown subtype-3a has a more favourable treatment outcome than genotype-1 infection (World Health Organisation, 1999). The reason for this is not known, but may relate directly to genotype specific viral sequence with a differential capacity to subvert the direct anti-viral effects of interferon (Fried et al., 2002). Alternatively, as genotypes-1 and -3 share limited sequence homology, together with the immunomodulatory affects of IFN , differential treatment outcomes may relate to the effect of therapy on genotype specific T-cell function or a distinct genotype specific T-cell repertoire in infected hosts - a hypothesis that is currently unexplored.

Whilst genotype-1 HCV-specific T-cell response has been extensively evaluated, very little is known about the nature of the T-cell responses that target other HCV genotypes. Whilst some studies of HCV-specific T-cell responses have included patients with

genotypes-2 and -3 infection (Diepolder, 2004), interpretation of these studies is confounded by the fact that the assessment has relied almost entirely on genotype-1 peptides that clearly do not represent the autologous circulating virus. To date, analysis of subtype-3a specific T-cell responses using subtype-3a peptides is confined to a single study that assessed responses to the NS3 protein (Hultgren et al., 2004).

A cross-reactive T-cell response would be beneficial in protecting a host, as a T-cell response to one genotype would also be protective against infection by other genotypes. Additionally identifying cross-reactive T-cell epitopes would be invaluable for vaccine design. However recent studies assessing HLA driven viral diversity between genotypes-1 and -3 has shown that there is likely to be limited T-cell cross-reactivity between these genotypes (Giugliano et al., 2009, Rauch et al., 2009).

A detailed analysis of subtype-3a specific T-cell immunity across the entire genome has not previously been performed and is clearly important for both vaccine development, and to further understand why IFN treatment outcomes dependent on genotype. Since few HCV subtype-3a viral sequences are currently available within the major HCV databases, full-length viral sequencing was first performed in a subtype-3a infected cohort in order to define a robust and relevant consensus sequence, from which a corresponding set of overlapping peptides were designed for T-cell analysis. Subtype-3a specific T-cell responses were then assessed in both chronic infection and during combination therapy, in association with autologous viral sequence.

5.2 Chapter Specific Materials and Methods

5.2.1 Full-length subtype-3a genomic sequencing to design a subtype-3a peptide set and sequencing of autologous virus

Full-length (aa 1-2929) viral sequencing was performed on 20 treatment naïve subtype-3a patients with chronic infection (John Radcliffe Hospital, Oxford). PCR fragments were gel purified (Qiagen) and sequenced bidirectionally using Prism Big Dye (Applied Biosystems) on an ABI 3100 DNA automated sequencer. Sequences were edited using X11 software. Accession numbers: GQ356200-GQ356215, GQ356217, JF509175-JF509177. A consensus sequence was determined in order to derive a subtype-3a peptide set (15-19 aa in length, overlapping by 11 aa, n=460) for T-cell analysis (Mimotopes, Australia). Subtype-1b J4 overlapping peptides (15-19aa) were obtained from BEI resources (http://www.beiresources.org/) (see Methods 2.5.1).

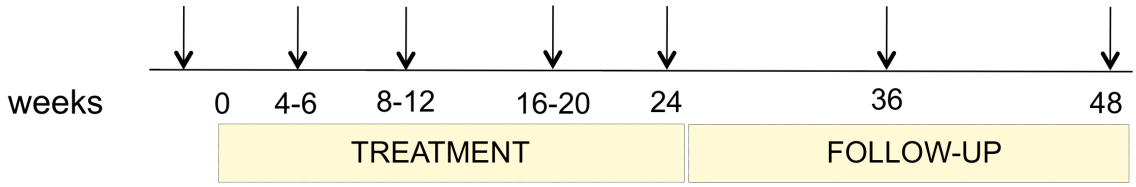
5.2.2 Diversity of subtype-3a full-length sequence

Phylogenetic tree models of evolution were assessed using JModelTest (http://www.beiresources.org/). The best-fit tree General time reversible was selected based on lowest likelihood score and AIC correction analysis. Trees were constructed using MEGA5 program (Posada, 2008) and bootstrap scores calculated based on 1000 replicates. Phylogenetic analysis included 8 patients followed longitudinally through combination therapy, in addition to subtype-3a reference sequence (D28917), and an out-group of H77 subtype-1a HCV nucleotide sequence (AF009606). Sequences were aligned using the ClustalX version 2.0.12 program.

5.2.3 Clinical cohort

Sixty-seven treatment naïve patients (36 genotype-1 – 16 subtype-1a, 12 subtype-1b, 6 genotype-1 (subtype not determined), 2 subtype-1a/b (subtype not determined) and 31 subtype-3a) were recruited to the study (John Radcliffe Hospital, Oxford, UK and Barts and the London NHS Trust, London, UK). A subset of 22 subtype-3a patients was further assessed immediately pre-treatment, during and after therapy (peg-IFN-α2b, 180μg/week and ribavirin 800-1200mg/day dependent on body weight given for 24 weeks). Ten healthy subjects were recruited in order to define a positive cut off for the subtype-3a ELISpot assay. Local ethical approval was obtained and all patients gave written informed consent for study participation. Treatment response is defined using standard definitions (see Methods 2.1.2) (Tamura et al., 2011). Genotype-1 ELISpots were done by Anthony Brown.

PBMC Sampling



5.2.4 ELISpot Assays and lymphocyte counts

PBMC were isolated from blood by density gradient centrifugation and frozen immediately before treatment, at multiple time points during and following treatment. Frozen PBMC enabled the concurrent assessment of T-cell responses sampled at different time points. Thawed PBMC were tested by IFN-γ ELISpot assays, as previously

described (See Methods 2.4.4) with peptide pools Core-NS5b II (3μg/ml), CMV lysate (0.05μg/ml), FEC CD8+ epitopes (3μg/ml). To define a cut-off, frozen and fresh PBMC from healthy volunteers (HCV negative) were used. For the subtype-3a ELISpot, a positive cut-off of 41 SFU/10^6 PBMC was defined in healthy volunteers using (mean of HCV specific responses SFU/10^6 PBMC - negative control wells) + 3 x standard deviation (SD). Under this cut-off, only 1 response in a single volunteer reached the cut-off. For genotype-1 a cut-off of 43 SFU/10^6 off was previously defined in healthy volunteers (unpublished) using an identical strategy.

To identify individual peptide responses, the above ELISpot cut-off was not applied. If a peptide had been identified with a positive ELISpot response, then the optimal peptide was used in further assays. Cross-reactivity was defined as a detectable T-cell response to both genotype-3a and equivalent subtype-1a and/or subtype-1b peptides.

Total lymphocyte counts were assessed from 200μL of blood pre-treatment and at treatment week 12 using the Sysmex Automated Hematology Analyzer XE-2100 (Sysmex Corporation).

5.2.5 Statistical Analysis

T-cells targeting structural and non-structural viral genomic regions in genotypes-1 and -3 HCV infected patients were assessed using Fisher's exact test. The magnitude of T-cell responses pre-treatment in relation to treatment outcome was assessed by Mann-Whitney test, and lymphocyte counts were assessed by un-paired two-tailed t-test. Comparisons between T-cell responses over time assessed using paired t-test. A p value <0.05 was considered significant.

5.3 Results

5.3.1 T-cells Responses in Chronic HCV Infection

In chronic genotype-1 HCV infection it has previously been shown that HCV specific CD8+ T-cell responses are rarely detected *ex-vivo*, though CD4+ T-cells that target multiple epitopes within core are frequently observed (Fried et al., 2002, Lechner et al., 2000c, Fleming et al., 2010, Semmo et al., 2005b, Harcourt et al., 2006, Semmo et al., 2007). In the Oxford genotype-1 patients *ex-vivo* T-cell responses targeting core were frequently detected (18/36 patients), whilst weak responses targeting the non-structural proteins could be seen in only 3/36 patients. In contrast, T-cells targeting the non-structural proteins were readily detectable in treatment naïve chronic subtype-3a infection (Figure 5.1). Whilst the total mean magnitudes of the HCV specific T-cell responses did not differ significantly between genotypes-1 and -3 (mean +/- SD; 103.9 +/- 156.6 genotype-1, vs. 192.5 +/- 642.2 genotype-3 SFU/10^6 PBMC), T-cells targeting the non-structural region were significantly more common in genotype-3 infection (3/36 geno-1 vs. 12/31 geno-3, p=0.0195) (Figure 5.1c).

5.3.2 Assessment of subtype-3a viral diversity

To ensure that the local Oxford cohort did not represent a single out-break and to account for the readily detectable CD8+ T-cell responses using peptides based on a consensus sequence (Figure 5.2a), viral diversity was assessed in the subtype-3a cohort using full-length HCV sequences. Phylogenetic analysis showed significant diversity within subtype-3a sequences used to generate the peptide set, and that a subtype-3a reference strain (Accession number D28917) fell within the Oxford subtype-3a cluster. An entropy map showed that significant viral variation was observed throughout the viral genome,

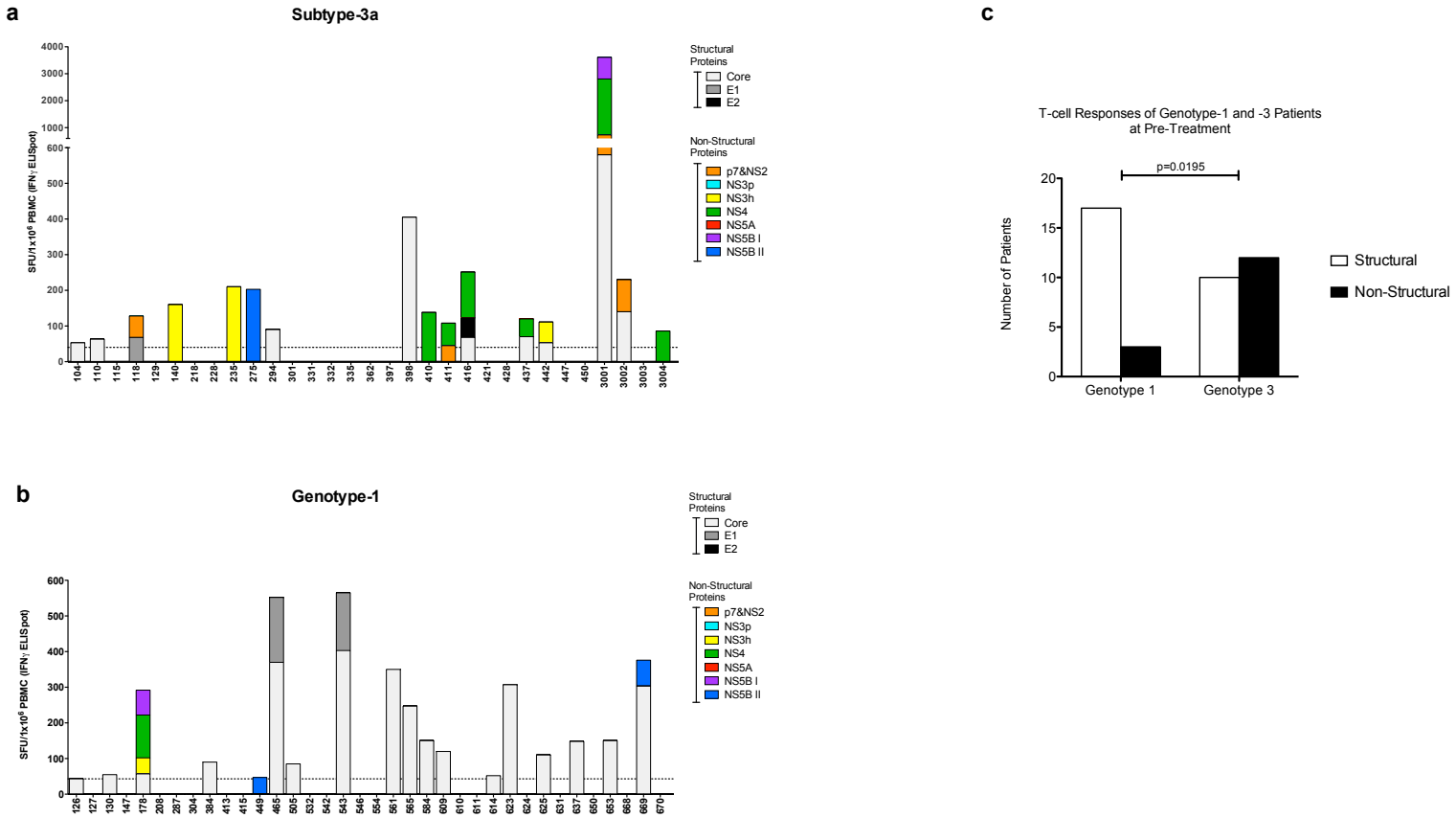


Figure 5.1 HCV-specific IFN-γ T-cell responses at pre-treatment

HCV specific IFN-γ T-cell responses of PBMC measured by ELISpot assay to (a) subtype-3a and (b) genotype-1 HCV peptides in treatment naïve chronically infected patients. HCV antigens are represented by a different colour. (c) Number of subtype-3a and genotype-1 patients with detectable HCV specific IFN-γ T-cell responses to structural and non-structural peptides (chi-squared test p=0.0051).

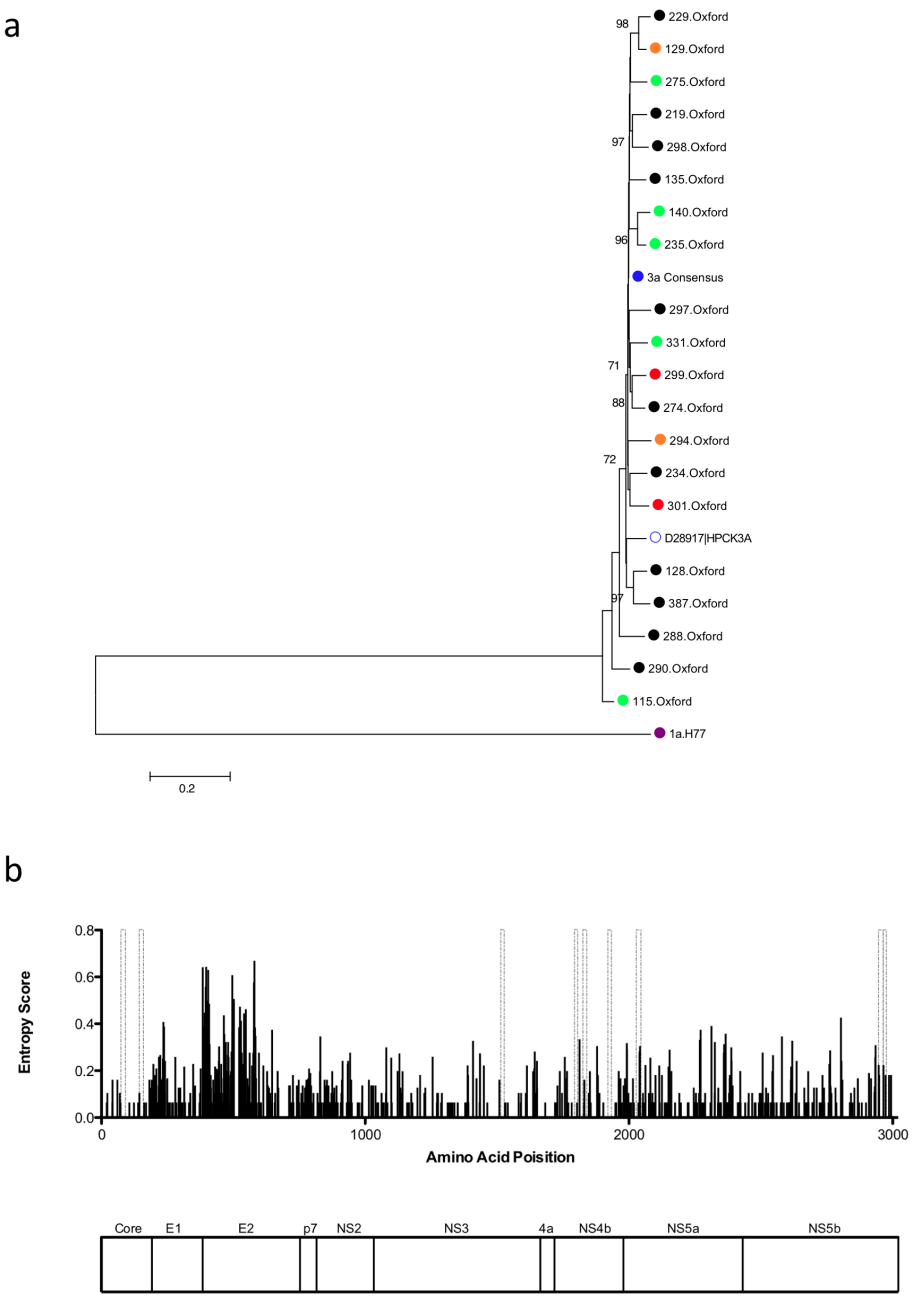


Figure 5.2 Subtype-3a diversity

(a) Phylogenetic tree of full-length nucleotide sequences from 20 subtype-3a infected chronic patients, including 8 patients followed longitudinally through combination therapy – SVR-green, REL-orange, and NR-red. Also included are the subtype-3a peptide consensus sequence (closed blue circle), a subtype-3a reference sequence (Accession number D28917) (open blue circle), together with H77 subtype-1a nucleotide sequence (Accession number AF009606) (purple circle) used as an out-group. Bootstrap scores >70% are shown. (b) Entropy score of full-length subtype-3a sequences from 20 chronic subtype-3a patients. Subtype-3a peptides positively identified by IFN-γ ELISpot assays are indicated by dashed grey bars. A map of HCV polyprotein is also shown.

particularly within E2 but also in the non-structural regions (Figure 5.2b).

5.3.3 Mapping of subtype-3a antigenic targets

The overlapping peptide set facilitated the identification of subtype-3a HCV specific T-cell antigenic targets (Table 5.1). Ten subtype-3a T-cell epitopes were identified, of which eight have not previously been described. CD4+ responses were detected to 3 core peptides. However, T-cell epitopes that mapped to the non-structural viral regions NS3, NS4b, NS5a and NS5b were exclusively CD8+ T-cell restricted. CD8+ T-cell responses targeting a single epitope in NS3 helicase (aa 1513-1529 RPSGMFDSVVL) were found in all three HLA B3501 patients (the equivalent viral region in genotype-1 infection RPSGMFDS**S**VL is a known B3501 epitope (Semmo et al., 2005a)). The IFN-γ ELISpot responses to 6 peptides in representative patients is shown in Figure 5.3.

Viral sequence analysis was used to determine whether identified T-cell antigenic targets represented the host circulating viral sequence. In nine of 13 cases the host viral sequence was identical to the target peptide. However amino acid differences between host viral sequence and peptide targets were found in 4 patients (Table 5.1, indicated in red). In patients 129 and 450, (targeting peptides NS5a aa 2029-2046 and NS5b aa 2965-2975, respectively) T-cell analysis using autologous variant and consensus peptides in IFN-γ ELISpot assays showed that the autologous variant peptide resulted in a reduction or total loss of T-cell response (129-REL consensus peptide 23 SFU/10^6 PBMC vs. autologous variant peptide 13 SFU/10^6 PBMC; and 450 consensus peptide 53 SFU/10^6 PBMC vs. autologous variant peptide 3 SFU/10^6 PBMC).

Table 5.1 Positively identified subtype-3a peptides

Peptide amino acid sequences of positively identified IFN-γ ELISpots of chronic subtype-3a patients. Patient viral sequence at pre-treatment is shown (amino acid differences shown in red). CD4/CD8 T-cell subset analysis was assessed using CD8 depleted PBMC in IFN-γ ELISpot assays. N/A-not available (PCR amplification not possible or insufficient sample); SVR-sustained virological response; REL-relapse. [a] epitopes where viral sequence is identical between subtypes-3a and -1a, [b] epitopes where viral sequence is different between subtype-3a and genotype-1, and [c] peptides were shortened to 11-aa based on previously published epitopes or sequence overlap between peptides.

