The effect of the HLA B27 allele on the immune response to acute HCV in HIV infected patients

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Abstract

In mono-infected individuals, the HLA-B27 allele is strongly associated with spontaneous clearance of HCV in association with a strong CD8+ response targeted against a single epitope within the HCV RNA-dependent RNA polymerase (NS5B). We studied variation across the whole HCV genome and T cell responses over time in a rare cohort of HLA-B27+ patients with acute HCV and HIV co-infection, the majority of whom progressed to chronicity.

We used next generation sequencing to detect changes within and outwith the immuno-dominant HLA-B27 restricted HCV-specific CD8+ T cell epitope $NS5B_{2841-2849}$ (ARMILMTHF) during evolving progression of early HCV infection. Within the Acute HCV UK cohort, 10 patients carried the HLA B27 allele. Of these, 3/8 patients (37.5%) with HIV infection and 2/2 (100%) without HIV spontaneously cleared HCV (p=0.44). Sequential samples from nine HLA-B27+ patients (2 with monoinfection and 7 with HIV co-infection) were available for analysis (four spontaneous clearers and five evolving progressors). Mutations identified using NGS were assessed using a replicon genotype 1a system to evaluate viral fitness. Multiple mutations within the HLA-B27 restricted $NS5B_{2841-2849}$ epitope were associated with progression to chroncity whereas patients who cleared the HCV infection spontaneously had no or only one mutation at this site (p=0.03). A triple $NS5B_{2841-2849}$ mutant observed during progression to chronicity was associated with restored replication when compared to wild-type virus while single or double mutants were significantly associated with impaired replication (p=0.0495).

T cell responses measured in these patients using ELISpot and flow cytometry. HLA-B27+ patients had significantly higher IFN-γ responses than patients who were HLA-B27- (p=0.0014). Those who progressed to chronicity had lower IFN-γ responses than those who cleared HCV (p=0.0011). Mono-infected patients had higher IFN-γ responses compared to co-infected patients (p=0.0015).

HIV co-infection is associated with a lower likelihood of spontaneous clearance of HCV in HLA B27+ patients and this is associated with impaired T cell

function in this group.

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Sultan Merja Alotaibi

سلطان بن مرجع الحمادي العتيبي

Author's declaration

This work was completed at the University of Glasgow between October 2012-2015 and has not been submitted for another degree. All work presented in this thesis was obtained by the author's own efforts, unless otherwise stated.

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Definitions/ Abbreviations

ATP	Adenosine triphosphate
aa	Amino acids
bp	Base pair(s)
cDNA	complementary DNA
°C	Degrees celsius
DAAs	Directly Acting Antivirals
DMEM	Dulbecco's modified Eagle's medium
EMCV	Encephalomyocarditis virus
E. coli	*Escherichia coli*
FCS	Foetal calf serum
HD	Hamming distance
HCVcc	Cell-culture-derived infectious HCV
HCV	Hepatitis C Virus
HCC	Hepatocellular carcinoma
HAART	Highly active antiretroviral therapy
Huh-7	Human hepatoma cell line
hVAP-33	human vesicle associated protein-33
H77	Hutchinson strain
HVR	Hypervariable region
IFN	Interferon
IRF-3	Interferon regulatory factor 3
ISDR	Interferon sensitivity-determining region
IRES	Internal ribosome entry site
ML tree	Maximum likelihood tree
MSM	Men who have sex with men
µ	Micro (10^{-6})
Min	Minutes
Neo	Neomycin phosphotransferase
NGS	Next Generation Sequencing
NS	Non-Structural
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nt	Nucleotides
ORF	Open reading frame
Pi	Pairwise diversity
PegIFNα	Pegylated Interferon-alpha
PWID	People who inject drugs
PBMC	Peripheral blood mononuclear cells

PCR	polymerase chain reaction
PI	Protease inhibitor
QD	Quasispecies diversity
RLU	Relative light units
RAMs	Resistance associated mutations
RAVs	Resistance associated variants
RIG-I	Retinoic acid inducible gene-I
RT	Reverse transcription
RBV	Ribavirin
RdRp	RNA-dependent RNA polymerase
Sec	Seconds
SBS	Sequencing by synthesis
SNP	Single nucleotide polymorphism
SGR	subgenomic replicons
SVR	Sustained virological response
TLR	Toll-like receptor
TRIF	Total reflection fluorescence
UTR	Untranslated region
WT	Wild type
WHO	World Health Organisation