Protein	Amino Acid	3a Peptide Sequence used in T cell assays	Patients	Patient Viral Sequence Pre-Treatment	HLA	Treatment Outcome	Defining CD4/ CD8 T cell subsets
Core	27-44	GGQIVGGVYVLPRRGPRL	362	GGQIVGGVYVLPRRGPRL	DRB1 0103, 0701	SVR	N/A
	73-90	GRSWAQPGYPWPLYGNEG[b]	416	GRSWAQPGYPWPLYGNEG	DRBI 0101, 0701 DQBI 0201, 0501	SVR	N/A
	143-158	PVGGVARALAHGVRAL[b]	110	P**A**GGVARALAHGVRAL	DRB1 0801, 1101	SVR	CD4
			331	PVGGVARALAHGVRAL	N/A	SVR	
			437	PVGGVARALAHGVRAL	DRB1 0103, 0501	SVR	
NS3 helicase	1513-1529	RPSGMFDSVVL[b,c]	140	RPSGMFDSVVLCECYDA	A2402, A3002, B0702, B3501	SVR	CD8
			235	RPSGMFDSVVLCECYDA	A0301, A3002, B0702, B3501, C0401, C0702	SVR	
			362	RPSGMFDSVVLCECYDA	A2402, B3501, B4402, C0401, C0409	SVR	
NS4b	1791-1806	PAVASLMAFTASVTSPL[b]	416	PAVASLMAFTASVTSPL	A0101, A0201, B0801, B5701, C0602, C0701 DRB1 0101, 0701	SVR	CD8
			437	N/A	A0201, A2601, B3801, B2702, C1203, C0102 DRB1 0103, 0101	SVR	
	1824-1841	THLAGPQSSSAFVVSGLA[b]	437	N/A	A0201, A2601, B3801, B2702, C1203, C0102 DRB1 0103, 0101	SVR	N/A
	1918-1933	EGAVQWMNRLIAFASR[a]	410	EGAVQWMNRLIAFASR	A0101, A3001, B1302, B4402, C0602, C0501 DRB1 0701, DQB1 0201	NR	CD8
NS5a	2029-2046	GVMSTRCPCGASIAGHVK[b]	129	GVMSTRCPCGASI**T**GHVK A2042T	A1101, A7401, B4403, B38, C04, C0702	REL	CD8
NS5b	2946-2963	GKAKICGLYLFNWAVRTK[b]	275	GKAKI**T**GLYLFNWAVRTK C2951T	A1101, B0702, B4402, C0501, C0702 DRB1 0401, 0407	SVR	CD8
	2965-2975	KLTPLPAAGQL[b,c]	450	KLTPLPAAG**L**L Q2974L	N/A	REL	CD8

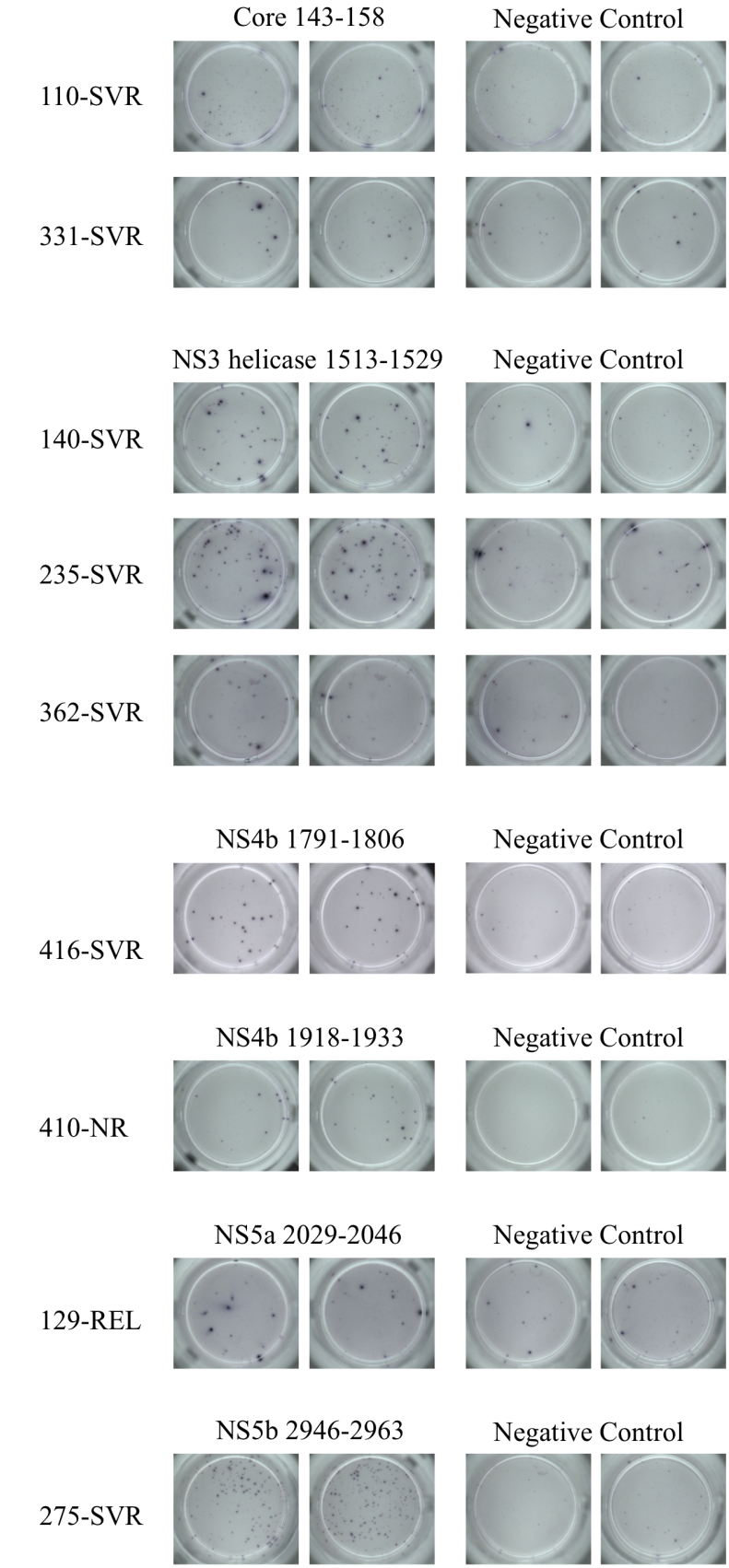


Figure 5.3 IFN-γ ELISpot responses to individual subtype-3a peptides
IFN-γ ELISpot responses in representative patients to individual subtype-3a peptides, negative control using DMSO. 200,000 PBMC were used per well.

5.3.4 Cross-Reactivity of subtype-3a and genotype-1 T-cell targets

Previous studies using a viral sequence-based approach in association with HLA type predicted little cross-reactivity between CD8+ T-cells that target genotypes-1 and -3 (Andri et al., 2009). However, this methodology can only identify T-cell targets that are subject to HLA-restricted viral escape. In contrast, consensus peptides that span the entire HCV polyprotein are likely to detect relatively conserved antigenic targets. In all cases where cross reactivity was assessed the equivalent viral region in genotype-1 infection differed from the subtype-3a T-cell epitopes (Table 5.2; indicated in bold). Using peptides derived from the equivalent regions in genotype-1, there was a total loss or reduction in T-cell recognition in all cases (with the exception of NS5a aa 2029-2046). In the aa 2965-2975 KLTPLPAAGQL NS5b genotype-3a epitope the host circulating viral variant (KLTPLPAAG**L**L) led to loss of recognition (Table 5.2).

Although DRB1*0103 and DRB1*1101 restricted CD4+ T-cells targeting aa 143-158 have been previously described in genotype-1 infection (Giugliano et al., 2009, Godkin et al., 2001, Barnes et al., 2002) there is significant sequence divergence between genotypes-1 and -3 at this region (PVGGVARALAHGVRAL vs. GAP**L**G**A**VARALAHGVR**V**L). I found that the dominant CD4+ T-cell response targeting the subtype-3a core epitope (aa 143-158) was found to be subtype-3a specific, in that the equivalent genotype-1 viral region was poorly recognised.

Table 5.2 Cross-reactivity of subtype-3a and genotype-1 peptides

T-cell responses were assessed in subtype-3a patients using the positively identified subtype-3a peptides and the equivalent 1a and 1b peptides (IFN-λ ELISpot assays). Amino acids that differ from the subtype-3a peptides are shown in bold.

- loss of cross-reactivity, ↓ decrease in reactivity, ↑ increase in reactivity.

Protein	Subtype-3a amino acid	3a peptide	3a Peptide SFU/10^6 PBMC	1a and 1b Equivalent Sequence		1a/1b Peptide SFU/10^6 PBMC	3a vs. 1a/1b Cross-Reactivity
Core	73-90	GRSWAQPGYPWPLYGNEG	33	1a	GR**T**WAQPGYPWPLYGNEG	28	↓
				1b	GR**A**WAQPGYPWPLYGNEG**L**	23	↓
	143-158	PVGGVARALAHGVRAL	50	1a=1b	**GA**P**L**G**A**VARALAHGVR**V**L	23	↓
NS3 helicase	1513-1523	RPSGMFDSVVL	30	1a=1b	RPSGMFDS**S**VL	8	↓
NS4b	1791-1806	PAVASLMAFTASVTSPL	65	1a	PA**I**ASLMAFTA**A**VTSPL	0	-
				1b	PA**I**ASLMAFTAS**I**TSPL	0	-
NS5a	2029-2046	GVMSTRCPCGASIAGHVK	23	1a	**I**V**H**STR**H**PCG**E**S**T**AGHVK	30	↑
				1b	G**I**M**Q**T**T**CPCGA**Q**IAGHVK	13	↓
NS5b	2965-2975	AVRTKTKLTPLPAAGQL	33	1a	VRTK**L**KLTP**IA**AAG**R**LDL	0	-
				1b	AVRTK**L**KLTP**I**PAA**S**QL	0	↓

5.3.5 Subtype-3a HCV-Specific T-cell Responses decrease during therapy

Twenty-one patients infected with subtype-3a were followed longitudinally during standard treatment with 6 months of PEG-IFN-α and ribavirin to assess whether therapy enhances subtype-3a specific T-cell immunity accounting for the preferential treatment response rates in this genotype. Of the 21 patients studied, 14 (67%) achieved SVR, 5 (24%) experienced virological relapse and 2 (9%) were non-responders. At pre-treatment, 8/14 SVR patients had detectable HCV specific T-cell responses (mean total magnitude 103 SFU/10^6 PBMC), higher than the detectable responses observed in 2/9 non-SVR patients, (mean total magnitude 32 SFU/10^6 PBMC, p=ns; Figure 5.4).

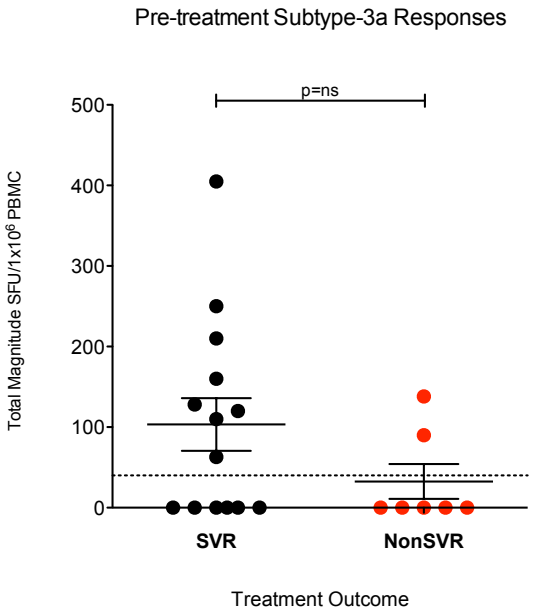


Figure 5.4 Pre-treatment T-cell responses and treatment outcome
Total magnitude of IFN-γ ELISpot responses of subtype-3a infected patients at pre-treatment in patients that achieved sustained virological response (SVR – black) and patients that failed treatment (NonSVR – red).

In the SVR patients, the total HCV specific T-cell response detected pre-treatment decreased during therapy (mean total HCV specific T-cell response pre-treatment 180 SFU/10^6 PBMC vs. 26 SFU/10^6 PBMC at week 8-12; p=0.0337). Responses increased after treatment (p=ns), when virus remained undetectable suggesting that the decline in T-cell responses was not merely due to decreasing levels of viremia (Figure 5.5).

In three patients, new weak HCV specific T-cell responses were first detected 3-6 months after the end of therapy and seem to be unrelated to the outcome of therapy (2 SVR and 1 non-SVR patients). This phenomenon has been observed in genotype-1 infection with an increase in proliferative and cytotoxic capacity in both SVR and non-SVR patients towards the end of therapy (Day et al., 2002, Barnes et al., 2002, Pilli et al., 2007).

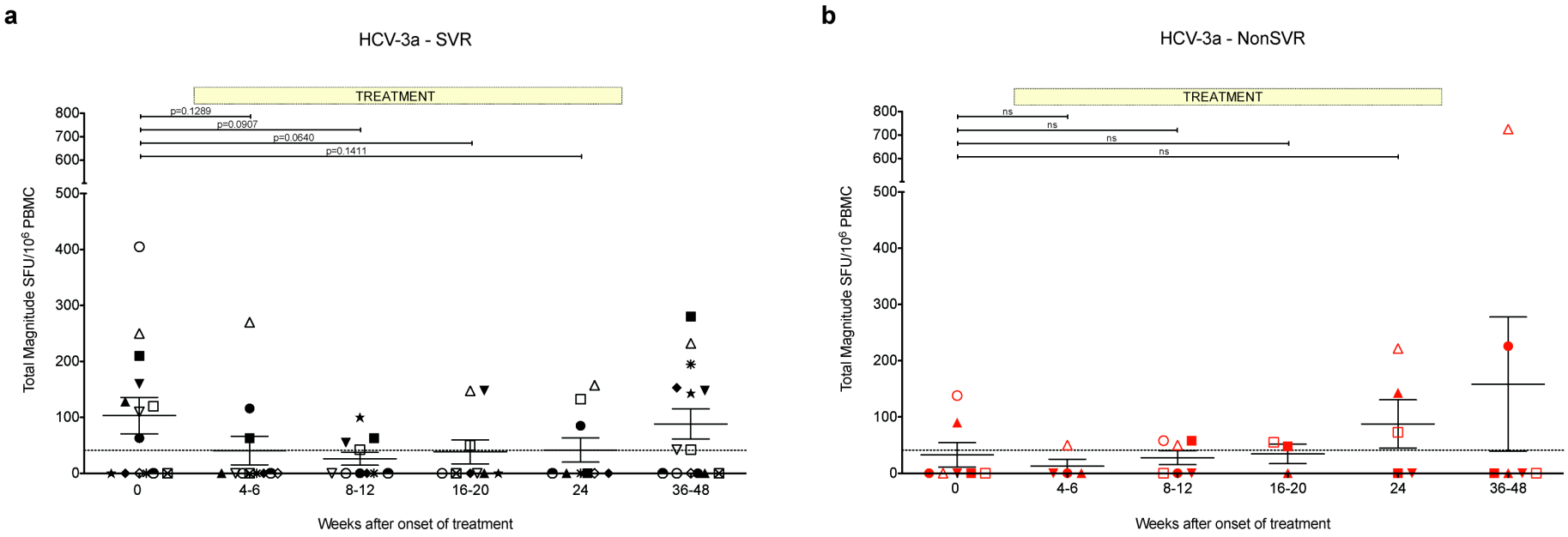


Figure 5.5 Magnitude of subtype-3a specific IFN-γ T-cell responses

Total magnitude of subtype-3a HCV specific IFN-γ T-cell responses measured by ELISpot assay in chronic subtype-3a patients. Responses to HCV subtype-3a peptides were measured before treatment, at multiple timepoints during treatment (treatment weeks 4-6, 8-12, 16-20) and post-treatment. a) Patients achieving sustained virological response (black symbols) and b) patients failing treatment red symbols. Treatment duration of 24 weeks is indicated.

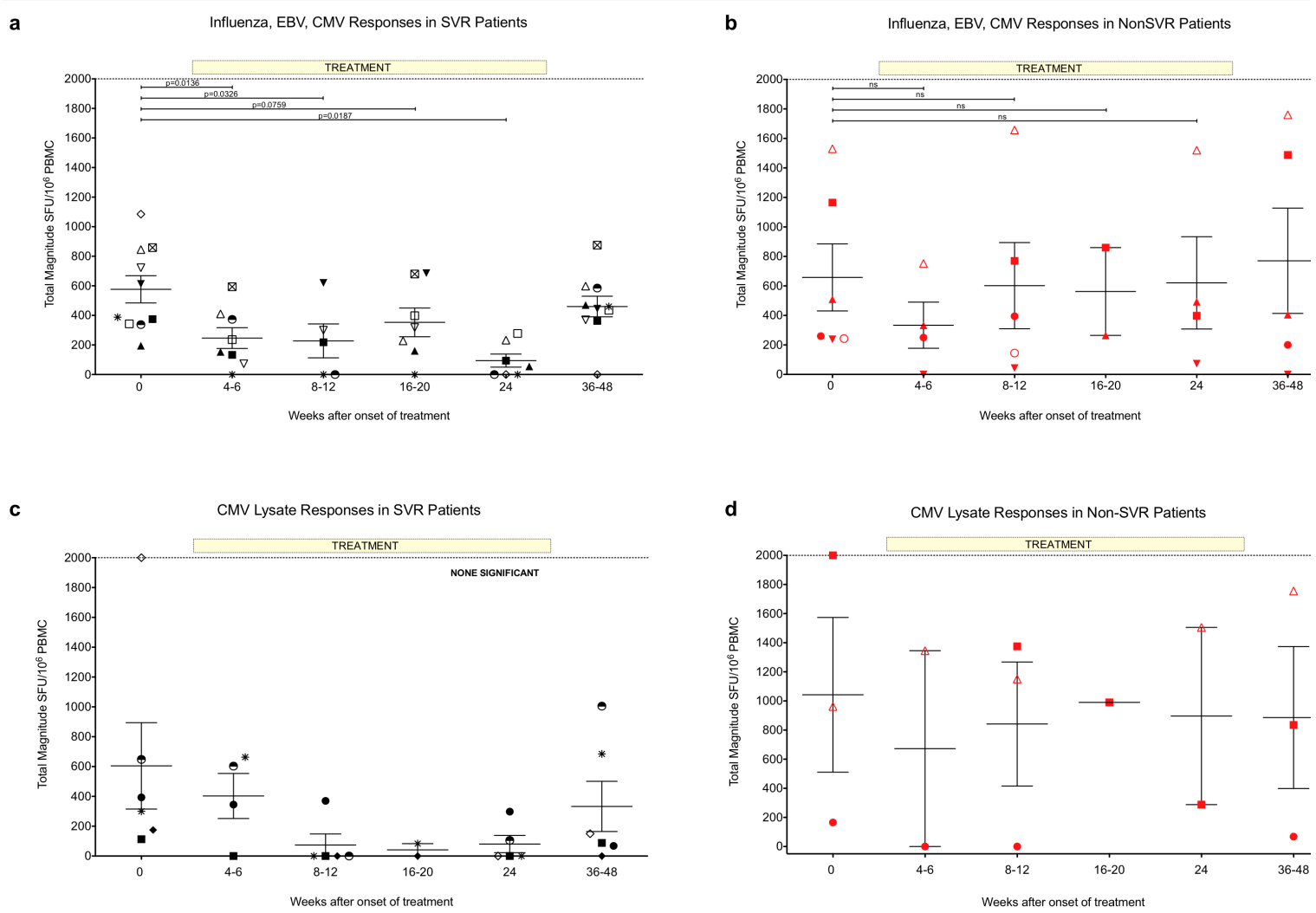
5.3.6 Non-HCV specific T-cell responses decline during therapy

To determine if the decrease in magnitude of T-cell IFN-γ responses during treatment applied to HCV specific T-cells only, IFN-γ T-cell responses to control viral antigens were also assessed. Control antigens included influenza, EBV and CMV (FEC) HLA CD8+ T-cell restricted epitopes, and CMV lysate that primarily induces CD4+ T-cells.

Pre-treatment, FEC responses were detected in 17 patients (10 SVR, 5 REL, 1 NR - magnitude range 195-1530 SFU/10^6 PBMC) (Figure 5.6). The FEC responses of the SVR patients decreased in magnitude throughout treatment and were significantly lower at all time points during treatment compared to pre-treatment (magnitude at TW4-6, TW8-12 and TW24 vs. pre-treatment p=0.0136, p=0.0326, p=0.0187 respectively) (Figure 5.6a). Following treatment response magnitude increased to magnitudes detected at pre-treatment. In contrast responses of non-responder patients did not change significantly during treatment (Figure 5.6b). Pre-treatment CMV lysate responses also decreased in magnitude during treatment in SVR patients (p=ns) (Figure 5.6c), whilst in non-SVR patients CMV responses were only detected in 3 patients and therefore the effect of treatment on responses could not be accurately assessed (Figure 5.6d).

Figure 5.6 Non-HCV specific T-cell responses in subtype-3a patients

Total magnitude of influenza/EBV/CMV (FEC) and CMV lysate specific IFN-γ T-cell responses measured by ELISpot assay in chronic subtype-3a patients measured before treatment, at multiple timepoints during treatment (weeks 4-6, 8-12, 16-20) and post-treatment. Patients achieving sustained virological response (SVR - black symbols) (a,c) and (b,d) patients failing treatment (NonSVR - red symbols). Threshold of positive HCV-specific responses is represented by dotted line (41 SFU/10^6 PBMC) defined in healthy controls (see Methods). Treatment duration of 24 weeks is indicated.



5.3.7 SVR with IFN-α and Ribavirin is associated with total lymphopenia

As the magnitude of T-cell responses to HCV, influenza, EBV and CMV decreased during treatment in 8 SVR patients, the effect of treatment on total lymphocyte number in 22 subtype-3a patients was assessed (14 SVR, 5 REL, 3 NR). The magnitude of the pre-treatment total lymphocyte count was not associated with treatment outcome (Figure 5.7a). The effect of treatment on lymphocyte count was assessed, by measuring the percentage change in the pre-treatment total lymphocyte count by week 12 of treatment (Figure 5.7b). The total lymphocyte number decreased by mean of 50% in SVR and relapse patients but decreased to only 90% of pre-treatment levels in non-responder patients (SVR vs. Nonresponder patients p=0.0010).

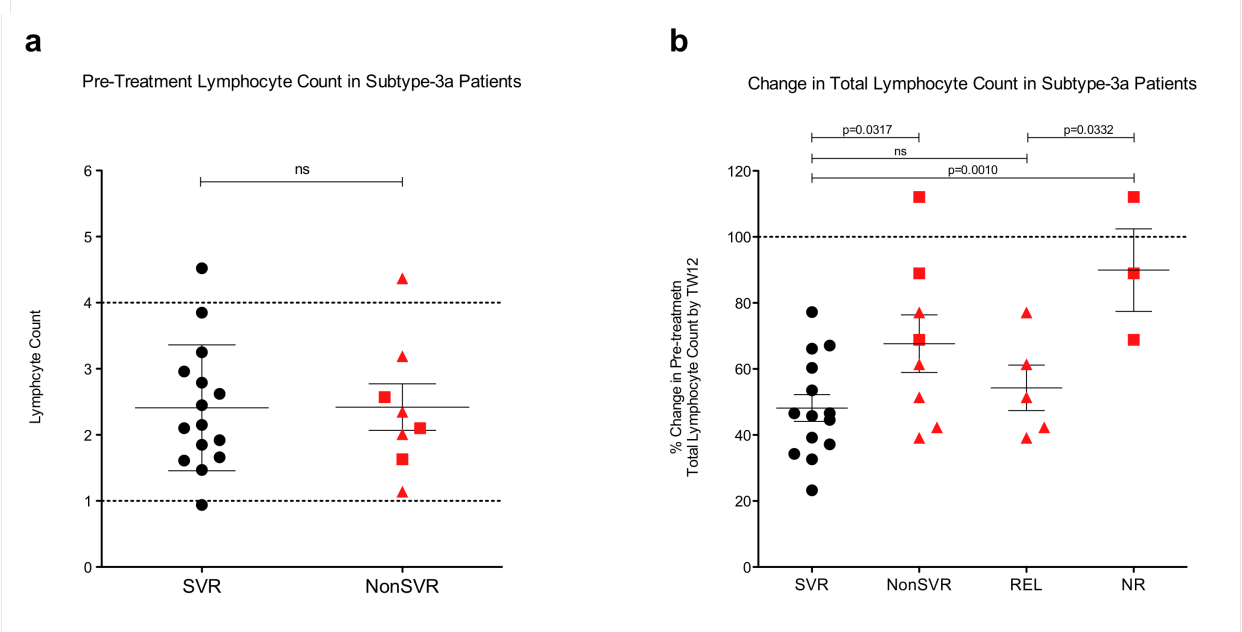


Figure 5.7 Lymphocyte counts of subtype-3a patients
(a) Pre-treatment lymphocyte counts of subtype-3a patients before initiation of treatment. Normal range of lymphocyte counts are indicated by dotted lines. (b) Percentage change in pre-treatment lymphocyte count by week 12 of treatment in 22 patients chronically infected with HCV subtype-3a. Pre-treatment level set at 100% is represented by dotted line. Patients achieving a sustained virologic response (SVR) are shown by black circles, relapse patients (REL) shown by red triangles and non-responders (NR) shown by red squares. NonSVR – Relapse and Nonresponder patients.

5.4 Discussion

Whilst T-cell immunity to HCV genotype-1 infection has been extensively studied and appears to play a key role in clinical outcome, very little is known about T-cell immunity to other HCV subtypes. Although many studies have included patients with non-genotype-1 infection, T-cell assessment has almost exclusively relied on genotype-1 peptides often with little knowledge of circulating host viral sequence. HCV subtype-3a is the dominant infecting subtype within the UK. This study focused on subtype-3a infection and assessed T-cell immunity targeting the entire subtype-3a polyprotein in association with viral sequence analysis and assessed these responses in association with treatment outcome. It is plausible that the distinct subtype-3a T-cell responses are induced by IFNs that are known to modulate T-cell function, in addition to the viral sequence diversity between different genotypes. An analysis of subtype-3a T-cell responses to the entire subtype-3a HCV polyprotein has not previously been performed. This study analysed subtype-3a specific T-cell responses during chronic infection, allowing comparison with genotype-1 responses and enabled the following hypothesis to be addressed; in chronic subtype-3a infection HCV specific T-cell responses are distinct to those in genotype-1 infection, and that the greater treatment response observed in subtype-3a chronic infection was due to either a greater magnitude of pre-existing T-cell responses or an enhancement of HCV specific T-cell responses during treatment.

As very little subtype-3a viral sequence data was available for major parts of the polyprotein, full-length viral sequencing was initially performed in a cohort of 20 treatment-naïve subtype-3a infected patients, enabling the design of a representative subtype-3a peptide set. Subtype-3a HCV specific T-cell responses were readily detectable in approximately half the patients with subtype-3a infection, with CD4+ T-cell subsets targeting the core protein and CD8+ T-cells targeting the non-structural HCV proteins.

This was significantly different to genotype-1 infection, where detectable responses almost exclusively targeted core - a protein shown previously to be a dominant CD4+ T-cell target (Barnes et al., 2002, Semmo et al., 2005b, Ruys et al., 2008). It has also previously been shown that proliferative T-cell responses to NS3 protein were more common in genotype-3 than -1 infection. However, proliferative responses usually represent CD4+ T-cells, this is in contrast to this study where responses to NS3 peptides were CD8 restricted (Fleming et al., 2010).

HCV-specific T-cell responses may have been more readily detectable in subtype-3a than genotype-1 infection due to peptide differences. The subtype-3a peptide set was based on a consensus of sequences and consequently was a cohort-adapted peptide set. Additionally, genotype-1 patients consisted of both subtype-1a and -1b infections, and assessed with peptides from a subtype-1b reference strain, which may account for the difference in the responses detected. Assessment of genotype-1 responses using subtype specific peptides may give a better representation of subtype-1a and -1b specific T-cell responses. Viral sequence differences between genotypes due to amino acid insertions or deletions in the polyprotein may also result in peptide differences. However, the polyprotein sequence was divided into individual proteins before peptide design, hence any amino acid insertions or deletions would only affect the individual protein e.g. E2, not the peptides of downstream proteins.

Responses to individual HCV core peptides were detected in five patients with subtype-3a infection. T-cell subset depletion assays confirmed that these were CD4+ T-cell responses and in 3/5 cases these responses were mapped down to aa 143-158; PVGGVARALAHGVRAL. The “equivalent” subtype-1a peptide that differs by 3 amino-acids is known to be a DRB1 1101 restricted CD4 T-cell target (Hultgren et al., 2004). In contrast to this study, CD4+ T-cells in genotype-1 infection are known to target a broad

range of epitopes within core (Day et al., 2002). Viral sequence analysis in 4/5 subtype-3a patients showed no evidence of viral escape supporting previous data in both humans (Schulze zur Wiesch et al., 2005) and chimpanzees (Fleming et al., 2010) that CD4+ T-cells, unlike CD8+ T-cells, rarely exert selection pressure on HCV.

Where T-cell subset analysis was possible I observed that subtype-3a CD8+ T-cells exclusively targeted peptides from non-structural proteins. Ten subtype-3a epitopes targeting core and NS3-NS5b are described. Interestingly four (NS4b 1791-1806, NS4b 1918-1933, NS5b 2946-2963, NS5b 2965-2975) of these epitopes were identified as CD8+ T-cell epitopes that overlap with CD4+ T-cell epitopes in the equivalent but non-identical subtype-1a viral regions (Fuller et al., 2010, Lamonaca et al., 1999, Godkin et al., 2001, Day et al., 2002). In only one epitope was the subtype-3a viral sequence identical to that in subtype-1a infection – NS4b aa 1918-1933 EGAVQWMNRLIAFASR. The majority of the subtype-3a epitopes assessed for cross-reactivity showed little or no cross-reactivity to the equivalent genotype-1 antigens, showing that the majority of T-cell targets in genotype-3a infection are subtype specific (Schulze zur Wiesch et al., 2005).

The effect of combination therapy on HCV specific T-cell responses in relation to outcome has been an area of investigation and controversy for some years in genotype-1 infection. It has been shown that HCV-specific T-cell responses were more common in non-genotype-1 infection and were related to a more rapid viral clearance (Giugliano et al., 2009). However this remains largely unexplored in subtype-3a infection. Exactly how therapy impacts on anti-viral T-cell responses is complicated by the fact that IFN has both direct anti-viral and also a broad range of immunomodulatory properties (Hultgren et al., 2004, Peters, 1996). Disparate results in genotype-1 infection may be due to technical challenges, such as weak T-cell responses at the detection limit of

immunological assays, and frequent use of peptides that may not represent the circulating viral strain therefore measuring historical responses. A number of studies have shown that T-cell responses that were undetectable before therapy can be detected during therapy at least in some individuals (Le Bon and Tough, 2002, Barnes et al., 2002). Recent observations have suggested that the level of pre-treatment HCV-specific immunity is associated with a SVR to combination therapy (Cramp et al., 2000, Pilli et al., 2007). In support of the latter, this study observed that the total HCV-specific T-cell responses pre-treatment were higher in SVR than non-SVR patients. However this difference did not reach statistical significance. Others have suggested that IFN-α therapy may in fact lead to a decline in HCV specific T-cell responses that remain undetectable in those achieving SVR, but that T-cells targeting control antigens are unaffected, suggesting that the decline in HCV specific T-cell responses mirrors the fall in HCV viral load (Rosen et al., 2007).

In this study, subtype-3a HCV-specific T-cell responses that were detectable before therapy declined significantly during therapy in individuals achieving SVR but responses recovered after the treatment period. In patients that did not achieve SVR the decline in HCV-specific responses was not apparent. However, the same pattern of decline was also observed in non-HCV specific T-cell responses suggesting a generic host response to IFN treatment. Moreover, when these observations were extended to include total lymphocyte counts, it was apparent that in subtype-3a infection lymphopenia was specifically associated with SVR to combination therapy whereas patients that failed treatment, non-responders in particular, were resistant to treatment induced lymphopenia. The mechanism of IFN induced lymphopenia is not known. However, there are a number of plausible explanations for this observation. The fact that total lymphocytes and non-HCV specific T-cells were reduced specifically in those with a subsequent SVR, and that these

responses recovered after treatment in spite of undetectable viremia, suggests that the decline in HCV-specific T-cell responses has little to do with changes in HCV viral load. It is more likely that there is a general host resistance to the effects of interferon therapy in subtype-3a infected hosts that are non-responsive to treatment.

Recent data showing that genetic polymorphisms linked to the IL28B/IFN-λ3 gene determine the outcome of IFN therapy strongly supports the concept that host genetics play a key role in genotype-1 infection (though data are weaker for subtype-3a infection) (Burton Jr et al., 2008, Ge et al., 2009, Mangia et al., 2010). It may be that the same interferon stimulated genes (ISG) both induce lymphopenia and successfully eradicate virus in the host. Certainly there are data supporting the idea that ISGs are stimulated in PBMC in IFN treated persons (McCarthy et al., 2010). However, host resistance to IFN may be independent of HCV viral infection i.e. genotype, and therefore alternatively a pre-activated host IFN system induced by specific viral proteins associated with a failure to respond to exogenous IFN, may also render the host resistant to exogenous IFN induced lymphopenia.

In conclusion, these data demonstrate that in subtype-3a infection CD4+ T-cells targeting structural proteins and CD8+ T-cells targeting non-structural proteins were readily detectable. Viral sequence analysis showed evidence of viral escape from CD8+ T-cells but not CD4+ T-cells. Paradoxically a successful treatment outcome in subtype-3a infection was associated with a decline in HCV and non-HCV specific T-cells, and with a decline in the total lymphocyte count. Further studies are required to assess subtype-3a T-cell responses in spontaneously resolved patients to compare with those detected in chronically infected subtype-3a patients.

Chapter 6 – Detection of Subtype-3a T-cell Responses using Class I HLA-Associated Putative Epitopes

This work was done in collaboration with Silvana Gaudieri, Royal Perth Hospital and Murdoch University, Perth, Australia.

Abstract

Viral escape mutations within HCV-specific CD8+ T-cell epitopes are associated with HCV persistence. In chronic genotype-1 infection, studies using sequence-based and predictive methods have identified associations between HCV sequence polymorphisms and HLA alleles, within known and putative CD8+ T-cell epitopes. However such associations have not been sought in subtype-3a.

Firstly, associations between HLA class-I alleles and HCV subtype-3a polymorphisms within NS2-NS5 were identified. Secondly, optimal CD8+ epitope sequence containing the polymorphism was predicted using HLA restriction allele and epitope prediction algorithms. T-cell recognition to the predicted epitopes were then assessed by IFN-γ ELISpot assays.

T-cell responses were weak and detected in very few patients *ex vivo* and following *in vitro* stimulation. Viral sequencing demonstrated that the majority of patients' viral sequence contained the HLA-associated escape residue. HLA-associated epitopes were not detected using overlapping peptides by IFN-γ ELISpot assays. In conclusion, the ability to detect T-cell responses is dependent on the design of the peptide set. HLA-associated putative peptides were based on T-cell driven polymorphisms, whilst overlapping peptides were based on consensus viral sequence and therefore likely to detect conserved viral regions.

6.1 Background

The high mutation rate of HCV results in the introduction of mutations along the genome during replication (Sarasin-Filipowicz et al., 2008). These mutations may occur within or flanking epitopes that are targeted by the adaptive and humoral immune response.

Succesful binding of epitopes to HLA molecules relies on the amino acid sequence of the peptide. Epitopes typically contain two anchor residues at position 2 and at the C-terminal, which tolerate only a few amino acids (Moradpour et al., 2007, Falk et al., 1990). A mutation that results in the abrogation of epitope recognition by T-cells is said to have "escaped" the immune response and is hence known as an escape mutation (Rammensee et al., 1999, Bowen and Walker, 2005b).

Mutations can also have varying affects on viral fitness. A mutation that causes a lower viral fitness but also enables an epitope to escape the immune response has an advantage over the wild-type residue and is selected. Escape mutations within HCV-specific CD8+ T-cell epitopes are associated with HCV persistence (Franco et al., 1995, Erickson et al., 2001, Weiner et al., 1995, Tester et al., 2005, Grakoui et al., 2003, Chang et al., 1997).

Within HCV sequences, polymorphic sites that contain wild-type and variant residues, have been associated with pressure exerted by HCV-specific T-cell responses (Cox et al., 2005b, Rauch et al., 2009). Previous studies of genotype-1 infection using population-based and predictive methods have found associations between HLA alleles and sequence polymorphisms within previously published and putative CD8+ T cell epitopes (Merani et al., 2011, Gaudieri et al., 2006, Timm et al., 2007). However HLA associated polymorphic sites may differ between genotypes due to the high degree of sequence diversity between genotypes (Rauch et al., 2009). Nevertheless areas of more conserved sequence such as the NS3 protein, may be the target of a cross-reactive response between genotypes-1 and -3 (Rauch et al., 2009).

The identification of crucial binding positions within epitopes, led to the development of prediction algorithms that can determine whether a peptide sequence matches the binding motif of a specific HLA allele (Neumann-Haefelin et al., 2008, Rotzschke et al., 1991). The use of viral sequencing, identification of HLA-associations together with epitope prediction software to identify putative epitopes indicate that the identified epitope is targeted by the host immune response, and also identifies the restricting HLA-allele and the escape residue. However this provides putative HLA-associated predicted epitopes and assessment of T-cell responses to these peptides is essential. A disadvantage to this method is that the identified epitope is capable of mutating and essentially escaping the immune response; therefore a T-cell response to this epitope may not be beneficial for viral elimination.

Using a novel method to detect subtype-3a T-cell epitopes, I have identified putative HLA class-I epitopes and their associated escape variants using viral genome sequencing and computer prediction programming. I have assessed *ex-vivo* T-cell responses to these putative epitopes and also to overlapping peptides spanning NS2-NS5b proteins, to determine if T-cell responses to putative HLA-class I epitopes can also be detected using overlapping peptides.

For the purpose of this chapter, HLA-associated peptide is defined as a peptide representing a putative epitope sequence identified using prediction software and containing the wild-type residue associated with a specific HLA allele.

6.2 Chapter Specific Materials and Methods

The following was done by both Silvana Gaudieri group and myself (Figure 6.1).

6.2.1 Viral Sequencing and HLA-typing of Subtype-3a Patients

HCV viral RNA was sequenced from 136 subtype-3a HCV chronically infected patients that spanned NS2 to NS5b proteins (see Methods 2.2).

The following was done by Silvana Gaudieri group.

6.2.2 Identification of polymorphic sites associated with HLA-alleles

HLA class I restricted epitopes were designed using a novel sequence-led approach (Sette et al., 1989). First, residues at polymorphic sites were assigned as consensus or non-consensus, and associations with HLA alleles were assessed by Fisher's exact test using S-Plus 8.0 (Insightful Corporation, Seattle, WA). Due to possible confounding effects such as founder effects, whereby a residue may be over-represented in a population due to a common ancestor instead of immune pressure, phylogenetic analysis stratified by clustering was performed to adjust the associations accordingly (Rauch et al., 2009). This enabled the identification of possible related sequences and their potential impact was assessed, based upon the hypothesis that HLA alleles would be distributed randomly within related sequences. Associations were then assessed by Mantel-Haenszel tests, further decreasing the risk of over-representation of alleles within a population (Kaufman and Rousseeuw, 1990). Associations with a p-value of ≤ 0.01 for both Fisher's exact test and Mantel-Haenszel test were reported. In addition, only sites with at least 5 non-consensus amino acids and 5 carriers of the HLA allele were reported (Mantel and Haenszel, 1959). The dataset was expanded to include all HCV sequences to reduce bias from excluding sequences that were more difficult to sequence (due to less conserved

sequence). Results were reported based on $p \leq 0.01$ and q value cut-off of ≤ 0.2 for the entire dataset (Rauch et al., 2009, Storey and Tibshirani, 2003).

The following work was performed by myself.

6.2.3 Identification of CD8 HLA-associated peptides

Putative epitopes were identified from viral sequences containing polymorphic sites by prediction software and selecting the associated HLA allele. The epitope prediction software allowed the prediction of all possible epitopes that may bind the specific HLA allele therefore multiple putative epitopes of varying length were predicted for some polymorphic residues. In total, 65 CD8 class-I associated peptides were predicted. Epitopes were predicted that contained the polymorphic sites *within* the peptide (55 peptides; Table 6.1) and also epitopes where the polymorphic site was *flanking* the predicted epitope (10 peptides; Table 6.2) as a mutation at flanking sites may affect the processing and presentation of the epitope and subsequent T-cell response.

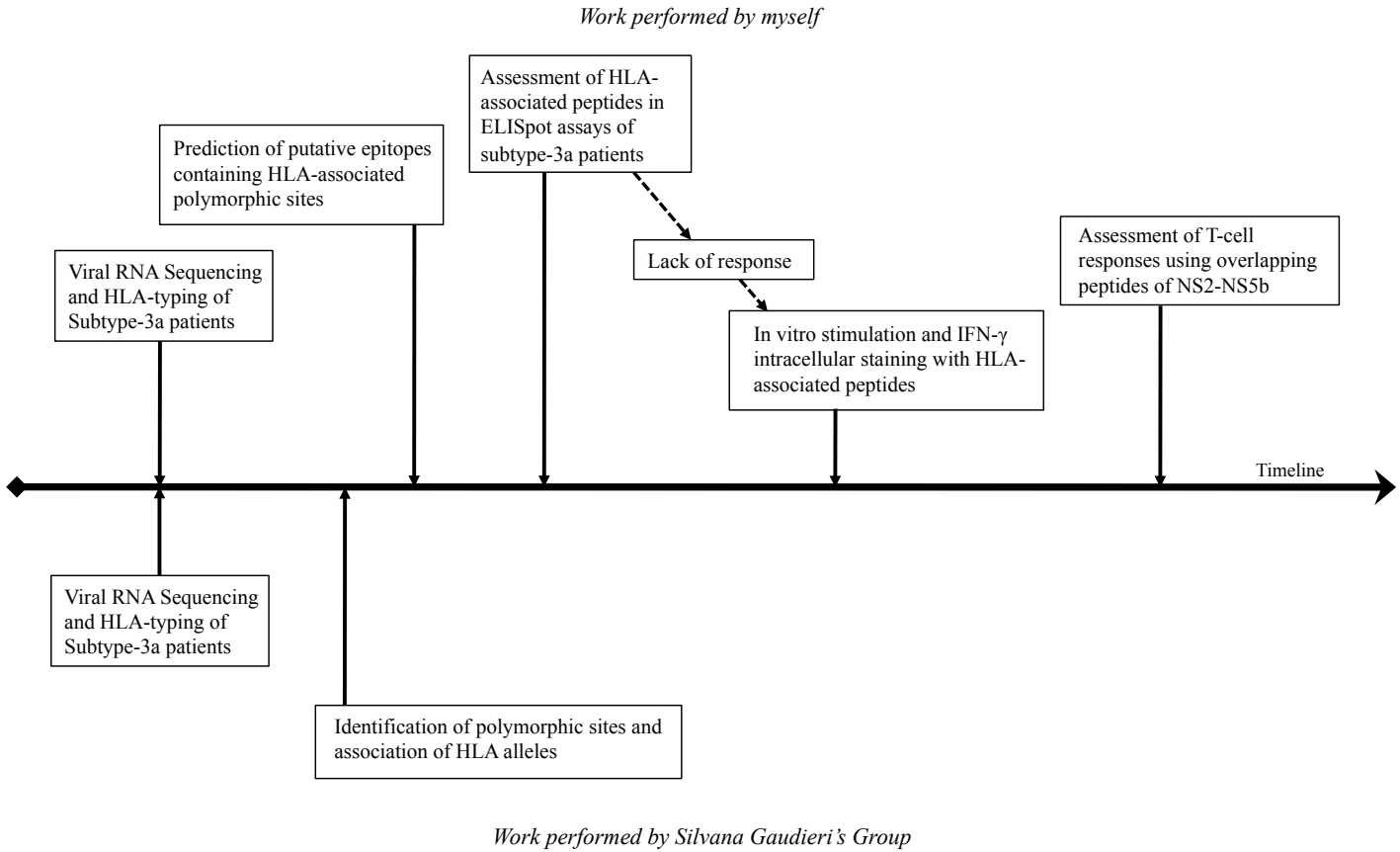


Figure 6.1 Timeline of experimental methods and assays performed
Methods and assays above the line were performed by myself, whilst those shown below the line were performed by Silvana Gaudieri's group.

Syfpeithi (www.syfpeithi.de) is an epitope prediction algorithm enabling the prediction of T-cell epitopes based on published T-cell epitopes and MHC ligands (Rauch et al., 2009). Prediction takes into account many of the epitope amino acids including anchor and auxillary anchor residues. An overall peptide score is obtained by scoring each amino acid from 1 for a slightly preferred amino acid to 15 for an optimal anchor residue. A negative value can also be given to amino acids that are disadvantageous to the peptides binding capacity. The scoring values are based on the frequency of the amino acids in published data. The expected reliability of obtaining an apt epitope using Syfpeithi is 80% (Rammensee et al., 1999). A cut off score of ≥ 20 was determined by assessing known HCV epitopes using Syfpeithi.

Bimas is an algorithm to search HLA peptide binding motifs of class I HLA molecules (http://www-bimas.cit.nih.gov/molbio/hla_bind/). The scoring algorithm is based on scoring each residue depending on amino acid type and location within the epitope for the selected HLA molecule (Rammensee et al., 1999). A cut off value of ≥ 50 was determined by assessing known HCV epitopes through Bimas.

The prediction software also allowed the prediction of epitopes to HLA alleles of 4 digit restrictions e.g. A0201, and also of 2 digit restriction e.g. A02. Therefore a patient that carried the A0201 allele was tested with peptides restricted to A0201 and also A02.The resulting putative epitopes are shown in Table 6.1 and 6.2.

6.2.4 Peptide Design of Overlapping peptides

Subtype-3a overlapping peptides spanning non-structural proteins were based on the Oxford consensus sequence (See Methods 2.5.1).