Amino Acid Name Abbreviations

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Publications

- Publications arising from this thesis

Alotaibi S *et al* 2014, Characterising the role of the HLA-B27 allele in spontaneous clearance and evolving progression of hepatitis C in a cohort of HIV-infected men, *HIV MEDICINE abstract*, Vol: 15, Pages: 144-144, ISSN: 1464-2662

Vattipally SB, **Alotaibi S**, Abdelrahman T, Brunker K, Orton R, Klymenko T, Wilkie G, Davison A, Thomson EC, TANOTI: a BLAST guided divergent read mapper for small genomes *under review BMC Bioinformatics*

Alotaibi S et al 2016, The protective effect of the HLA-B27 allele is reduced in HIV-infected men with acute hepatitis C infection *manuscript in preparation* for *AIDS*.

Chapter 1: Introduction – overview of Hepatitis C virus infection

1.1 Discovery of the Hepatitis C Virus

In the early 1970's, new viral and antigenic markers were discovered for two viruses causing hepatitis; hepatitis A (HAV) and hepatitis B (HBV). The subsequent development of new sensitive serologic methods allowed rapid diagnosis of these infections in many patients with abnormal liver function. However, a significant number of patients with clinical hepatitis, including individuals that had had a blood transfusion, could not be diagnosed as having either HAV or HBV (Prince et al., 1974, Feinstone et al., 1975); at this time the agent responsible, considered to be infectious in nature, was termed non-A, non-B hepatitis (NANBH). Injecting chimpanzees with plasma or serum from human patients with NANBH resulted in elevated serum alanine aminotransferase (ALT) levels, demonstrating that the NANBH agent was able to cause infection and was capable of establishing chronic infection (Alter et al., 1978, Hollinger et al., 1978). Infectious plasma from human patients that had been treated with chloroform was injected into chimpanzees but did not cause infection, revealing that the NANBH agent was sensitive to this lipid solvent (Feinstone et al., 1983). Injection of human plasma from infected patients into chimpanzees, having first passed it through an 80nm filter (Bradley et al., 1985) also resulted in infection, suggesting the presence of an infectious agent smaller than a bacterium. Together, all these data suggested that the cause of NANBH was a small, enveloped virus. Further studies that attempted to culture the NANBH agent were inconclusive due to a lack of cell culture models as well as the inability to infect animals other than chimpanzees.

The development of new, highly-sensitive molecular biology techniques in the 1980s enabled scientists to further characterise the cause of NANBH. At this time, all available immunological methods were ineffective in detecting viral antigens and antibodies, as there was a low concentration of viral antigen in NANBH infection (Choo et al., 1989). To overcome this limitation, nucleic acid extracted from infected plasma was used to make complementary DNA (cDNA)

using a bacteriophage (strain λgt11) that aided sequencing of the virus and expression of the polypeptide encoded by cDNA (Choo et al., 1989).

Sera were collected from patients with NANBH. A bacteriophage-containing colony called 5-1-1 was found to express polypeptide that was later recognized by the serum from NANBH patients but not with serum from control individuals (Choo et al., 1989).

Subsequent experiments showed that RNA obtained from infected animals (chimpanzees) combined with cloned cDNA and was present as a single strand. This hybridisation of RNA and cDNA was not dependent on treatment with ribonuclease. Based on this, it became clear that the cause of most cases of NANBH was a single-stranded RNA virus. Sequence analysis revealed that the cDNA from colony 5-1-1 contained a single open reading frame (ORF); this newly identified single stranded RNA virus was named hepatitis C virus (HCV) (Choo et al., 1989).

1.1.1 Classification and genotypes of HCV

The HCV genome is organised in a similar way to other members of the Flavivirus family (Choo et al., 1991, Miller and Purcell, 1990), namely, monopartite single stranded RNA genomes of positive polarity that encode a single polyprotein that is subsequently broken down into individual functional proteins by host and virus proteases. The HCV genome has a high level of genetic diversity and 7 different major genotypes.

New genotypes are defined by 1) having one or more complete coding region sequence(s) available following sequencing; 2) belonging to a phylogenetic group distinct from any previously described sequence by 30-35%; 3) the exclusion of intergenotypic or intersubtypic recombinations (Simmonds, 2004, Smith et al., 2014).

Variability between the 7 different genotypes occurs unevenly across the genome. The viral E1 and E2 (glycoproteins) genes represent the most diverse

regions of the HCV genome while the 5' untranslated region (5' UTR) shows the lowest variability. The 7 genotypes can be further split into 67 subtypes that differ in genome sequence by an average of 20-25% (Simmonds, 2004, Smith et al., 2014).

The high diversity of HCV, like all other positive-strand RNA viruses, is due to an RNA polymerase that contains no proofreading or 5'-3' exonuclease activity, resulting in a high rate of mutations introduced into progeny genomes during replication. A comparison of different studies, following sequencing of the complete genome over eight and thirteen years in chimpanzees and humans respectively, showed an average frequency of mutation of $1.4*10^{-3}$ – $1.9*10^{-3}$ changes per single nucleotide yearly (Ogata et al., 1991, Okamoto et al., 1992). There is a high rate of genetic variation in viral strains infecting individuals (within host variation) and within populations; the mix of circulating mutation(s) within viral strains in an infected host is referred to as a quasispecies (Martell et al., 1992).

1.1.2 Global prevalence and incidence of HCV

The current estimate of global HCV prevalence is around 3% of the world population, equivalent to 170 million people (Thomson, 2009). Accurate figures of the prevalence of HCV are difficult to determine, however, due to the absence of screening programmes in most countries. As HCV infection is usually asymptomatic for a long period of time, it remains unsuspected by those infected who may not seek medical attention until they develop advanced disease. There is variability in its prevalence in different geographical areas and genotypes and distributed variably around the world.

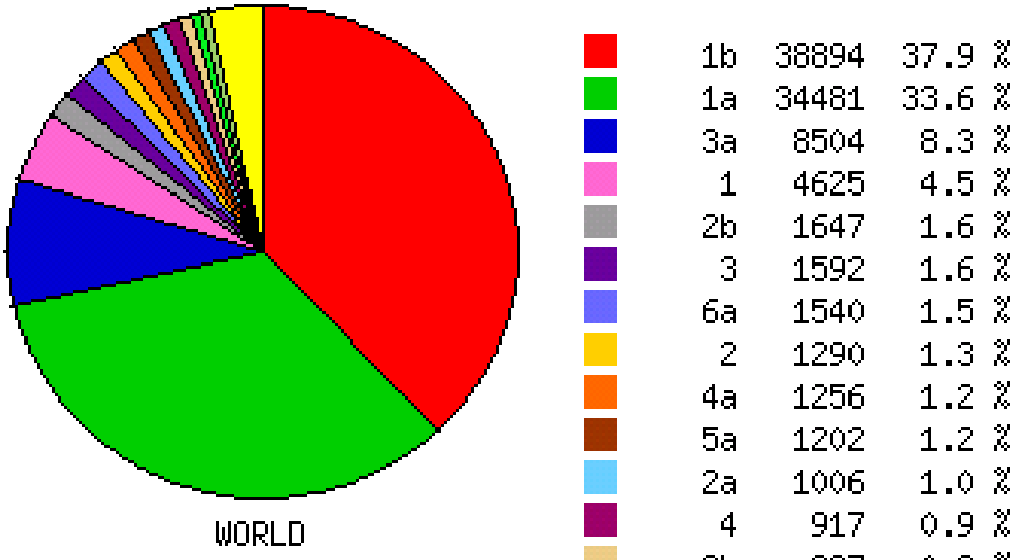
HCV is estimated to infect about 0.6% of the population in the UK, while almost 2% of the population in the USA are infected with HCV (Thomson, 2009). In low-income countries, scant amounts of data are available, despite the potential for very high prevalence levels, largely due to iatrogenic transmission. In Egypt, for example, between 15-20% of the population have positive antibodies (Frank et al., 2000), following parental antischistosomal therapy

(PAT) campaigns between the 1960s and 1980s. HCV genotypes can be classified, linked and mapped geographically (Nguyen and Keeffe, 2005). For example, the predominant and most prevalent genotype found across Europe, the USA and China is genotype 1, while in East Africa (including Egypt), genotype 4 is most prevalent. South Africa has predominantly genotype 5 infection while genotype 6 occurs in South East Asia. Global genotype distribution is illustrated in (Figure 1-1 and Figure 1-2).