Table 6.1 HLA-associated peptides – *within* epitope polymorphisms

Protein	HLA	Wild-type Residue	Amino Acids	Peptide	Predicted Epitope
NS2	A0101	V	879-887	001-A0101	**V**ILLTSLLY
	A02	L	862-871	049-A02	ALQVWVPPL**L**
		L	870-879	048-A02	L**L**ARGSRDGV
		Y	881-890	052-A02	LLTSLL**Y**PSL
		Y	885-894	047-A02	LL**Y**PSLIFDI
	A0301	V	926-935	006-A0301	RLCMLVRS**V**M
		V	929-938	004-A0301	MLVRS**V**MGGK
		V	930-939	005-A0301	LVRS**V**MGGKY
	A2402	Y	886-894	007-A2402	L**Y**PSLIFDI
	B1501	T	878-887	008-B1501	GVILL**T**SLLY
	B15	I	946-954	055-B15	S**I**GRWFNTY
	B2705	R	940-949	010-B2705	FQMIILSIG**R**
		R	948-956	009-B2705	G**R**WFNTYLY
		H	962-971	011-B2705	MQ**H**WAAAGLK
	B4402	V	822-831	012-B4402	ATLGAG**I**LVL
	B44	V	826-835	057-B44	AG**I**LVLFGFF
	B5101	S	871-880	013-B5101	LARG**S**RDGVI
	C03	L	829-838	050-C03	I**L**VLFGFFTL
	C04	V	981-990	051-C04	IFSPMEIK**V**I
NS3	A0101	Y	1442-1450	014-A0101	ATDALMTG**Y**
	A0201	A	1389-1398	015-A0201	**A**LLKGGRHLI
	A0301	V	1138-1146	016-A0301	L**V**TRDADVI
	B1501	K	1296-1305	017-B1501	**K**LTYSTYGKF
	B2705	I	1379-1388	018-B2705	IPFYGKAIP**L**
		V	1632-1641	019-B2705	YRLGP**V**QNEI
	B4402	G	1407-1416	020-B4402	DEIASKLR**G**M
	B4403	L	1639-1647	021-B4403	NEIC**L**THPI
	B5101	A	1388-1397	023-B5101	I**A**LLKGGRHL
NS4B	A02	A	1873-1882	062-A02	KIMGGELPT**A**
		A	1880-1889	065-A02	PT**A**EDMVNLL
	A0301	I	1901-1910	026-A0301	GV**I**CAAILRR
	A2601	R	1852-1861	027-A2601	**R**VLLDILAGY
	A68	V	1736-1744	067-A68	EK**A**LGLLQR
	B27	R	1948-1957	061-B27	A**R**VTALLSSL
	B4001	I	1847-1855	028-B4001	G**I**GLGRVLL
	B51	A	1879-1888	066-B51	LPT**A**EDMVNL
		A	1881-1889	064-B51	T**A**EDMVNLL
	C0401	V	1733-1742	025-C0401	QFKEK**V**LGLL
NS5A	A0201	G	2321-2330	029-A0201	ALPPR**G**APPV
	A0301 A03	V, P	2382-2391	030-A0301	K**V**PPS**P**GGES
	A2601	D	2268-2276	033-A2601	ET**D**AELSVA
	A68	S	1989-1998	070-A68	WVC**T**VLSDFK

	B0702	V	2309-2317	036-B0702	APDY**V**PPTV
		V	2313-2322	035-B0702	**V**PPTVHGCAL
		T	2332-2341	034-B0702	PPRRKR**T**IQL
	B0801	I	2251-2259	037-B0801	ESETKVV**I**L
	B44	R	2097-2105	071-B44	VEVR**R**VGDF
NS5B	A0201	T	2489-2498	038-A0201	VLDDHYK**T**AL
		N	2540-2549	040-A0201	SLSSKAI**N**QI
		N	2544-2552	039-A0201	KAI**N**QIRSV
	A1101	R	2500-2509	041-A1101	EVKERAS**R**VK
	A2601	K	2537-2545	042-A2601	DVRSLSS**K**A
	B0801	I	2507-2515	043-A2601	RVKARMLT**I**
	B1501	Q	2476-2484	044-B1501	S**Q**RQKKVTF
	B5101	K	2474-2482	045-B5101	SASQRQ**K**KV

Table 6.2 HLA-associated peptides – flanking polymorphic sites

Protein	HLA	Polymorphic Site	Peptide	Amino Acids	Predicted Epitope and Wild-type Residue ()	Variant Residue
NS2	A0201	943	002-A0201	934-942	VMGGKYFQM(**I**)	V
		943	003-A0201	944-953	(**I**)ILSIGRWFNT	V
	A02	871	054-A02	861-870	AALQVWVPPL(**L**)	P
		887	053-A02	878-886	GVILLTSLL(**Y**)	H
NS3	A68	1046	058-A68	1047-1056	(**L**)GTIVTSLTGR	F
	B5101	1389	022-B5101	1379-1388	IPFYGKAIPI(**A**)	D
		1389	024-B5101	1390-1398	(**A**)QLKGGRHLI	D
NS5A	A1101	2383	031-A1101	2374-2382	DTQSSTTSK(**V**)	A
	A2402	2148	032-A2402	2138-2147	RYAPPCKPLL(**D**)	E
NS5B	B5101	2855	046-B5101	2846-2854	APTIWVRMV(**M**)	L

Polymorphic site flanking epitope is indicated in bold within brackets ().

6.2.5 Patients

HCV specific T-cell responses to HLA-associated peptides were assessed *ex vivo* in 23 subtype-3a infected patients (Table 6.3) using frozen PBMC. T-cell responses to the overlapping peptides were also assessed using PBMC from 21 of the 23 patients. Sixteen patients were tested with samples from the same timepoint; for 7 patients PBMC from the same timepoint were not available therefore a later timepoint was tested. ELISpot assays of 14 patients using overlapping peptides were performed by Annette von Delft.

Table 6.3 Subtype-3a patients

Patient	HLA					
	A1	**A2**	**B1**	**B2**	**C1**	**C2**
101	A0205	A2402	B1302	B4901	C0602	C0701
105	A1101	A1101	B3501	B5101	C0401	C1402
111	A0101	A0201	B14	B35	C0401	C0802
114	A0101	A0201	B4001	B5501	C0303	C0304
115	A0302	A2601	B3503	B5501	C01	C0401
128	A0201	A6801	B0801	B4402	C0701	C05
135	A0201	A0301	B4001	B5101	C0102	C0304
149	A0301	A2402	B1302	B1801	C0602	C05
154	A1101	A1101	B1517	B3501	C0401	C0701
216	A0201	A6801	B5101	B5108	C1502	C1602
235	A0301	A3002	B0702	B3501	C0401	C0702
236	A0301	A2601	B0801	B5201	C0702	C1202
266	A3007	A3101	B5101	B5801	C0701	C1502
267	A0201	A0301	B1402	B1801	C0501	C0802
275	A1101	A1101	B0702	B4402	C0501	C0702
281	A1101	A2910	B1402	B5201	C0802	C1202
288	A2601	A2901	B3801	B3801	C1203	C1203
291	A0101	A2901	B3501	B4403	C0401	C1601
295	A0301	A2402	B3501	B5501	C0303	C0401
297	A0101	A0301	B1801	B5501	C0102	C0602
298	A1101	A2902	B2705	B4403	C0202	C1601
437	A0201	A2601	B3801	B2702	C1203	C0102
442	A0101	A0301	B0801	B4403	C0401	C0701

6.2.6 Interferon-γ ELISpot Assays

HCV specific T-cell responses were assessed by IFN-γ ELISpot assays (see Methods 2.4.4). Peptides were pooled according to HLA type. A positive response was further tested with single peptides contained within the positive peptide pool. Final concentration of peptides was 3μg/ml. Where insufficient PBMC were available, peptides were tested in pools according to HLA alleles e.g. A02, sub-pool 1 (A02-1) contained peptides 002, 003, 047 and 048. Only HLA-associated peptides containing the wild-type residue were assessed.

6.2.7 T-Cell Lines

To assess T-cell responses that were undetectable by ELISpot assay, PBMC were stimulated with HLA-associated peptides *in vitro*. PBMCs were resuspended in R10 and plated in 24-well plate at $1x10^6$/ml. Individual peptides and pools (10μg/ml), FEC (0.3μg/ml) were added, and R10 served as unstimulated control. Pure costimulatory antibodies anti-CD28 and anti-CD49d (BD Biosciences) were added (1μg/ml). Plates were incubated at 37°C and 5% CO_2 and recombinant IL-2 was added on days 3 and 10 (final concentration 50g/ml, Sigma Aldrich). Cells were re-stimulated on day 7. On days 12-14 cells were harvested.

6.2.8 IFN-gamma Intracellular Cytokine Staining (ICS)

IFN-γ T-cell responses of cell lines were assessed using IFN-γ ICS. One million PBMC were incubated with medium alone, HCV peptides/pools (final concentration 10μg/ml), FEC (1μg/ml) or PMA (phorbol 12-myristate 13-acetate) (50ng/ml) and ionomycin (0.5μg/ml). Antibodies CD28 (1μg/ml), CD49d (1μg/ml) and Brefeldin A (10μg/ml) were added and incubated at 37°C and 5% CO_2 for 6 hours. Samples were washed, and

stained with Viaprobe to exclude dead cells (Cell viability solution, BD Biosciences; 1:25 dilution) and phycoerythrin (PE)-conjugated mouse anti-human CD8 (Miltenyi Biotech; 1:50 dilution) and incubated at 4°C for 30 mins. Samples were fixed with 2% formaldehyde and incubated at room temperature for 15 mins. Samples were permeabilised (Permeabilisation solution, BD Biosciences) and incubated at room temperature for 15 mins, prior to staining with allophycocyanin (APC)-conjugated mouse anti-human CD3 (R&D Systems; 1:50 dilution) and fluorescein isothiocyante (FITC)-conjugate mouse anti-human IFN-γ (BD Biosciences; 1:25 dilution), and incubated at 4°C for 30 mins. Samples were fixed in 1% formaldehyde, and analysed on the FACSCalibur flow cytometer. A minimum of 100,000 events in the lymphocyte gate was collected prior to analysis.

6.3 Results

6.3.1 IFN-γ Responses to CD8 HLA-Associated peptides

IFN-γ T-cell responses of 23 subtype-3a patients to CD8 HLA-associated peptides were assessed using IFN-γ ELISpot assays. CD8 peptides were matched according to each patients' specific HLA type. For analysis of polymorphic sites *within* epitopes, patients were tested with a mean of 11 peptides (total 121; range 3-22 peptides) and for polymorphic sites *flanking* T-cell epitopes a total of 21 peptides (mean 3.5, range 1-7 peptides) were assessed.

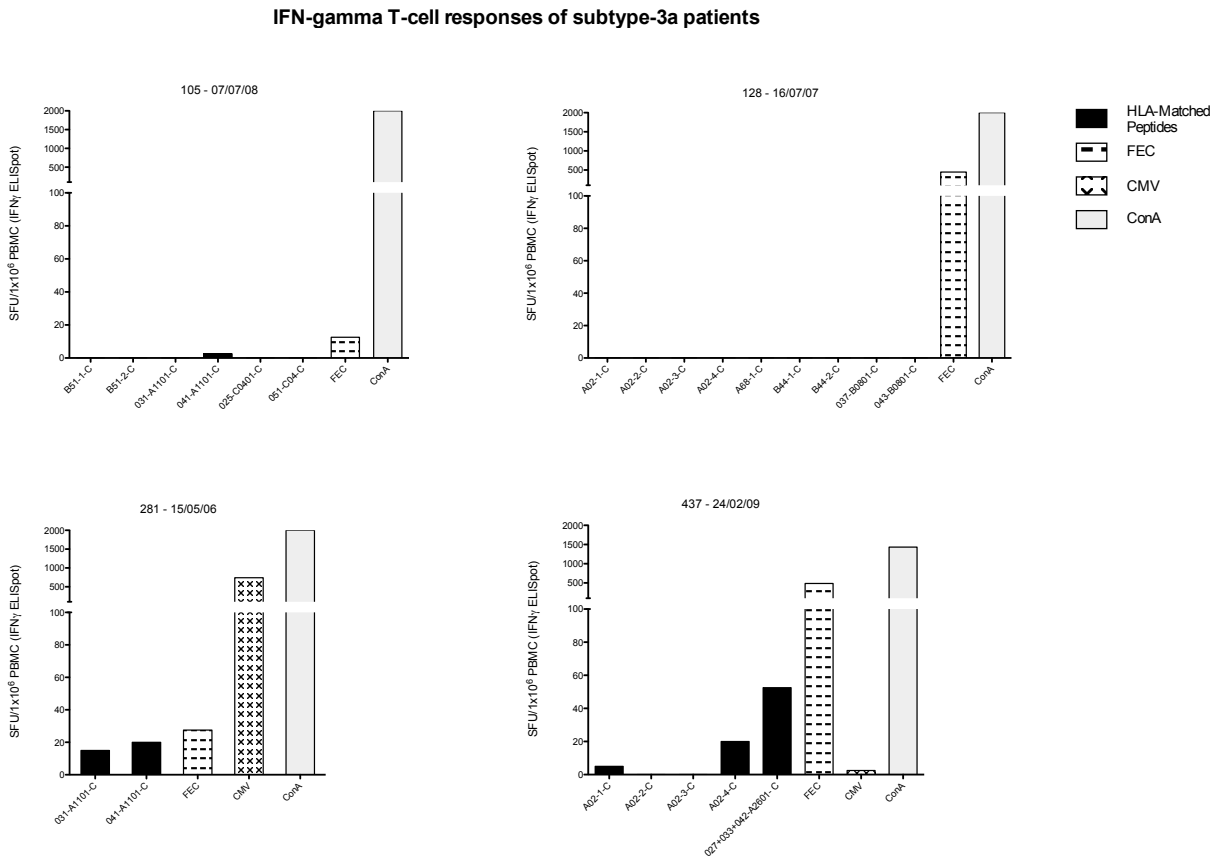
Of the 23 patients assessed, IFN-γ T-cell responses to CD8 HLA-associated peptides were detected in 6 patients (27%). Responses were very weak, with the greatest response detected in patient 437 to HLA*A2601 peptides 027, 033 and 042 with a total magnitude of 53 SFU/10^6 PBMC (See Appendix III). Examples of the responses detected are shown in Figure 6.2.

Sufficient PBMC were available for 12 patients to assess IFN-γ T-cell responses by ICS following *in vitro* expansion, as T-cell responses may have been too weak to assess *ex vivo* by ELISpot assay. The percentage of $CD3^+CD8^+IFN\text{-}\gamma^+$ lymphocytes were assessed. IFN-γ production was not detected in response to HLA-matched peptide stimulation (Figure 6.3) but was detected to control antigens PMA and ionomycin, and FEC (influenza/EBV/CMV) peptides (Figure 6.3).

Figure 6.2 IFN-γ ELISpot responses to CD8 HLA-associated peptides

IFN-γ T-cell responses to CD8 HLA-associated peptides (shown in black) in 4 representative subtype-3a patients. Dates of the samples are shown.

B51-1 - peptides 031, 022, 023, 024; B51-2 - peptides 063, 064, 066, 045, 046; A02-1 - peptides 002, 003, 047, 048; A02-2 - peptides 049, 052, 053, 054; A02-3 - peptides 015, 062, 065; A02-4 - peptides 068, 029, 038, 039, 040; A68-1 - peptides 058, 059, 067, 070; B44-1 - peptides 012, 020, 056, 057; B44-2 - peptides 060, 071, 021; A2601 – peptides 027, 033, 042. Responses to test antigens – influenza/EBV/CMV (FEC) CD8+ epitopes (shown as white bars with black hatched lines), CMV lysate (CMV) (shown as white bars with black crosses) and Concanavalin A (ConA) (grey bars), are shown when tested.



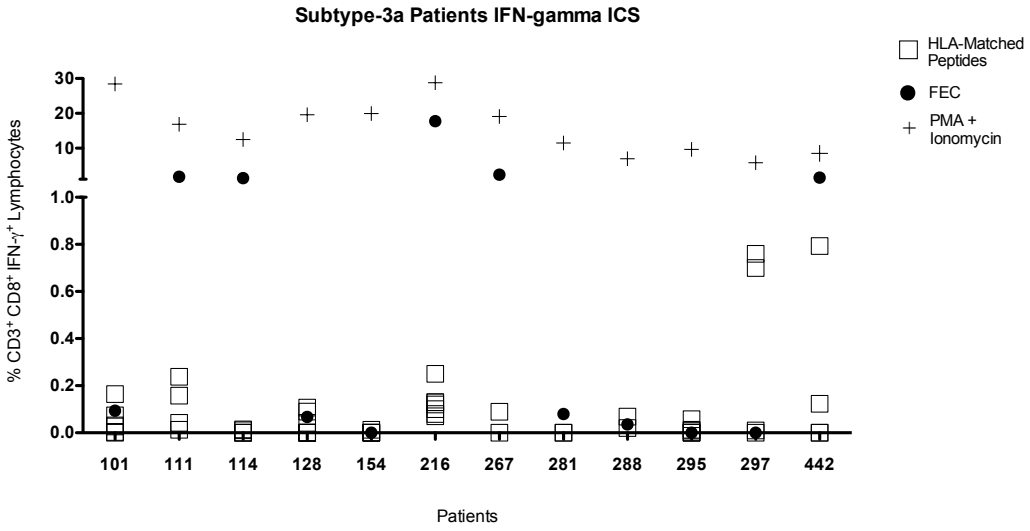


Figure 6.3 IFN-γ production after in vitro stimulation with HLA-associated peptides
Percentage of $CD3^+CD8^+$ lymphocytes producing IFN-γ following in vitro stimulation and intracellular cytokine staining. HLA-associated peptides are indicated as squares; FEC (Influenza/EBV/CMV peptides) as black circles; PMA (phorbol 12-myristate 13-acetate) and ionomycin as crosses.

6.3.2 Patient viral sequences and HLA-associated peptide sequences

The lack of T-cell responses to HLA-associated peptides may have been due to amino acid differences between peptides and circulating host viral sequence. The variant residue may be present within the circulating virus due to it being transmitted to the patient, and having already escaped the T-cell response.

Viral sequence was available for 11 patients, and was compared with 104 HLA-associated peptides tested. Of the 11 patients, the percentage of HLA-associated peptides identical to patient viral sequence ranged from 33% to 85% (mean 57.97%). Viral sequence mutations were observed in 41 peptide sequences (Table 6.4), of which 20 contained the HLA-associated variant amino acid (Table 6.4; indicated in red). The viral sequence corresponding to 7 peptides contained the wild-type HLA-associated residue, however mutations at other sites within the epitope were detected (Table 6.4; indicated in *italics*). Of the seven patients with detectable, albeit weak IFN-γ T-cell responses, the viral sequence of two patients contained the HLA-associated variant residue (indicated by asterisk; Table 6.4).

The sequences of *flanking* T-cell epitopes were assessed in six patients with available viral sequence. The viral sequence of 16/20 peptides contained the wild-type residue, with two sequences containing the variant residue (Table 6.5; indicated in red). Additional mutations within epitopes were also detected (patients 111 and 128; Table 6.5).

Table 6.4 Comparison of HLA-associated polymorphism *within* peptides and patient viral sequences

Patient	Peptide Tested	HLA-associated Peptide Sequence	Variant Residue	Patient Viral Sequence
101	068-A0205	ALPPR**G**APPV	S	ALPPR**S**APPV
111	014-A0101	ATDALMTG**Y***	F	ATDALMTG**F**
115	030-A03	K**V**PPS**P**GGES	S	KVPPS***SEE***ES
	027-A2601	**R**VLLDILAGY*	K	**K**VLLDILAGY
	033-A2601	ET**D**AELSVA	G	ETDD*T*ELSVA
	042-A2601	DVRSLSS**K**A	R	D*A*RSLSSKA
128	029-A0201	ALPPR**G**APPV	S	ALPPR**S**APPV
	062-A0201	KIMGGELPT**A**	T	KIMGGE*C*PT**T**
	065-A0201	PT**A**EDMVNLL	T	PT**T**EDMVNLL
	067-A6801	EK**A**LGLLQR	I	EK**L**LGLLQR
	070-A6801	WVC**T**VLSDFK	S	WVC**S**VLSDFK
	043-B0801	RVKARMLT**I**	M	*K*VKARMLTI
	012-B4402	ATLGAG**I**LVL	V	ATLGAG**V**L*A*L
	020-B4402	DEIASKLR**G**M	S	DE*V*ASKLRGM
	057-B4402	AG**I**LVLFGFF	V	AG**V**L*A*LFGFF
135	015-A0201	**A**LLKGGRHLI	D	A*Q*LKGGRHLI
	048-A0201	L**L**ARGSRDGV	P	LLARG*G*RDGV
	062-A0201	KIMGGELPT**A**	T	KIMGGE*F*PTA
	016-A0301	L**V**TRDADVI	I	LVTR*E*ADVI
	030-A0301	K**V**PPS**P**GGES	A, S	KVPPS**S**GGES
	013-B5101	LARG**S**RDGVI	G	LARG**G**RDGVI
	023-B5101	I**A**LLKGGRHL	D	IA*Q*LKGGRHL
	050-C0302	I**L**VLFGFFTL	M	IL*A*LFGFFTL
235	004-A0301	MLVRS**V**MGGK	A	MLVRS**I**MGGK
	006-A0301	RLCMLVRS**V**M	A	RLCMLVRS**I**M
	035-B0702	**V**PPTVHGCAL	E	**T**PPTVHGCAL
	036-B0702	APDY**V**PPTV	E	APDY**T**PPTV
236	004-A0301	MLVRS**V**MGGK	A	MLVRS**M**MGGK
	006-A0301	RLCMLVRS**V**M	A	RLCMLVRS**M**M
275	041-A1101	EVKERAS**R**VK	K	E*I*KERAS**K**VK
	020-B4402	DEIASKLR**G**M	S	DE*M*ASKLRGM
	060-B4402	NEIC**L**THPI	S	NEIC**S**THPI
	071-B4402	VEVR**R**VGDF	Q	VEVR**Q**VGDF
288	027-A2601	**R**VLLDILAGY	K	RVL*I*DILAGY
	033-A2601	ET**D**AELSVA	G	ETDD*N*ELSVA
297	014-A0101	ATDALMTG**Y**	F	ATDALMTG**F**
	030-A0301	K**V**PPSPGGES	A	KV*LPP*PGGES
298	018-B2705	IPFYGKAIP**L**	I	IPFYGKAIP**I**
	020-B4403	DEIASKLR**G**M	S	DE*M*ASKLRGM
	060-B4403	NEIC**L**THPI	S	NEIC**S**THPI
	071-B4403	VEVR**R**VGDF	Q	VEVR**Q**VGDF

The HLA-associated polymorphic residue is indicated in bold. Within the patient viral sequence, an amino acid mutation at the polymorphic sites are shown in bold and red, and additional amino acid mutations are indicated in italics. Asterisk indicates IFN-γ T-cell response detected by the HLA-associated peptide using IFN-γ ELISpot assay.