Age-specific analysis of HCV prevalence varies geographically (Wasley and Alter, 2000). In countries such as Australia and USA, a high rate of infection is found in patients between 30 and 49 years of age, showing that the risk of infection occurs mainly in young adults (Armstrong et al., 2006). In Japan, China and parts of Europe, including Italy and Spain, most HCV infections are found in older patients, suggesting that the establishment of HCV risk infection in these countries may have been at its highest rate about 40-60 years ago (Campello et al., 2002, Dominguez et al., 2001, Zhang et al., 2005).

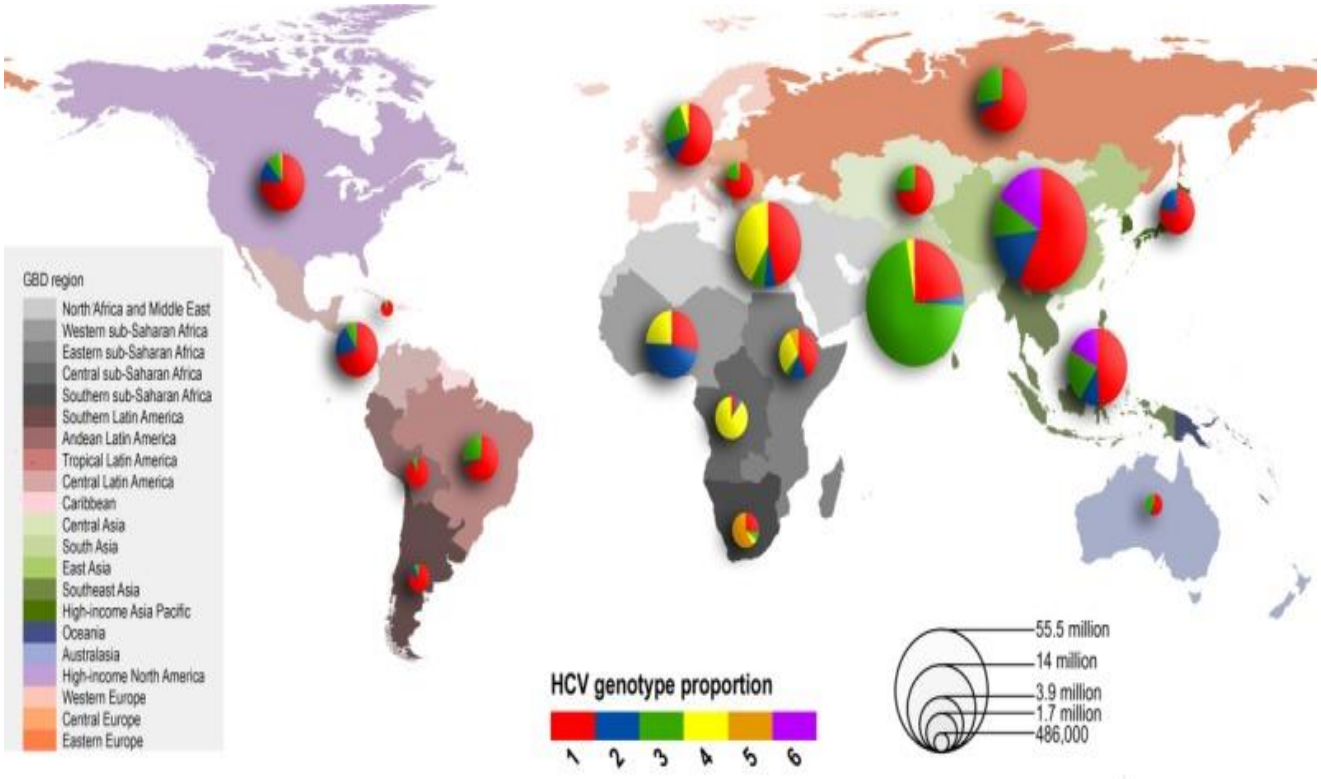
In Egypt, the prevalence of HCV infection increases with age, and high rates of infection are found in all age groups (Frank *et al.*, 2000). This suggests that as well as an increased risk of HCV in the past, there is also an ongoing risk of infection (Wasley and Alter, 2000). Much of this regional variability can be attributed to the frequency and extent to which different risk factors have contributed to HCV transmission.

Figure 1-1: Prevalence of hepatitis C virus genotypes based on the NCBI HCV database.



The diagram shows the frequency of different HCV subtypes in the NCBI HCV Database. The subtype distribution is shown for 102516 sequences, accessed 01/12/2015 http://hcv.lanl.gov/components/sequence/HCV/geo/geo.comp.

Figure 1-2: Global distribution of hepatitis C virus genotypes.



Global prevalence of different HCV genotypes. Size of pie charts is proportional to the number of seroprevalent cases (Messina et al., 2015).

The majority of HCV infections are transmitted through direct percutaneous exposure to blood, occurring via three main routes: first, the transfusion of blood products from infected donors, second, non-sterile therapeutic injections, and third, injecting intravenously in recreational drug users (Shepard et al., 2005). The testing of nucleic acid and antibody screening of donated blood has essentially eradicated new cases of transfusion-related HCV infection in high income countries. Transmission of HCV this way is a huge problem in low income countries that do not have nucleic acid testing. Unsafe medical practices contribute to a vast number of HCV infections around the world. One study estimated that in one year (2000), approximately 2 million new cases of HCV infection were caused worldwide as a result of using unsterile medical injections (Hauri et al., 2004). Similarly, in Egypt most cases of HCV transmission happened because of poor medical practices. For example, during the PAT campaign, new cases of HCV infection were recorded as a result of contaminated glass syringes being re-used (Frank et al., 2000).

In medium and high income countries including the USA, the UK and Australia, intravenous drug use is the most common factor associated with new HCV infections (Thomson, 2009). For instance, in 2003, 90% of those diagnosed with a new HCV diagnosis in Scotland had used intravenous drugs (Hutchinson et al., 2005). HCV infection can also be transmitted perinatally and via sexual activity, particularly in HIV-infected men who have sex with men (MSM) (van de Laar et al., 2009{van de Laar, 2010 #910, van de Laar et al., 2010, Danta and Dusheiko, 2008).

1.2 Clinical features of HCV infection

The hepatocyte is the main target of HCV infection. HCV infects and replicates within hepatocytes over many years resulting in liver cirrhosis and hepatocellular carcinoma (HCC) in a significant number of those infected. It is now one of the most common reasons for a liver transplant (Sharma and Lok, 2006). The first phase of the infection is the acute phase (within 6 months of infection), which is then followed by the chronic phase (more than 6 months of infection).

1.2.1 Acute HCV infection

As a result of the initial exposure to HCV, the majority of new HCV infections are mild or asymptomatic; this means that new infections are rarely recognized (Thomson, 2009). Nonetheless, during the acute phase, about 20% of individuals infected with HCV will exhibit malaise, nausea and jaundice, although 80% of the cases will show no symptoms (Pawlotsky, 2004). Fulminant hepatitis can cause liver failure during early infection although this is extremely rare. Regardless of asymptomatic signs during the acute phase, a high level of ALT can be detected in many HCV cases if blood testing is carried out routinely for other reasons (for example co-infection with HIV) (Thomson et al., 2009).

Some patients (15-30% of cases) will spontaneously eliminate the virus, usually during the first 6 months of infection. The mechanism behind spontaneous clearance of HCV is not fully understood but factors contributing to clearance include the host and individual's immune response, age, sex, and alcohol intake (Hezode et al., 2003, Peters and Terrault, 2002). The presence of HLA-B27 and HLA-B57 are strongly associated with spontaneous elimination of the virus and these are linked to early multi-specific T-cell responses. Patients who progress to chronicity have weak immune responses (Heim and Thimme, 2014).

1.2.2 Chronic HCV infection

Most (70-85%) of individuals infected with HCV progress to chronicity. During this stage, the HCV continues to replicate and viremia is detected, but is not usually at as high a level as during the acute phase. During the chronic phase, up to 10^{12} new virions are produced every day (Neumann et al., 1998).