Table 6.5 Comparison of HLA-associated polymorphisms *flanking* peptides and patient viral Sequence

Patient	Peptide Tested	Amino Acids	HLA-associated Peptide Sequence and Wild-type Residue ()	HLA-Associated Variant Residue	Patient Viral Sequence
101	053-A02	878-886	GVILLTSLL(Y)	H	Y
	054-A02	861-870	AALQVWVPPL(Y)	P	L
111	002-A0201	934-942	VMGGKYFQM(I)	V	I V*T*GGKYFQM
	003-A0201	944-953	(I)ILSIGRWFNT	V	I
	053-A02	878-886	GVILLTSLL(Y)	H	Y
	054-A02	861-870	AALQVWVPPL(Y)	P	L
128	002-A0201	934-942	VMGGKYFQM(I)	V	I
	003-A0201	944-953	(I)ILSIGRWFNT	V	I
	053-A02	878-886	GVILLTSLL(Y)	H	Y
	054-A02	861-870	AALQVWVPPL(Y)	P	L *S*ALQVWVPPL
	058-A6801	1047-1056	(L)GTIVTSLTGR	F	L
135	002-A0201	934-942	VMGGKYFQM(I)	V	A
	003-A0201	944-953	(I)ILSIGRWFNT	V	A
	053-A02	878-886	GVILLTSLL(Y)	H	Y
	054-A02	861-870	AALQVWVPPL(Y)	P	L
	022-B5101	1379-1388	IPFYGKAIPI(A)	D	A
	024-B5101	1390-1398	(A)QLKGGRHLI	D	A
	046-B5101	2846-2854	APTIWVRMV(M)	L	M
275	031-A1101	2374-2382	DTQSSTTSK(V)	A	**A**
298	031-A1101	2374-2382	DTQSSTTSK(V)	A	**A**

The polymorphic residue is indicated in brackets (). The patient viral sequence at the polymorphic site is shown. Epitopes containing additional mutations are also shown in italics.

6.3.3 Overlapping peptide IFN-γ ELISpots

IFN-γ T-cell responses of 20 patients were assessed using subtype-3a overlapping peptides, representing peptides from NS2 to NS5b proteins to determine whether the responses to HLA-associated peptides could also be predicted using overlapping peptides. Subtype-3a specific IFN-γ T-cell responses to overlapping peptides were detected in 8/20 patients with responses ranging from 40-273 SFU/10^6 PBMC (mean magnitude 116 SFU/10^6 PBMC) (See Appendix IV). Responses were detected to more than 1 peptide pool in 50% of patients, with the majority of responses detected to NS3 helicase. Responses were also detected to NS5a, NS5b protein (amino acids 2431-2736, NS5BI) and NS3 protease. Examples of the IFN-γ T-cell responses detected in 4 patients are shown in Figure 6.4. Of the 8 patients with a response to overlapping peptides, only patient 115 also had a T-cell response to CD8 HLA-associated peptides. However the HLA-associated peptide represented an NS4b epitope whereas the overlapping peptides were from NS5a and NS5b (aa 2431-2736).

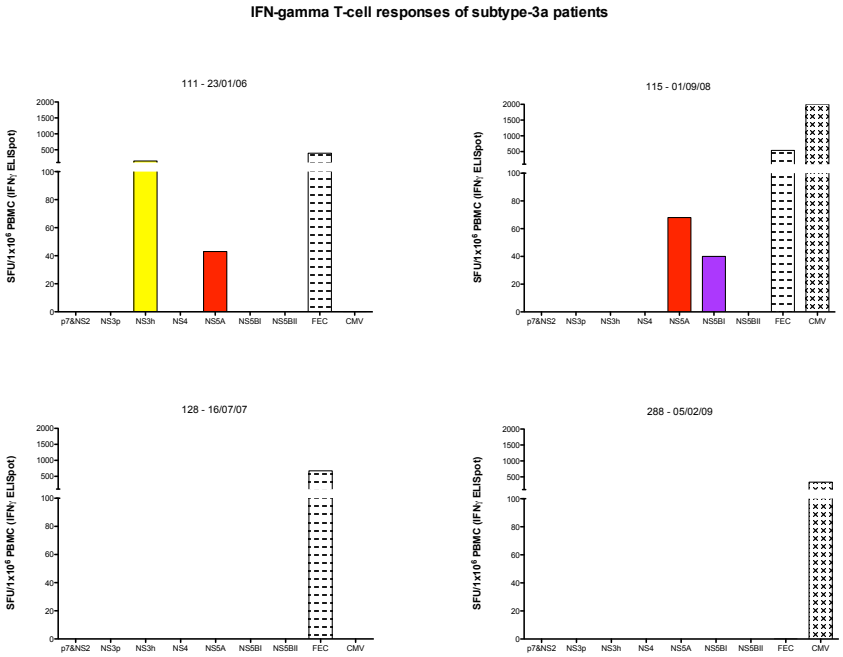


Figure 6.4 IFN-γ ELISpots to subtype-3a overlapping peptides
IFN-γ ELISpot responses to overlapping peptides in 4 representative patients. Dates of samples are shown. Responses to control antigens FEC (influenza/EBV/CMV) peptides and CMV lysate are also shown.

6.3.4 Comparison of HLA-matched and Overlapping Peptide Sequences

The overlapping peptide set was designed based on consensus viral sequences and therefore may contain variant amino acids i.e. escape mutations. As a result, T-cell responses to these peptides may not be detected. The sequences of the HLA-associated peptides and overlapping peptides were compared to determine sequence differences.

Only 2 overlapping peptides contained amino acid differences at polymorphic sites. Overlapping peptide P.209 contained the variant isoleucine residue associated with HLA 018-B2705, (Table 6.6; indicated in red), whilst overlapping peptide P.262 corresponding to HLA 067-A68 contained neither the consensus nor variant residue. Additional mutations were found within the overlapping peptides when compared with the HLA-associated peptide sequence (Table 6.6; indicated by *italics*). All the overlapping peptides contained the wild-type *flanking* polymorphic residues.

Table 6.6 Comparison of HLA-associated and overlapping peptide sequences

Protein	Polymorphic Residue	HLA Peptide	HLA-Associated Peptide Sequence	Overlapping Peptide	Overlapping Peptide Sequence	HLA-Associated Consensus or Variant amino acid	Additional Mutations
NS2	828	012-B4402	ATLGAG**I**LVL	P.124 P.125	ATLGAGI**L***A*LFGFFTL GI**L***A*LFGFFTLSPWYKHW	Consensus	V→A at position 9
		057-B44	AG**I**LVLFGFF				
		050-C03	I**L**VLFGFFTL				
NS3	1388	018-B2705	IPFYGKAIP**L**	P.209	EGEIPFYGKAIP**I**AQLK	Variant	None
	1389	015-A0201	**A**LLKGGRHLI	P.210 P.211	YGKAIPI**A***Q*LKGGRHLIF **A***Q*LKGGRHLIFCHSKKK	Consensus	L→Q mutation
		023-B5101	I**A**LLKGGRHL				
NS4	1738	067-A68	EK**A**LGLLQR	P.262	HQFKEK**V**LGLLQRATQQQA	Neither	None
NS5a	2270	033-A2601	ET**D**AELSVA	P.344	PLRAET**D**DAELS*A*AAECF	Consensus	V→A mutation. Extra amino acid.

Polymorphic sites are indicated in bold. Variant residues within overlapping peptides are indicated in red. Additional mutations are indicated in italics.

6.4 Discussion

HCV-specific T-cells are important in eliminating HCV infection, with acute infection characterised by strong and multi-specific responses. However progression to chronic infection is characterised by weak and narrowly focused HCV-specific T-cell responses. Viral escape mutations within HCV-specific CD8+ T-cell epitopes have been associated with HCV persistence (Parker et al., 1994, Erickson et al., 2001, Weiner et al., 1995, Cox et al., 2005b, Tester et al., 2005).

In this study, I have used a novel approach to identify subtype-3a T-cell responses using predicted epitopes containing HLA-associated polymorphic sites. The ability to detect these epitope responses using subtype-3a overlapping peptides covering NS2-NS5b proteins was also assessed. HCV-specific T-cell responses to HLA-associated peptides were very weak and detected in few subtype-3a patients.

The lack of T-cell responses to the HLA-associated peptides may have been due to T-cell responses that were too weak to detect *ex vivo*, due to exhausted function or impaired IFN-γ secretion, as previously found in genotype-1 infection (Bowen and Walker, 2005b, Penna et al., 2007, Urbani et al., 2006). However the inability to recover HLA-associated responses after *in vitro* peptide stimulation suggests that responses may have been exhausted irreversibly, deleted within the tolerogenic liver environment or were no longer able to proliferate (Radziewicz et al., 2007, Gruener et al., 2001, Wedemeyer et al., 2002, Bowen and Walker, 2005b). A lack of response may also be due to T-cells sequestered in the liver and hence not detectable within the blood, as previously seen (Penna et al., 2007, He et al., 1999). Alternatively, the lack of responses may be a result of assessing an insufficient number of peptides, as for some patients only a limited number of HLA-associated peptides were available (Valiante et al., 2000).

As the HLA-associated peptides contained sites of polymorphism, patients' circulating virus was assessed to determine whether the virus contained the variant, i.e. escaped amino acid. Viral sequencing revealed that 70% of patient viral sequences contained the HLA-associated variant, suggesting that immune pressure by weak and ultimately undetectable CD8+ T-cell responses result in an escape mutation although are incapable of clearing HCV infection. A T-cell response was detected in a subtype-3a patient to the NS3 1442-1450 HLA-A*0101 epitope. However sequencing of the circulating virus revealed the presence of the variant amino acid within the epitope, suggesting that within this patient the NS3 1442-1450 epitope had escaped the immune response, and the detectable T-cell response is not beneficial. In the chimpanzee model, the emergence of escape mutations within CD8+ epitopes were observed in the presence of a weak CD8+ response due to a lack of CD4+ T-cell help (Lauer et al., 2002). Additionally, the transmission of an escape residue to a patient without the restricting allele would result in reversion of the residue to the wild-type amino acid (Grakoui et al., 2003, Tester et al., 2005, Timm et al., 2004, Ray et al., 2005, Cox et al., 2005b). Therefore viral sequencing is important in determining whether detectable immune responses are effective against the circulating virus or if an epitope has escaped the T-cell response, rendering it ineffective (Merani et al., 2011).

Viral sequencing also revealed additional mutations within HLA-associated epitopes. These mutations may be as a result to the increased immune pressure exerted on the epitope compared with other non-targeted regions of the polyprotein, as previously found in genotype-1 and -3 (Neumann-Haefelin et al., 2008). The additional mutations may be compensatory mutations that can occur within or flanking an epitope and facilitate the generation of an escape mutation at a region that is constrained, seen previously in HCV,

HIV and simian immunodeficiency virus (SIV) infections (Rauch et al., 2009, Fitzmaurice et al., 2011, Kelleher et al., 2001).

T-cell responses to overlapping peptides did not correspond with responses to HLA-associated peptides. Only 1 overlapping peptide contained the associated escape mutation, indicating its restriction by a common HLA-allele, and only 7 overlapping peptides contained non-HLA-ssociated amino acid differences. However the lack of responses to both peptide sets may be due to the design of the peptide sets. The HLA-associated peptides respresented optimal epitopes whilst the overlapping peptides, designed to be 15-19aa may have resulted in weaker i.e. less optimal and possibly undetectable T-cell responses. Nevertheless differences in detectable responses were likely as each technique was designed to detect different types of epitopes, such that HLA-associated peptides were based on viral polymorphisms driven by T-cell immune pressure, whereas the overlapping peptides were based on a consensus sequence and therefore more likely to detect responses to conserved regions of the polyprotein.

Unfortunately there were limitations to the study design and statistical analysis of the initial HLA-associations, that may have resulted in the detection of very few and weak T-cell responses. The correct use of statistical methods is important to avoid both false-positive and false-negative associations.

The identified HLA-polymorphic site associations may have been due to a founder effect of a common viral ancestor instead of immune pressure exerted by a specific allele. The phylogenetic correction method to adjust for this confounding effect was based on clustering the data (Peyerl et al., 2003). However limitations of this correction is that if the sample is already biased then the results may still be biased after the correction, and that the efficiency of this method is dependent on the sample size (Kaufman and Rousseeuw, 1990). Additionally, the corrections are based on estimations and therefore

an element of the confounding founder effect may remain (Kaufman and Rousseeuw, 1990).

Multiple statistical tests were also applied in identyfing significant associations, which therefore requires the application of a correction or compensation step to account for the multiple inferences made. One such method of correction is to determine the q-value. The q-value is the expected proportion of false positives of all the significant associations identified (Rauch et al., 2009). The q-value aims to provide a balance between the identification of as many true positive associations whilst including a relatively low number of false positives. In this study, the q-value was ≤0.2, indicating that up to 20% of the significant associations identified may be false positive and hence may account for the lack of T-cell responses detected in this study. It is likely that the 0.2 q-value allowed the identification of all possible true associations, which could then be assessed in further studies, such as the T-cell ELISpots in this study (Storey and Tibshirani, 2003).

Another limitation is due to the linkage disequilibrium of the MHC, defined as the non-random association of alleles at two or more loci. Therefore an identified HLA-association may in effect be true for another allele through linkage disequilibrium (Storey and Tibshirani, 2003), and as such it is usually not possible to identify the specific allele that drives immune escape. It has been suggested that HLA linkage disequilibrium is as important as phylogenetic relatedness in avoiding false positive associations (Gaudieri et al., 2006).

Associations were determined for HLA alleles at 2-digit and 4-digit resolutions. However certain HLA alleles, even those that differ by a single amino acid as A*0201 and A*0204 have different binding preferences and hence bind different peptides (Carlson et al., 2008, Sudo et al., 1995). Therefore to classify alleles as 2-digit would confound results, as shown previously in studies of HIV infection (Barouch et al., 1995). To overcome this

problem, prior knowledge of binding motifs would be needed to identify alleles that could be assessed at either 2-digit or 4-digit resolution, or if this is not possible, all HLA-alleles should be assessed at 4-digit resolution (Carlson et al., 2008).

Following the identification of HLA-associated polymorphisms, potential epitopes containing polymorphic sites were predicted using prediction algorithms, SYFPEITHI (Leslie et al., 2008) and BIMAS (Rammensee et al., 1999). However there are limitations for these prediction algorithms also. These prediction algorithms are matrix based and score residues within the peptide based on previous publications of T-cell epitopes and MHC ligands. SYFPEITHI takes into consideration anchor positions and also assigns negative scores to amino acids that are disadvantageous for the binding capacity of a specific position (Parker et al., 1994). However a limitation of the BIMAS scoring system is that it is based on the assumption that each amino acid contributes independently to the binding capacity of the peptide, when in fact the binding ability of one amino acid, can affect the binding of amino acids within the peptide (Rammensee et al., 1999, Buus, 1999). Consequently BIMAS may not be able to predict optimal peptide sequences. I determined the epitopes based on scores obtained from both prediction algorithms, allowing a greater efficiency in predicting potential epitopes. Nevertheless due to the limitations above, inefficient epitopes may have been generated, resulting in lack of responses. As these prediction algorithms are based on the most common previously identified epitopes, they are limited to predicting similar lengths of peptides (Leggatt et al., 1998) and their reliance on published data results in the inclusion of MHC alleles for which a large amount of data is available.

Recently more accurate prediction algorithms have been developed that also take into consideration other steps of the antigen processing and presentation pathway, such as MAPP (http://www.mpiib-berlin.mpg.de/MAPPP/) (Lundegaard et al., 2010) and

NetCTL (http://www.cbs.dtu.dk/services/NetCTL) (Hakenberg et al., 2003), which have been shown to outperform SYFPEITHI and BIMAS (Larsen et al., 2005, Lundegaard et al., 2006). Therefore use of more recent prediction program such as MAPP, or a combination of prediction algorithms would obtain a greater efficiency of epitope predictions.

In conclusion, overlapping peptides detected a greater number of HCV-specific T-cell responses and therefore may be more efficient to identify new T-cell responses. Whilst HLA-associated polymorphisms provide valuable information on viral escape from the immune response, the methods used to identify these associations and subsequent peptide predictions are crucial in obtaining accurate associations and peptides for use in further assays. Additional studies of T-cell responses to both peptide sets during acute infection and in spontaneously resolved patients would also be valuable.

Chapter 7 - Conclusions

Subtype-3a HCV infection is now the dominant genotype in newly diagnosed infections within the UK. Its increasing prevalence and its favourable treatment outcome emphasises the importance of gaining further insight into this subtype and the reasons(s) for the higher response rate to treatment. Subtype-3a HCV infection is largely unexplored therefore the reason(s) for the favourable response to treatment may relate to viral genetic and phenotypic differences between strains, to differences in the hosts' ability to exert an effective immune response against particular viral sequences, or a combination of both factors. In this thesis I have assessed both viral and host factors that may be involved in the subtype-3a treatment response.

Firstly I have identified novel hypervariable regions within the E2 protein that are unique for subtype-3a HCV (chapter 3). These sites may act as antigenic determinants and as such provide an additional target for the antibody response. Additionally, the lack of sequence variability within these sites suggests they may not be capable of escaping the immune response as efficiently as HVR1, resulting in a greater effectiveness in the antibody clearance of HCV. These novel hypervariable sites are under early selection pressure suggesting possible unique function for subtype-3a E2 protein. The 5-amino acid insertion within HVR575 is likely to have an effect on the structure and conformation of subtype-3a E2 protein, and consequently may affect the formation of E1E2 heterodimers, binding to cellular proteins and cell entry. These novel regions suggest that the E2 protein of subtype-3a infection is distinct in structure and function. Further studies are needed to elucidate the function of the hypervariable regions, and the efficiency of antibodies in recognising these regions.

Viral subtype-3a quasispecies before initiation of treatment does not predict treatment outcome and was not associated with a specific viral sequence, in contrast to a previous study of subtype-3a patients and studies of genotype-1 patients. However I observed a trend towards significantly lower viral complexity in patients that cleared virus. Viral persistence was not associated with a specific viral sequence. Hence viral quasispecies should be assessed in a larger cohort of subtype-3a patients, including a greater number of patients that have failed treatment. However due to the favourable treatment rates in subtype-3a, a cohort of patients will unlikely contain more than 30% of patients that fail treatment.

Interferon insensitive strains were present in only a subset of patients that failed treatment, suggesting that failure to clear infection is not exclusively due to the selection of a resistant strain during treatment. This also suggests that there are different mechanisms of viral resistance present within subtype-3a infection. However analysis of the HVR1 sequence does not provide information on the mechanism of resistance, which may be due to specific sequence found elsewhere in the genome. Further longitudinal studies are needed of patients undergoing treatment to determine the pattern of resistance.

The continual variation of quasispecies during natural infection demonstrates that the viral population changes in the absence of treatment suggesting that there is a greater viral diversity within the host than that detected within the plasma, or that the ongoing immune response despite being incapable of clearing virus, exerts pressure resulting in sequence change. Studies of quasispecies within a subtype-3a infected liver would provide a better understanding of viral diversity in subtype-3a infection, as the continual change of strains in the plasma may represent variants within the liver.

Another viral factor that has been controversially associated with treatment outcome is the E2 PePHD sequence. I did not observe such an association in this subtype-3a cohort

suggesting that PePHD in subtype-3a is not exclusively responsible for the higher treatment response rates and that other viral factors are also involved. As mutations were predominantly found at 2 different sites within the PePHD sequence, further studies are needed to determine the effect of different mutations on the binding ability of subtype-3a E2 protein to PKR.

Host factors such as HCV-specific T-cell responses may also be responsible for the higher response rate of treatment of subtype-3a infection. T-cell responses were readily detectable in subtype-3a chronic infection. The majority of CD8 responses targeted non-structural proteins, in contrast to the predominant core responses in genotype-1 infection, suggesting that T-cell responses target different viral proteins in different genotypes. The responses of acutely infected and spontaneously resolved subtype-3a patients should also be assessed to compare the magnitude of responses and epitopes targeted within these patients. Pre-treatment responses were higher in SVR patients but did not reach statistical significance supporting the hypothesis that the magnitude of the pre-treatment response determines treatment outcome. During treatment, subtype-3a T-cell responses decreased, in contrast to the hypothesis that favourable treatment response was due to an enhancement of subtype-3a T-cell responses during treatment. The decline was unlikely due to the treatment-induced decrease in viral load due to a decrease in non-specific T-cell responses and total lymphocyte counts in SVR patients, and suggests that patients achieving SVR may be more sensitive to interferon. It is known that a pre-activated host response i.e. activation of interferon stimulated genes, results in treatment failure as the host response does not respond to exogenous interferon. This may also be true for treatment-induced lymphopenia that was only observed in patients that successfully

cleared viral infection and corroborates the importance of identifying host factors that may be predictive of treatment such as the IL28B allele.

HLA-associated and overlapping peptides identified different T-cell responses in subtype-3a patients. However the identification of HLA-assocations with polymorphic sites and subsequent peptide design are vital in obtaining accurate results. It would be valuable to assess the phenotype of the HLA-associated T-cell responses, to determine if they are exhausted or impaired. HLA-associated peptides should also be assessed in acutely infected patients where T-cell responses are known to be stronger in magnitude and target numerous epitopes.

I have assessed both viral and host factors that may be predictive of treatment outcome of subtype-3a infection and have found that subtype-3a has distinct characteristics to other HCV genotypes, such as hypervariable regions within E2, and in terms of host immune response to this prevalent HCV subtype. These results also emphasise the complex interplay between virus and host factors that are involved in the response to HCV treatment.

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Appendix

Appendix I

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Full-Length Characterization of Hepatitis C Virus Subtype 3a Reveals Novel Hypervariable Regions under Positive Selection during Acute Infection▽

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Hepatitis C virus subtype 3a is a highly prevalent and globally distributed strain that is often associated with infection via injection drug use. This subtype exhibits particular phenotypic characteristics. In spite of this, detailed genetic analysis of this subtype has rarely been performed. We performed full-length viral sequence analysis in 18 patients with chronic HCV subtype 3a infection and assessed genomic viral variability in comparison to other HCV subtypes. Two novel regions of intragenotypic hypervariability within the envelope protein E2, of HCV genotype 3a, were identified. We named these regions HVR495 and HVR575. They consisted of flanking conserved hydrophobic amino acids and central variable residues. A 5-amino-acid insertion found only in genotype 3a and a putative glycosylation site is contained within HVR575. Evolutionary analysis of E2 showed that positively selected sites within genotype 3a infection were largely restricted to HVR1, HVR495, and HVR575. Further analysis of clonal viral populations within single hosts showed that viral variation within HVR495 and HVR575 were subject to intrahost positive selecting forces. Longitudinal analysis of four patients with acute HCV subtype 3a infection sampled at multiple time points showed that positively selected mutations within HVR495 and HVR575 arose early during primary infection. HVR495 and HVR575 were not present in HCV subtypes 1a, 1b, 2a, or 6a. Some variability that was not subject to positive selection was present in subtype 4a HVR575. Further defining the functional significance of these regions may have important implications for genotype 3a E2 virus-receptor interactions and for vaccine studies that aim to induce cross-reactive anti-E2 antibodies.

Hepatitis C virus (HCV) infection is a major global health issue leading to persistent viral infection in the majority of those infected and is associated with progressive liver disease, cirrhosis, and hepatocellular carcinoma. Six major genotypes of HCV have been described that have evolved in geographically distinct regions and that share approximately. 80% nucleotide homology with one another. HCV viral genotypes have been further classified into subtypes (25). HCV subtype 3a infection is now the most common subtype in the United Kingdom (11), although it is globally distributed and frequently associated with intravenous drug use.

The classification of HCV viral strains by genotype and subtype has proven informative not only in terms of the epidemic and evolutionary history of the virus but also in terms of clinical outcomes. In particular, the response rates to current gold standard therapy (9) and the prevalence of hepatic steatosis (20) are significantly higher for subtype 3a than for genotype 1 infections. The reasons for this are not understood but must relate to viral genetic and phenotypic differences between strains, or to differences in the ability of hosts to exert an effective immune response against particular viral sequences, or to a combination of both factors.

To date, detailed assessment of the HCV genome has largely focused on HCV genotype 1. Indeed, only a few full-length HCV subtype 3a viral sequences are currently published and available within the major HCV databases (Los Alamos; http://hcv.lanl.gov/components/hcv-db/combined_search/searchi.html and euHCVdb; http://euhcvdb.ibcp.fr/euHCVdb/) (16).

To characterize HCV subtype 3a in detail, we performed whole-genome analysis of a cohort of patients with persistent HCV subtype 3a infection. We subsequently focus on the highly variable regions observed in the envelope protein E2 in both acute and chronic infection, since it was apparent that these regions were not restricted to the well-documented hypervariable region 1 (HVR1) that is found at the 5′ end of E2 in all HCV genotypes.

Viral genomic variability can be assessed at a number of different levels; first, intergenotypic variability may arise in genomic regions that are conserved within the same subtype but are distinct between subtypes. Second, there is intragenotypic variability, which may be defined as regions of viral variability within the same genotype or subtype. Finally, intrahost

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TABLE 1. Primers used to obtain viral sequence of HCV subtype 3a

Primer	Sequence (5′–3′)	Binding site (nt)[a]	Source or reference
F4 For	TGGGATGGGCGCTGAAATGGGA	2470	In-house
F4 Rev	CTGGGTAGCCGTAGAAAGCACCT	3520	In-house
F5 For	ACAGCATACGCCCAGCAAACTAGG	3429	In-house
F5 Rev	TAGAATGTGGCACAGTGATGCTGC	4399	In-house
F6 For	GATGAATGTCATGCCCAAGACGCTAC	4287	In-house
F6 Rev	GCCATGATGTATTTTGTGATGGGGTGTG	5254	In-house
F7 For	TGTCTCGTGCGGCTTAAGCCAA	5169	In-house
F7 Rev	GTGACAGTTAGAGAACTCAGCAATG	6178	In-house
F8 For	GGAGGGAGCGGTACAGTGGATGA	6068	In-house
F8 Rev	CACAACCTTTGTTTCAGACTCCACCCG	7068	In-house
F9 For	TGAGCTAGTGGACGCCAACTTGTTATG	7013	In-house
F9 Rev	GTTCTTCGCCATGATGGTGGTTGGAAT	8001	In-house
F10 For	CGAAGTTCGGGTATAGTGCGAAGGA	7897	In-house
F10 Rev	TGCCCGATGTCTCCAAGCTCGTA	9091	In-house
HCV_2412 F2	CACCTCCACCARAACATYGT	2412	In-house
HCV_9192R	GGAGTGAGTTTGAGCTTGGT	9195	In-house
UTR_277-For	CCTTGTGGTACTGCCTGATAG	279	2
977-Rev	GTCHTCRGCCTCATACACAAT	975	2
745-For	TACATCCCGCTCGTCGGC	747	In-house
E2-1585-Rev	ATGTGCCACGAGCCATTGGT	1587	2
1435-For	GGCAACTGGGCCAAGGTCGC	1437	In-house
2982-Rev	ATAAAGCAGGCTTGTTAG	2967	In-house
2237-For	TCAAGGTGAGGATGTTTGTG	2221	In-house
2340-Rev	GAATGCAGCAGCGGATGTTGC	2324	In-house
Utr-246.for	GACTGCTAGCCGAGTAGTGTTG	248	17
NS4a-5315.rev	CGACCTCYARGTCNGCYCACATRC	5310	17
outerCE1.for	ATGATGATGAACTGGTCNCCYAC	1308	In-house
BlyR1.4	CTAYCAGCARCATCATCC	2251	31

[a] nt, nucleotide.

variability is where viral genomic variability occurs within the same viral subtype and also the same host when individual clonal sequences are assessed. Although intergenotypic variability may simply be a feature of the existence of geographically distinct HCV subtypes, intragenotypic and intrahost variability may reflect viral regions subject to specific selection pressures, with important functional implications.

We observed two distinct regions of intrahost and intragenotypic hypervariability within genotype 3a envelope 2 (E2)—in addition to the previously described HVR1—that we have named HVR495 and HVR575. We show that these regions are subject to positive selection pressure, sometimes very early in acute infection. Although HVR575 has been previously recognized as a site of intergenotypic variation (18), the identification of this region as a hypervariable site within genotype 3a and as a site under early selection pressure leading to variability within the same host has not been previously described.

MATERIALS AND METHODS

Patients. Plasma samples were obtained and immediately stored at −80°C from 40 treatment naive patients with chronic HCV infection; 18 with subtype 3a, 13 with subtype 1a, and 9 with subtype 1b (John Radcliffe Hospital, Oxford, United Kingdom) and from 4 patients with acute subtype 3a infection sampled at multiple time points longitudinally (San Bortolo Hospital, Vicenza, Italy). Acute HCV was defined as an alanine transaminase (ALT) of >1,000 IU/ml with detectable HCV RNA in the presence of specific risk factors for acute HCV infection (three patients reported a history of recent intravenous drug use, and one had recently undergone orthopedic surgery) and the absence of any other cause of an acute hepatitis. In two of four patients the development of HCV antibody seroconversion was also demonstrated. Local ethical approval was obtained, and all patients gave written informed consent for study participation.

Viral RNA extraction and sequencing. Plasma (500 μl) was concentrated by high-speed centrifugation (23,600 × *g* for 1 h) at 4°C. Viral RNA was extracted by using a QIAmp viral RNA minikit (Qiagen). Reverse transcription (RT) and first-round PCR were performed in a single reaction (Superscript III OneStep RT-PCR system with Platinum *Taq* enzyme; Invitrogen). Subtype 3a, 1a, and 1b specific primers included both previously described and newly designed in-house primers (Table 1). For subtype 3a, primers 277-For and F4-Rev amplified a 4-kb product that coded Core, E1, and E2 structural proteins. Primers 2412F and 9192R amplified a 7-kb product that coded nonstructural proteins (NS2 to NS5). The RT-PCR cycling conditions were as follows: 55°C for 30 min and 94°C for 2 min, followed by 39 cycles of 94°C for 15 s, 60°C and 58°C for 30 s (7- and 4-kb reactions, respectively), 68°C for 1 min/kb, and a final extension of 68°C for 10 min. Second-round PCR used High Fidelity *Taq* DNA polymerase (Roche), in nested PCRs (Core, 277-For and 977-Re [600 bp]; E1-745-For and 1585-Rev [840 bp]; E2-1435-For and 2982-Rev [1,547 bp]; F4–F10 primer pairs [~1 kb each]). PCR conditions were set according to the manufacturer's instructions. For genotype 1 samples, primers UTR-246.for and NS4a-5315.rev amplified a 5,063-bp fragment (17). A second-round PCR was performed to produce a 1,260-bp fragment of E2 using the inner primers outerCE1.for and BlyR1.4 (31). Second-round PCR fragments were gel purified (Qiagen) and sequenced bidirectionally with second-round PCR inner primers and additional inner primers using Prism BigDye (Applied Biosystems) on an ABI 3100 DNA automated sequencer. Each sequence was edited by using X11 software and aligned by using Se-Al (http://tree.bio.ed.ac.uk) to obtain full-length genomic sequence for each subtype 3a patient and E2 sequences of genotype 1 patients.

E2 cloning. The entire E2 PCR product (1,105 nucleotides) was cloned (TOPO TA cloning kit [Invitrogen]) for patients with acute genotype 3a HCV infection (It13, It14, It16, and It17) at multiple (n = 4 to 6) time points (It13 [total 75 clones, mean number of clones per time point = 19]; It14 [total 91 clones, mean number clones per time point = 20.5; It16 [total 85 clones, mean number clones per time point = 22.3]; It17 [total 109 clones, mean number clones per time point 18.1]). The entire E2 protein was also cloned for patients with chronic HCV infection; 9 with subtype 3a, 9 with subtype 1a, and 7 with subtype 1b (6 to 26 clones per patient; mean = 18.7 clones). Colonies were grown overnight and plasmid DNA purified by using a Montage plasmid miniprep kit

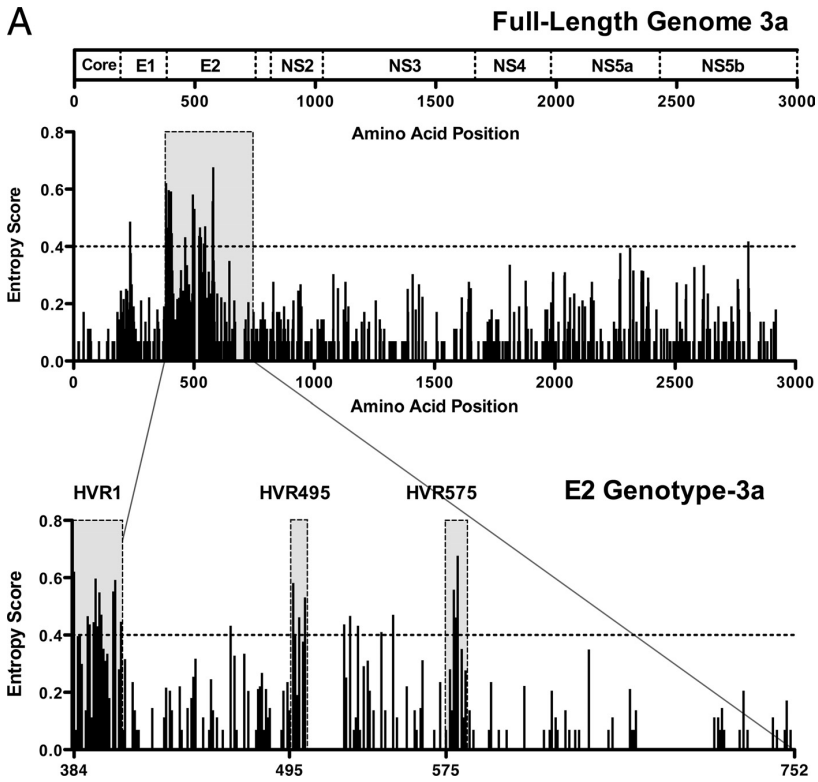

FIG. 1. Sequence variability across the HCV subtype 3a genome identifies two novel HVRs within E2. (A) The entropy score (a mathematical measure of variability) at each amino acid site following full-length viral genome sequence analysis of 18 patients with chronic HCV genotype 3a infection is shown. Each bar represents variability at a single amino acid site. The corresponding HCV subtype 3a genome map is given above. Analysis of E2 subtype 3a shows the HVR1 at the N-terminal domain of E2, in addition to two novel hypervariable regions (HVR495 and HVR575). (B) For comparison, we show the E2 entropy scores from 31 patients with HCV subtype 1a, 27 patients with subtype 1b, 20 patients with subtype 2a, 15 patients with subtype 4a, and 15 patients with subtype 6a infection (sequences were determined in house with additional sequences derived from the Los Alamos and euHCVdb HCV databases).

(Millipore). EcoRI digestion identified positive clones, which were sequenced bidirectionally using M13For, M13Rev, and additional internal primers. Sequences were aligned and edited by using Sequencher software (GeneCodes Corp.).

Entropy and diversity measurement. A mathematical measure of entropy was used to evaluate the sequence diversity of the full-length HCV sequences from 18 chronically infected genotype 3a patients and the E2 sequences from 32 patients with subtype 1a, 27 patients with subtype 1b, 20 patients with subtype 2a, 15 patients with subtype 4a, and 15 patients with subtype 6a sequences (derived from in-house bulk sequencing or consensus sequence from multiple clones, with additional sequences from the Los Alamos and euHCVdb HCV databases). Entropy values for each amino acid position were calculated by using the Shannon Heterogeneity In Alignments Tool v1.0 (http://evolve.zoo.ox.ac.uk/software). This program computes the Shannon (23) information entropy score, E, for each codon as follows:

$$E = -\sum_{i=1}^{n} p_i \log_n p_i$$

where p_i is the proportion of sequences that contain residue i at the codon in question. In this analysis there are $n = 21$ types of residue (20 amino acids plus the stop codons).

Selection analysis. The program CODEML was used to identify amino-acid sites that had undergone positive selection (Yang et al. [30]; http://abacus.gene.ucl.ac.uk), both in the patients with chronic infection through analysis of E2 bulk sequences and in each acutely infected individual, through analysis of all E2 clonal sequences obtained from each individual patient. The maximum-likelihood method implemented in the CODEML program fits various models of codon evolution to sequence data connected by a phylogenetic tree and considers selection pressures at individual codon sites. The models of codon evolution differ in their distribution of dN/dS values among codons. CODEML detects selection by calculating ω, the ratio of nonsynonymous to synonymous nucleotide substitution. Positive selection at a codon is signified by a ω value that is greater than 1. A likelihood ratio test between the M7 and M8 models was performed in order to test for the presence of selected sites (χ^2 distribution with 2 degrees of freedom and $P \leq 0.05$) (30). An empirical Bayesian approach, also available in CODEML, was then used to identify individual codons subject to positive selection, with a posterior probability of >90% taken to indicate positive selection.

Nomenclature. The amino acid nomenclature throughout the manuscript conforms to the system proposed by the Los Alamos database group (15). Amino acids are numbered relative to genotype 1a H77, and genotype 3a insertions are designated with a lowercase letter.

Accession numbers. Full-length HCV subtype 3a sequences (GQ356200 to GQ356217), E2 subtype 3a clones from chronically infected patients (GQ356218 to GQ356422), subtype 3a clones from acutely infected patients (GQ356423 to GQ356779), E2 subtype 1a and 1b chronically infected patients (bulk and clonal sequencing) (GQ370065 to GQ370362) were evaluated.

RESULTS

Cross-sectional analysis of full-length subtype 3a viral genomes. Full-length viral genomic sequences were obtained

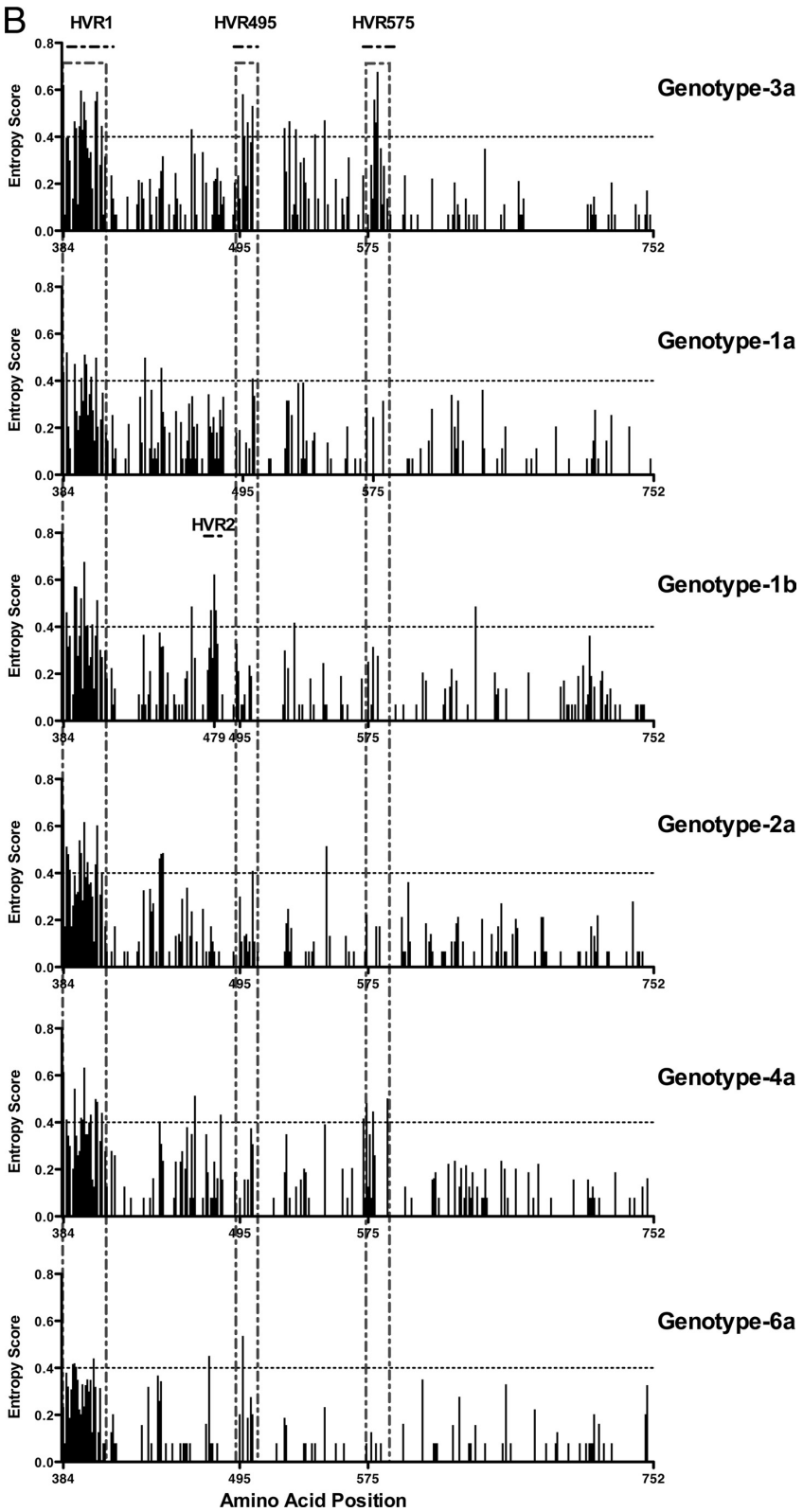

FIG. 1—*Continued.*

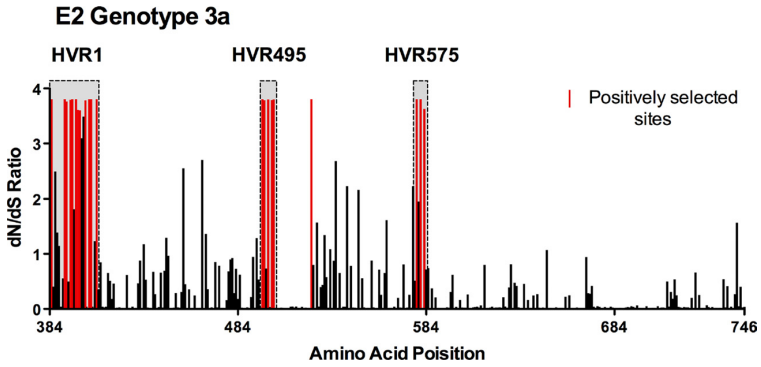

FIG. 2. Assessment of positive selection within HCV subtype 3a E2. Assessment of positive selection was performed by using CODEML analysis in 18 patients with chronic subtype 3a infection. *dN*/*dS* ratios within E2 are shown; positively selected sites, with a posterior probability of >90%, are highlighted in red.

from 18 treatment-naive patients with chronic HCV subtype 3a infection. A mathematical measure of entropy (Shannon information entropy score) was used to evaluate the sequence diversity of the full-length sequences from these 18 patients. Analysis of aligned sequences showed, as expected, that the regions of highest variability (which we define here as entropy scores of >0.4) were predominantly located in the genomic region coding for the envelope proteins, particularly E2 (Fig. 1A, upper panel). Further analysis of the E2 region of subtype 3a revealed three distinct regions of genomic variability including not only the HVR1 at the N-terminal end of E2 (which is known to be present in all HCV genotypes) but also two further regions. The first of these we named HVR495; this region spans amino acids 495 to 501 and is 7 amino acids long. The second we named HVR575, which represents amino acids 575 to 578e and is 9 amino acids long (Fig. 1A, lower panel).