HCV patients in the chronic phase can remain without symptoms for up to 35 years, although around 1 in 5 patients develop serious liver disease including compensated and decompensated cirrhosis, steatosis (fat accumulation within hepatocytes) and hepatic fibrosis (as an outcome of chronic inflammation).

Around 20% of HCV chronic patients will develop liver cirrhosis over a period of 20 years (Ishii et al., 2008).

Liver cirrhosis may lead to portal hypertension with consequent risks of gastro-intestinal haemorrhage and hepatic encephalopathy (Thomson and Finch, 2005). Following the onset of cirrhosis, hepatocellular carcinoma (HCC) can occur in a small percentage of patients, about 1-3% per year in cirrhotic individuals (Ishii and Koziel, 2008). It is thought that HCC may occur as the result of a long-term inflammatory response, resulting in oxidative stress, possibly leading to DNA cellular damage (Okuda et al., 2002).

Other studies have shown evidence that the HCV core protein can play a role in oncogenesis (Moriya et al., 1998). Interestingly, different HCV genotypes and periods of infection have variable impacts and outcomes (Zein, 2000). For instance, HCV genotype 3 can cause a rapid onset of hepatic steatosis and fibrosis; it has been shown that genotype 3 leads to significantly faster fibrosis progression compared with the other genotypes (Probst et al., 2011). It is also more likely to result in chronic infection, compared with HCV genotype 1 (Lehmann et al., 2004, Rubbia-Brandt et al., 2000) but is more sensitive to interferon-based treatment. Response rates to direct-acting antivirals (DAAs) are lower than in genotype 1.

1.3 Diagnosis of HCV infection

HCV infection may be diagnosed using serological and molecular virological methods.

1.3.1 Serological Assays

The main screening assay for detecting anti-HCV antibodies is the enzyme immunoassay (EIA), that identifies antibodies targeting different epitopes within the HCV genome. It has many advantages, including ease of use, ease of automation and low cost. There are three different generations of serology assay.

First generation assays include a single HCV antigen recombinant that was obtained from the non-structural protein 4 (NS4) of the HCV genome.

This first generation of EIA lacked sensitivity and specificity. For instance, in high prevalence centres such as tertiary care centres, only 80% of patients with evidence of HCV were detected as positive with anti-HCV using first generation assays (Gretch et al., 1992). Individuals from a low prevalence population were tested and of the 70% who tested positive using EIA-1, many were not infected by HCV. Based on this, a supplementary test had to be used to confirm infection.

In 1992 a new version of EIA was launched and called EIA-2 (Alter, 1992) . This assay contained antigen from different regions of HCV including the core and NS3 genes in addition to NS4. The sensitivity of the test was improved and the specificity relative to EIA-1 also slightly improved (Han et al., 1991, Houghton et al., 1991). Using antigen from two different regions (the core and NS3) in EIA-2 helped to shorten the average detection time for HCV seroconversion: instead of 16 weeks with EIA-1 it became 10 weeks. Furthermore, it allowed for the detection of 95% of individuals with molecular evidence of HCV.

A new third-generation of serological assay (EIA-3) was subsequently developed. In this generation, in addition to core and NS3 antigens a new NS5A antigen was added which was not present in the EIA-2 generation. With this generation the seroconversion detection time was reduced to 2-3 weeks (Kwok and Higuchi, 1989, Miller and Purcell, 1990). Sensitivity also increased to 97% and specificity was slightly better compared to EIA-2 (Kao et al., 1996). A summary of all the different generation assays is given in Table 1-1.

Table 1-1: Sensitivity and positive predictive values of EIA for HCV detection

Assay	Sensitivity* (%)	Positive Predictive Value** (%)	
		Low Prevalence	High Prevalence
EIA-1	70-80	30-50	70-85
EIA-2	92-95	50-61	88-95
EIA-3	97	25	Not Done

* Based on clinical findings and detection of HCV RNA by PCR.

** Compared with RIBA

1.3.2 Molecular virological assays:

Virological assays for the detection of HCV RNA allow both quantitative and qualitative analysis of viral load. Both assays have more advantages over the serological assay as they can detect the virus before seroconversion and distinguish between those with chronic infection and those who have been treated or spontaneously cleared infection. RNA, extracted from either serum or plasma, is reverse transcribed into cDNA that is then amplified using polymerase chain reaction (PCR) to create either double stranded DNA (dsDNA) PCR product or amplified by transcription-mediated amplification (TMA) that yields ssRNA products (Chevaliez and Pawlotsky, 2007).