Comparative analysis of E2 HCV subtype 3a with other viral subtypes. Next, we assessed whether HVR495 and HVR575 were found in other HCV genotypes. In addition to the HCV subtype 3a patients, E2 sequence was determined in-house in 22 patients with genotype 1 infection (13 subtype 1a and 9 subtype 1b). In addition, 85 patients with full-length genomic sequences—including 18 patients with each of the HCV subtypes 1a and 1b, 9 patients with subtype 2a, 15 patients with subtype 4a, and 15 patients with subtype 6a—were randomly selected from the Los Alamos and euHCVdb databases after exclusion of related and synthetic sequences (Fig. 1B). These were aligned within each subtype. Summing the entropy scores for each amino acid position within each subtype showed that the total entropy score for E2 subtype 3a (30.15) was higher than that for subtypes 1a (26.51), 2a (25.23), 4a (26.86), and 6a (17.33) and similar to subtype 1b (that contains the additional HVR2). HVR495 and HVR575 were not observed in the analysis of the 1a, 1b, 2a, or 6a subtypes. There was a single polymorphic amino acid at position 495 in subtype 6a, and variability (less marked than that observed in subtype 3a infection) was observed within HVR575 in subtype 4a infection with an entropy score of >0.4 at position 575b (Fig. 1B).

Evolutionary analysis of positive selection within HCV subtype 3a E2. In theory, regions of high variability may arise because some viral genomic regions are simply functionally unconstrained or because variation is induced by selective forces. We therefore performed a selection analysis using the program CODEML to ascertain whether HVR495 and HVR575 were also subject to positive selection. Evolutionary analysis of E2 by CODEML revealed 21 positively selected sites where $\omega > 1$ and with a posterior probability of >90% (Fig. 2, highlighted in red; Table 2), which were concentrated predominantly within HVR1, HVR495, and HVR575. Positively selected sites included amino acids 495, 496, 498, 500, and 501 within HVR495, and 577, 578a, and 578c within HVR575. Only 1 of 21 selected sites detected were located outside these three regions. Neither the polymorphic site at position 495 in subtype 6a nor HVR575 in subtype 4a was subject to positive selection by CODEML analysis.

TABLE 2. Position and residue of selected amino acids

HVR[a]	Amino acid position	Amino acid residue
HVR1	384	E
	392	S
	392	A
	394	H
	395	S
	397	S
	398	G
	399	I
	402	L
	404	S
	405	P
	408	R
HVR495	495	D
	496	T
	498	P
	500	L
	501	N
	521	T
HVR575	577	D
	578a	N
	578c	G

[a] HVR1 spans amino acid positions 384 to 408, HVR495 spans amino acid positions 495 to 501, and HVR575 spans amino acid positions 577 to 578c.

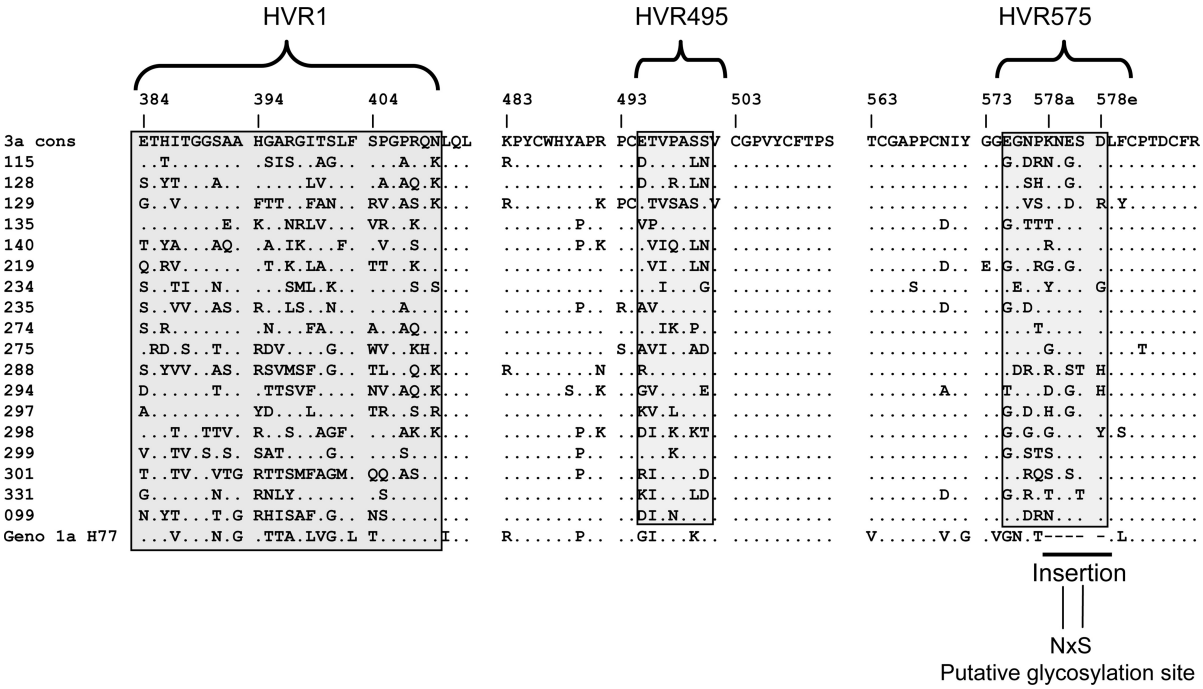

FIG. 3. The amino acid sequence of HVR1, HVR495, and HVR575 within HCV subtype 3a E2. Amino acids within the E2 HVR1, HVR495, and HVR575 are shown. The 5-amino-acid insertion is shown with a bar and is contained within HVR575. The E2 H77 1a sequence is shown for comparison.

HVR575 contains a genomic insertion unique to HCV subtype 3a. HVR575 contains a 5-amino-acid insertion that is found only in genotype 3a infection. The viral genomes that constitute HVR1, HVR495, and HVR575 in the 18 patients with chronic infection are shown (Fig. 3). Within the 5-amino-acid insertion lies at a putative N-linked glycosylation site (N-X-S/T-X; where X represents any amino acid except proline, N represents asparagine, and S/T represents serine/threonine). Detailed analysis of this insertion shows that, in all 18 patients, the only conserved sites are those absolutely required for glycosylation (i.e., amino acids 578b and 578d), which are always asparagine and serine/threonine, respectively (Fig. 3).

HVR495 and HVR575 show clonal variability within a single host that occurs independently of variability observed in HVR1. Having shown that HVR495 and HVR575 are highly variable among individuals infected with the same subtype, we next assessed the intrahost variability of these regions. E2 clonal sequence analysis was performed in 22 patients with chronic HCV infection (9 subtype 3a, 9 with subtype 1a, and 7 with subtype 1b). A total of 6 to 26 (mean 18.7) clones were derived from each patient. In subtype 3a patients HVR495 and HVR575 contained multiple variants within a single host; of the nine patients evaluated, all showed clonal variation within HVR575, and seven of nine patients showed clonal variation within HVR495. In contrast, in genotype 1a and 1b these regions were highly conserved except in a single patient (patient 381) that had an aspartic acid to asparagine mutation at position 576 in 5 of the 10 clones sequenced. Clonal analysis of two representative patients of subtype 3a (patients 129 and 299) and one patient with subtype 1a (patient 396) are shown (Fig. 4).

The structure of HCV E2 is currently not known. In theory, then, it is possible that HVR495 and HVR575 lie in close proximity to HVR1 and form a common functional unit. We therefore assessed whether sequence variation within HVR1 was connected to variation seen within HVR495 and HVR575. In patient 129 amino acid variation is seen at multiple sites within HVR575 that is not connected to the variation seen in HVR1 within the same clones. Similarly, patient 299 shows amino acid variation at multiple sites within HVR495 that is not connected to the variability seen in HVR1 within the same clones.

Amino acid compositions of HVR495 and HVR575. We assessed the amino acid composition of HVR495 and HVR575 in terms of hydropathicity and hydrophilicity. The percentage of individuals expressing a particular amino acid at each site within HVR495 and HVR575 was determined, and amino acids were classified into hydrophobic, neutral, and hydrophilic categories. HVR495 and HVR575 show highly variable central amino acids that are largely hydrophilic or neutral, surrounded by highly conserved hydrophobic amino acids (Fig. 5). The central, highly variable amino acids are those that are identified as being under positive selection by the evolutionary analysis performed by CODEML (indicated by a star in Fig. 5) and include amino acids 495, 498, 500, and 501 within HVR495 and amino acids 577, 578a, and 578c within HVR575.

HVR495 and HVR575 are under positive selection during acute infection. Next, we investigated the evolution of the

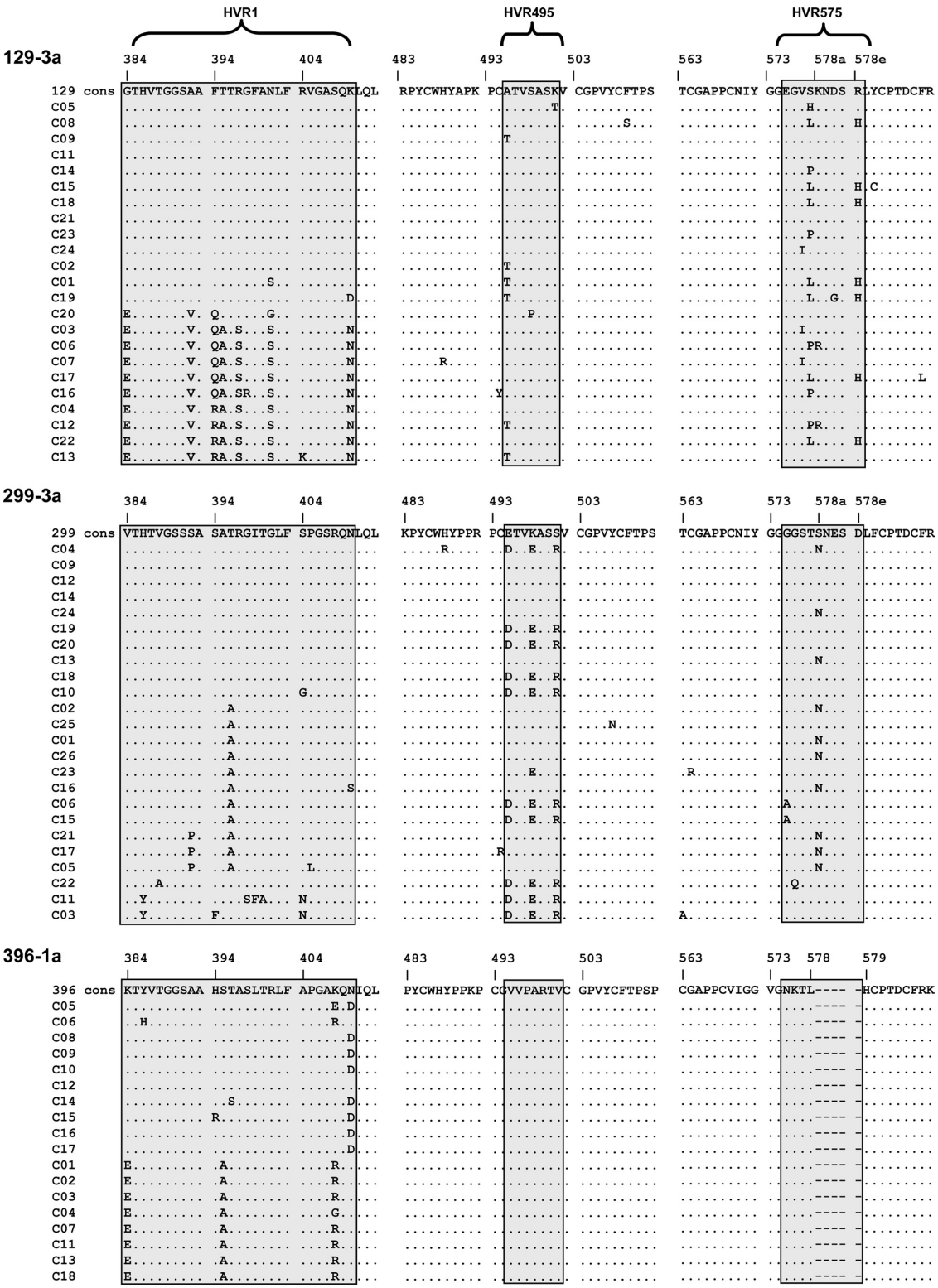

FIG. 4. Intrahost variability of HVR495 and HVR575. Amino acids within HVR1, HVR495, and HVR575 are derived from 18 to 25 clones after E2 sequencing in three representative patients: patient 129, subtype 3a; patient 299, subtype 3a; and patient 396, subtype 1a. The consensus sequence is given in the top line. A dot indicates that the amino acid within a single clone is identical to the consensus amino acid.

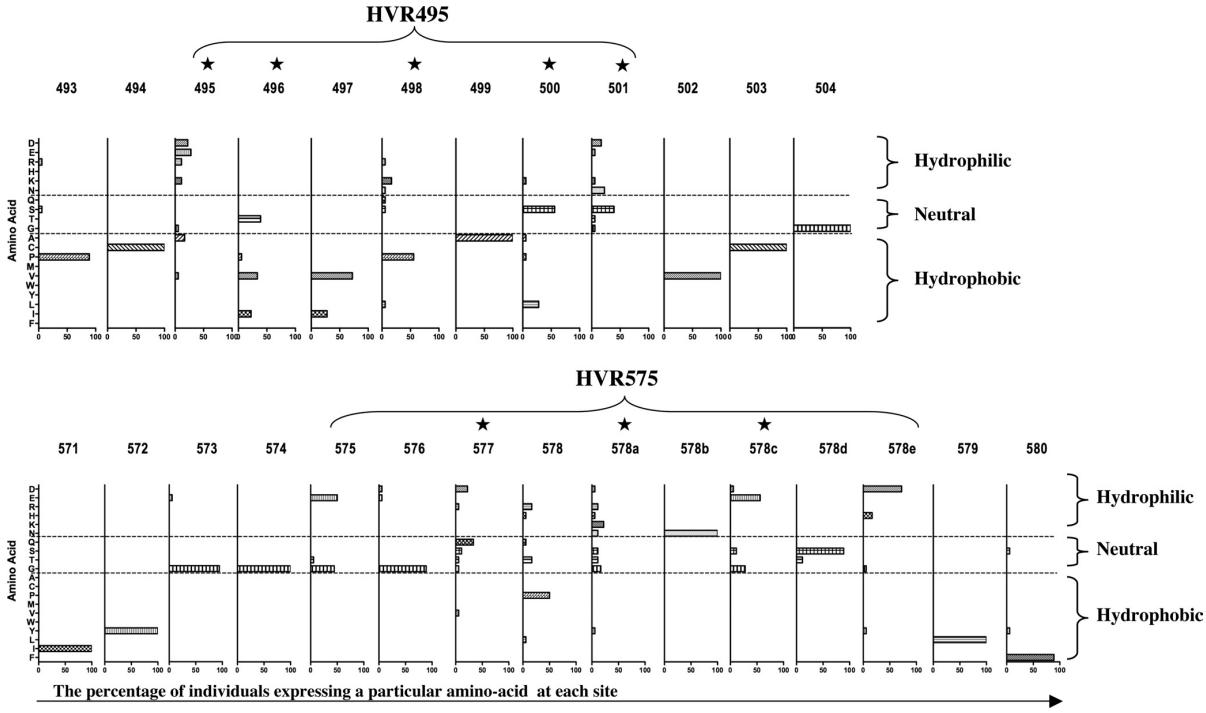

FIG. 5. Hydrophobicity or hydrophilicity of the amino acids that comprise the novel HVRs. The data are derived from E2 amino acid sequence in 18 patients with chronic HCV infection. The HVR495 and HVR575 regions are shown. The numbers given are amino acids relative to the genotype 1a H77 sequence. The percentage of individuals expressing a particular amino acid at each site is given. Amino acids are classified as hydrophobic, neutral, and hydrophilic. The star graphic highlights sites under positive selection as defined by CODEML analysis.

HVR495 and HVR575 in four individuals with primary HCV genotype3a infection (It13, It14, It16, and It17) presenting with acute hepatitis. Three individuals acquired acute HCV through intravenous drug use, and one acquired acute HCV 70 days after orthopedic surgery. The clinical course of infection is described in Fig. 6A (upper panel graphs). Viral samples were collected at multiple (4–6) time points, and analysis of clonal E2 sequences was performed. Patients It14, It16, and It17 acquired mutations within HVR575 that were detectable in the majority of clones analyzed at 39, 309, and 88 days, respectively, after acute presentation. Dominant mutations were also observed in HVR495 in patients It13 and It16 at 163 and 309 days, respectively, after acute presentation (Fig. 6A, lower panel). Furthermore, CODEML analysis (of all clones derived from all time points) for each acutely infected patient confirmed that mutations within both the HVR495 (amino acid 113 in patient It16) and the HVR575 (amino acids 195, 196, and 198 in patient It17) were under positive selection during acute infection. Although mutations within HVR495 and HVR575 clearly arose early in acute infection, analysis of patient It16 at day 888 after acute infection showed that mutations continued to accumulate within HVR575 after almost a year (day 309) of infection.

Although sporadic mutations outside HVR1, HVR495, or HVR575 (relative to the earliest time point studied) were observed in a low proportion of sequences during acute infection (Fig. 6A), very few dominant mutations (i.e., found within the majority of clones) within E2 were observed outside HVR1 or HVR495 and HVR575 (Fig. 6B) at any time studied.

DISCUSSION

HCV subtype 3a is the predominant infecting strain in the United Kingdom (11) and is the endemic subtype in parts of Asia. Somewhat surprisingly then, in light of the fact that millions of people are infected with this subtype worldwide, little information is known about the sequence of HCV subtype 3a.

Following full-length sequencing of 18 patients with chronic HCV subtype 3a, we focused our analysis here on the E2 protein. We found that HCV subtype 3a contains not only the common HVR1 at the 5′ end of E2 but also two additional regions of hypervariability, which we have termed HVR495 and HVR575, that are not present in subtypes 1a, 1b, 2a, or 6a. The 15-amino-acid insertion in HCV genotype 3a is contained within the HVR575, and this region, in addition to HVR495, appears to be under selection pressure early in acute genotype 3a HCV infection. Analysis of more datasets may be required to confirm or refute the presence of HVR575 in subtype 4a HCV, where we have demonstrated some variability but were unable to show evidence of positive selection.

The fact that the viral sequence within HVR495 and HVR575 is different in every individual with chronic genotype 3a infection studied makes it highly unlikely that viral variabil-

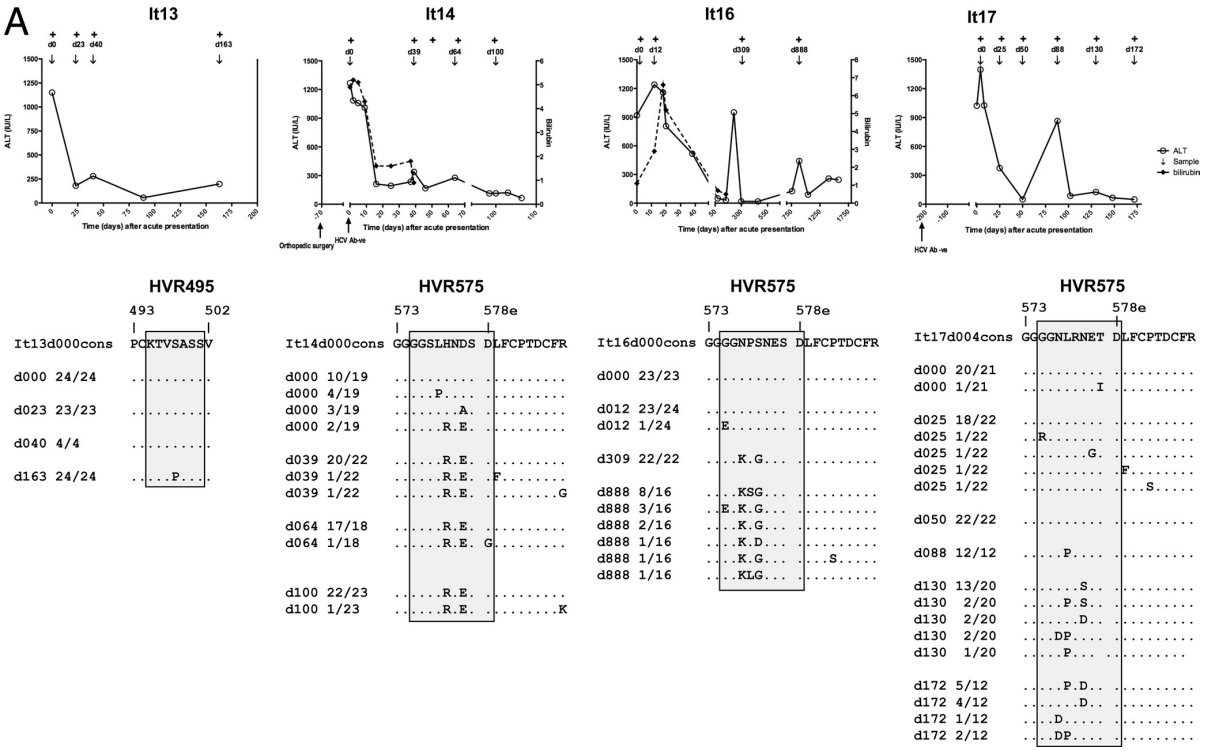

FIG. 6. Evolution of HVR1 and HVR495 and HVR575 during primary HCV infection. (A) The clinical courses of four patients (It13, It14, It16, and It17) with an acute hepatitis secondary to primary HCV infection are shown. HCV RNA was detected by PCR at the time points shown (+). Patients were sampled at multiple time points when HCV RNA positive as indicated by " ↓ ". E2 quasispecies analysis was performed at each time point. HVR495 or HVR575 are shaded. The amino acid sequence of each variant is shown relative to the consensus sequence at the earliest time point (day 0). A dot indicates that the amino acid of the variant is identical to the consensus amino acid at day 0. The proportion of each variant/total number of variants is given. (B) The upper panel maps the position of the HVRs (shaded regions) derived from 18 patients with chronic HCV subtype 3a infection to the panels below. The data from four patients (It13, It14, It16, and It17) with acute infection are shown in the lower panels. These show mutations (represented by a dotted vertical bar) in the consensus sequence at each time point studied, relative to the consensus sequence at the earliest time point (day 0).

ity in this region is driven by T cells, where a T-cell "footprint" will be directed by human leukocyte antigens, and viral variation will be limited to individuals of a particular human leukocyte antigen type. Although speculative, the hydrophobicity profile and associated constant/variable amino acid distribution within these sites suggest the presence of external loops that may act as an antigenic determinant (21, 27) or play a role in viral attachment or entry.

HCV envelope proteins are known to be glycosylated. The E2 proteins of genotypes 1a and 3a contain seven and six potential glycosylation sites, respectively (24). HVR575 contains a putative glycosylation site. N-linked glycosylation has been shown to be required for E2 protein folding (26) and for the formation of E1/E2 complexes (19). Glycosylation also has an important role in shaping immunogenicity against a protein by the shielding of epitopes against targeting antibodies and in maintaining antigenic structure. A number of studies have shown that N-linked glycosylation can limit the antibody response to HCV envelope proteins, and removal of these glycans increases the ability of antibodies to neutralize their target (4, 8, 12). In acute human immunodeficiency virus infection, envelope mutations may lead to the development of new glycosylation sites (that reduce but do not abrogate completely antibody recognition of antigenic determinants in close proximity) and also to the loss of glycosylation sites (29). In HCV infection, glycosylation sites are thought to be highly conserved (18), although, interestingly, we show a dominant mutation developing in the HVR575 glycosylation site (patient It17) 130 days after presentation. Subtypes 1a, 1b, 2b, 4a, and 6a also have a glycosylation site in close proximity to HVR575 (18), suggesting a critical role of the glycosylation site in this region.

Other regions of genotype-specific variability within E2 have been described in chronic HCV infection; this includes a 9-amino-acid region of variability seen in HCV genotype 2 (5) and a 7-amino-acid variable sequence 75 amino acids downstream of HVR1 in HCV genotype 1b infections, termed HVR2 (14). A third region (HVR3) of variability has been described between HVR1 and HVR2 (28), although this is confounded by an analysis that grouped patients of mixed genotypes. The HVR1 region has been intensively studied over recent years and shown to be particularly important as a target for antibody recognition, and there is also some evidence to show that the HVR1 and HVR2 (22) and also the igVR (18) may interplay in a complex fashion to modulate E2 receptor

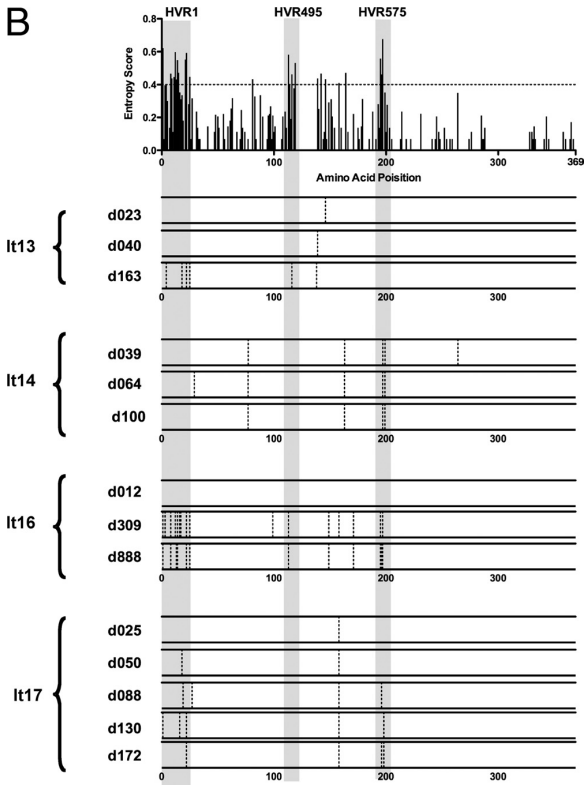

FIG. 6—*Continued.*

binding. The HVR1 has also been used as a genetic "marker" to identify and quantify circulating viral genetic diversity (quasispecies) within infected hosts and to correlate with various clinical outcomes. Quasispecies dynamics based on HVR1 analysis have been linked to interferon treatment responses (1, 7, 10), spontaneous virus resolution after primary infection (3, 6), and HCV liver pathology (13). Due to the very real practical difficulties in performing full-length viral genome clonal analysis, it is not clear whether differences in HVR1 evolution and clinical outcome relate directly to this viral region or whether this region is serving as a marker for sequence differences elsewhere along the viral genome. However, the finding of HVR495 and HVR575 suggests that these regions should be taken into account in analyses of quasispecies diversity that focus on HCV genotype 3a.

In conclusion, we have identified two regions of hypervariability within E2 in HCV subtype 3a chronically infected individuals. Further analysis shows that these regions are subject to strong intrahost selective pressure that arises early during acute infection. Future studies will need to address the functional significance of these specific regions in subtype 3a infection.

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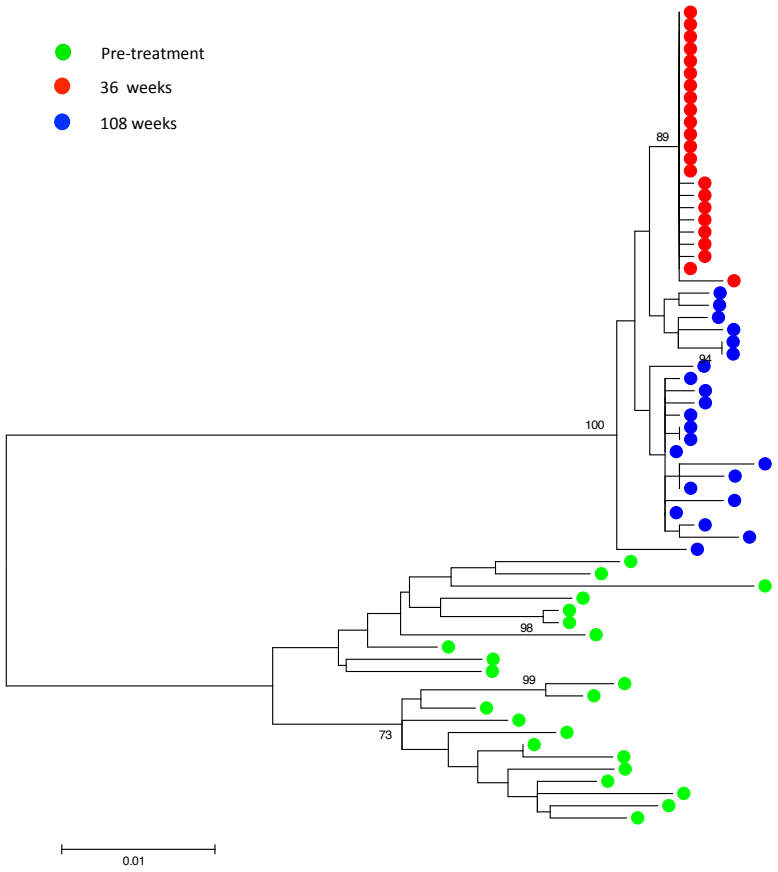
Appendix II

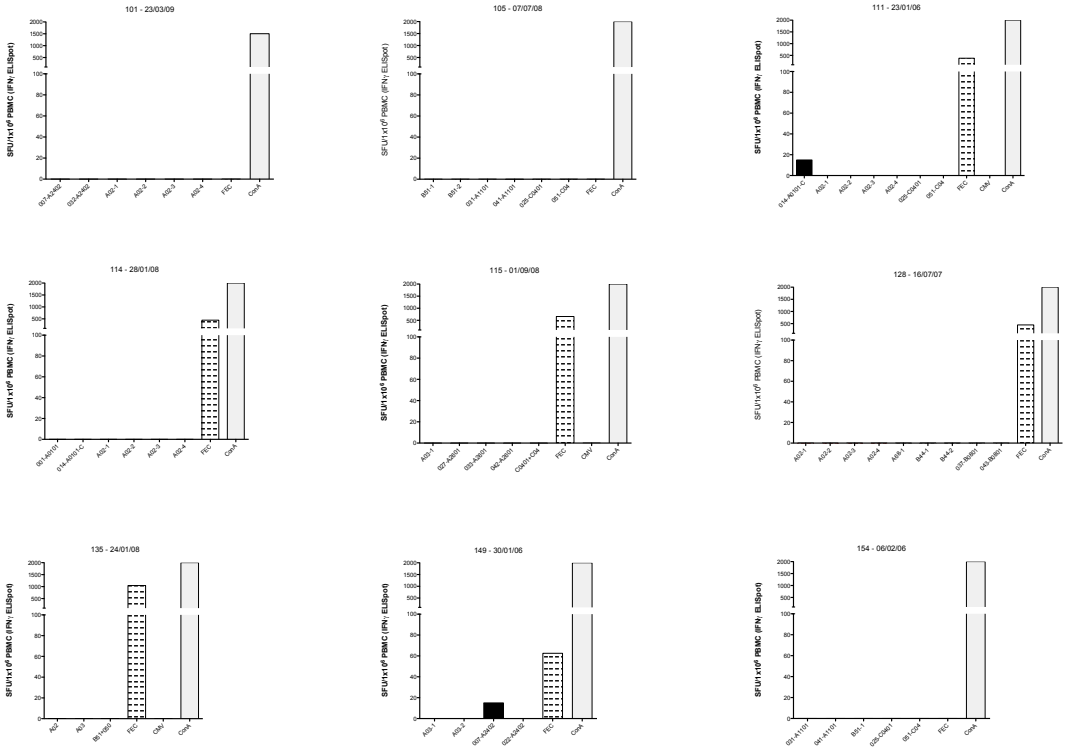
Chapter 4 - E2 Sequence Variation and Treatment of Chronic Subtype 3a Infection

129-REL

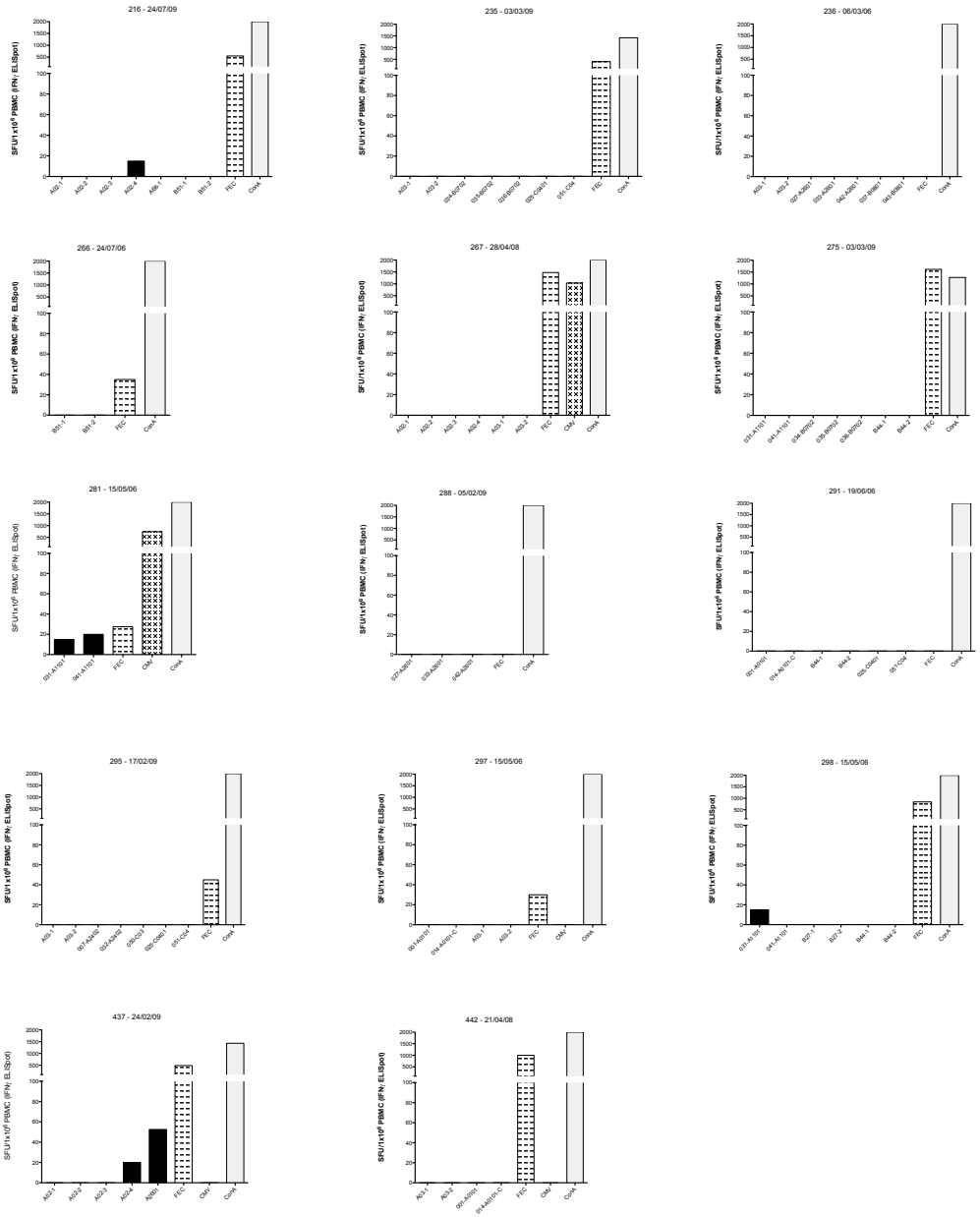


Phylogenetic tree of E2 clones of patient 129 Relapser.
General time reversible plus Gamma distribution tree of entire E2 clonal sequences at pre-treatment, point of relapse and 108 weeks. Bootstrap scores >70% are shown based on 1000 replicates. Pre-treatment clones are shown in green, point of relapse (week 36) in red and week 108 in blue.

Appendix III
Chapter 6 - Class I HLA-Associated Peptides to Detect T-cell responses in Chronic Subtype-3a Infection



HLA-Associated Peptide ELISpots
IFN-γ T-cell responses measured by ELISpot of HLA-matched peptides, and control antigens, FEC (Influenza/EBV/CMV), CMV lysate and ConA (Concanavalin A). ELISpot responses for patients 101, 105, 111, 114, 115, 128, 135, 149, 154. Dates shown are sample dates.

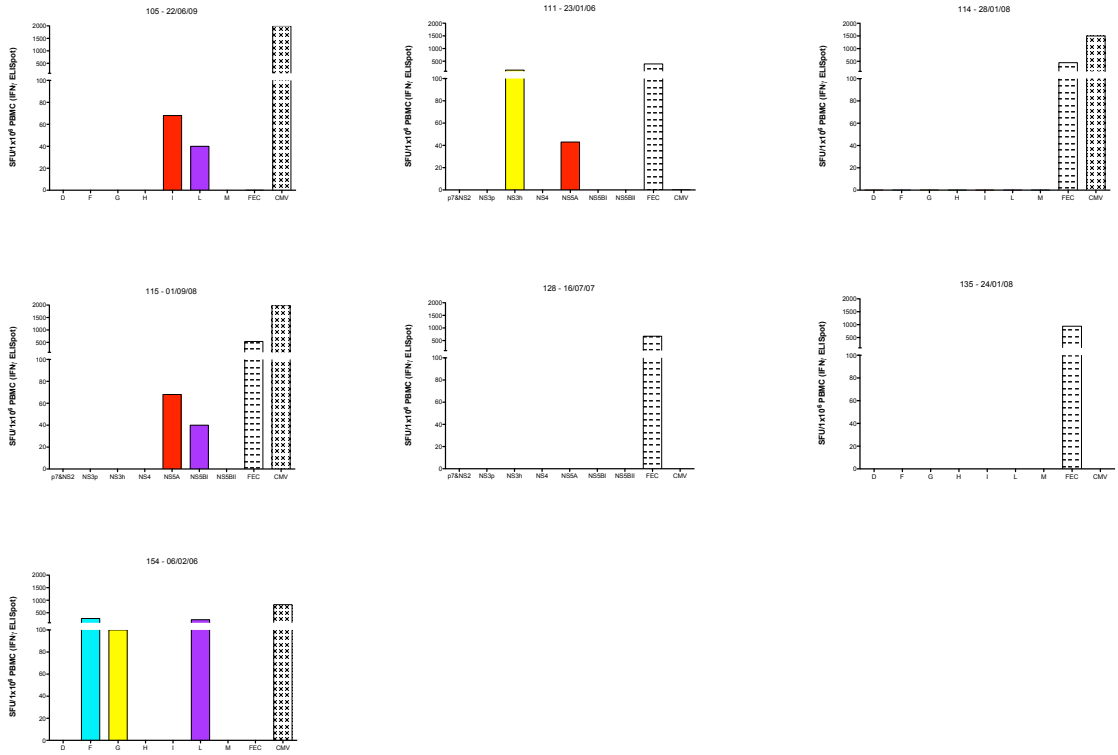


HLA-Associated Peptide ELISpots cont.

IFN-γ T-cell responses measured by ELISpot of HLA-matched peptides, and control antigens, FEC (Influenza/EBV/CMV), CMV lysate and ConA (Concanavalin A). ELISpot responses for patients 216, 235, 236, 266, 267, 275, 281, 288, 291. Dates shown are sample date.

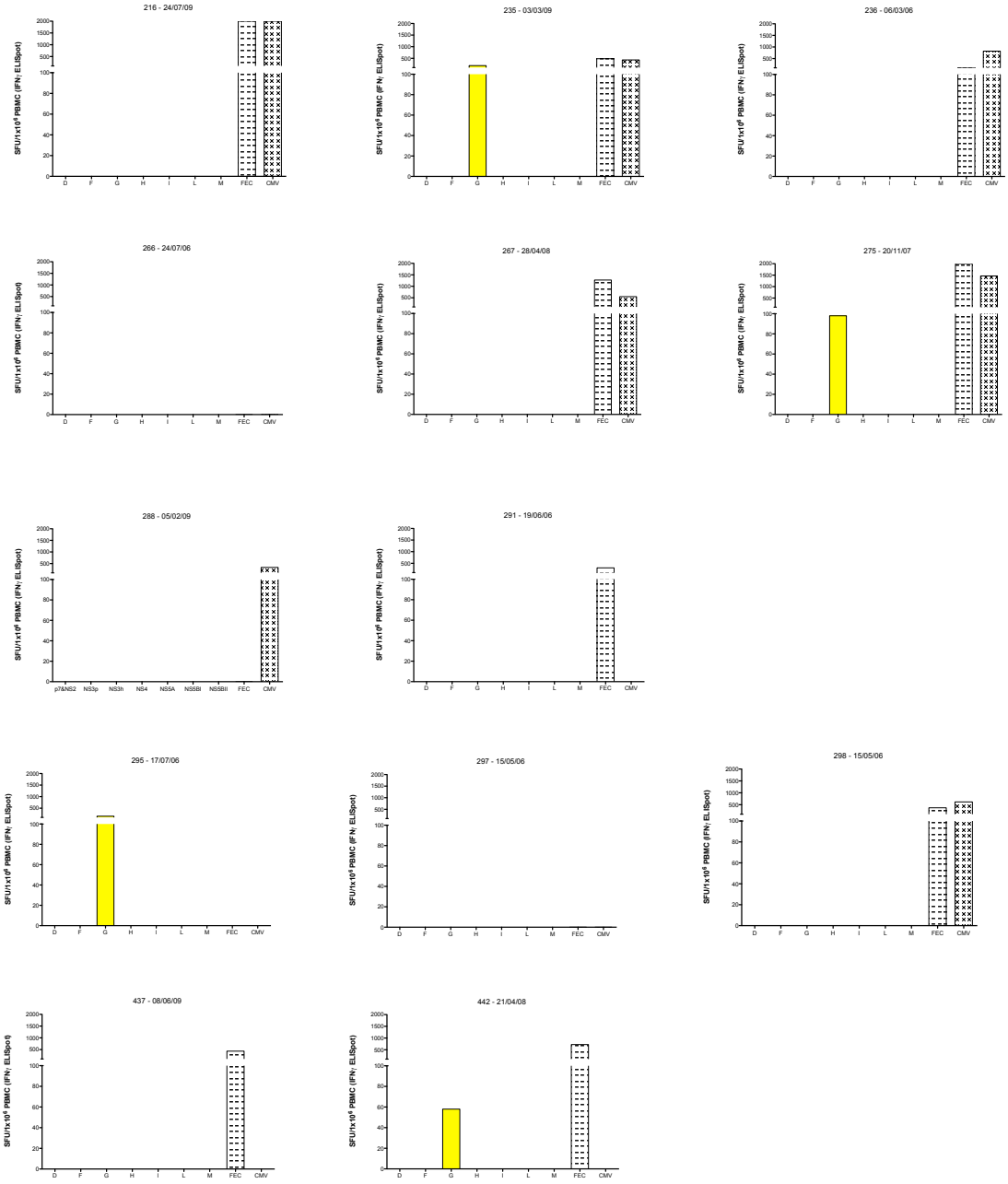
Appendix IV

Chapter 6 - Class I HLA-Associated Peptides to Detect T-cell responses in Chronic Subtype-3a Infection



Overlapping peptide ELISpots

IFN-γ T-cell responses measured by ELISpot of overlapping peptides, and control antigens, FEC (Influenza/EBV/CMV), CMV lysate and ConA (Concanavalin A). ELISpot responses for patients 105, 111, 114, 115, 128, 135, 154. Dates shown are sample dates.



Overlapping peptide ELISpots cont.

IFN-γ T-cell responses measured by ELISpot of overlapping peptides, and control antigens, FEC (Influenza/EBV/CMV), CMV lysate and ConA (Concanavalin A). ELISpot responses for patients 216, 235, 236, 266, 267, 275, 288, 291. Dates shown are sample dates.