These assays have the advantage that they give qualitative results. HCV RNA can be quantitatively measured using RT-PCR or a signal amplification method such as the branched DNA (bDNA) assay.

Branched DNA (bDNA) utilizes oligonucleotides harbouring enzymes that work to catalyse a colour change; these bind to HCV RNA molecules by hybridisation.

This technique is different from RT-PCR, as this technique does not need the reverse transcription step. Virological assays can also work to detect different genotypes of HCV using PCR, by using specific PCR primers that attach to well-characterized polymorphisms in 5' UTR (Tsongalis, 2006).

1.4 Treatment of co-infected individuals

Responses to PegIFN alpha plus RBV treatment are lower in patients with co-infection (HCV/HIV) than in those with mono-infection (HCV) (Bhagani, 2011, Torriani et al., 2004a). Clearance of HCV infection using PegIFN/RBV therapy is likely to be related to immunogenic factors, as IFN alpha stimulates both innate and adaptive immune responses (Feld and Hoofnagle, 2005). Host interleukin 28B genotype predicts the outcome of treatment of chronic HCV genotypes 1 or 4 with PegIFN/RBV (Corchado et al., 2014, Chen et al., 2012) and may also predict the outcome of treatment with direct acting antiviral drugs (DAAs) (De Nicola et al., 2012).

While IFN-based therapies are associated with lower sustained virological response (SVR) rates in HIV-infected individuals (Torriani et al., 2004b), the use of DAAs is equal to that from the same agents used with mono-infection patients (Poordad et al., 2011a, Jacobson et al., 2011, Cotte et al., 2014, 2015). Newer DAAs treatments target the NS3/4A serine protease, NS5A and the NS5B RNA polymerase (Table 1-2).

Table 1-2: Recommendation on treatment of HCV (EASL guidelines 2016)

IFN-free regimens	
Options	Genotype
SOF+RBV	2,3 (Suboptimal)
SOF/LDV (±RBV)	1,4,5,6
Sofosbuvir/Velpatasvir (± RBV)	All
OBV/Paritaprevir/Ritonavir+ DSV (±RBV)	1
Grazoprevir/Elbasvir (± RBV)	1,4
SOF + SMV (± RBV)	1,4
SOF + DCV (± RBV)	All
OBV/ Paritaprevir/ Ritonavir (±RBV)	4

The combination of two or more DAAs is important to reduce the possibility of resistance (Chung and Baumert, 2014, Rockstroh and Bhagani, 2013). Currently licensed DAAs include sofosbuvir which competitively inhibits NS5B, protease inhibitors such as simeprevir and paritaprevir that act on NS3/4A and daclatasvir and ledipasvir that inhibit NS5A. Interferon-free DAA combination treatments are associated with >90% SVR rates in HIV infected and uninfected individuals and have a shorter time-period of treatment regimen than IFN-based therapies. The side effect profile of these compounds is minimal.

Patients infected with genotype 3 HCV and those with cirrhosis are less likely to respond to treatment with DAAs than other groups. Genotype 3 HCV has a higher prevalence in PWIDs and in HIV –infected individuals (Ampuero et al., 2014, Morice et al., 2006).

As many trials have shown there is no difference in the effectiveness of DAAs between individuals infected with mono- or co-infection (Poordad et al., 2011b, Jacobson et al., 2011, Sulkowski et al., 2013a, Sulkowski et al., 2013b, Lawitz et al., 2013, Jacobson et al., 2014), physicians recommend that patients with mono- or co-infected infection can be treated with the same regime (Kohli

et al., 2014) although drug-drug interactions with ARVs may limit the use of certain combinations.

HCV patients treated within the acute phase with PegIFN have a higher SVR rate than patients with chronic infection in both HIV infected and uninfected populations although HIV-infected patients have lower SVR rates overall {Gilleece, 2005 #1670}(Jaeckel et al., 2001). Recent trials show that DAA treatment of a shorter duration (for example 6 weeks of sofosbuvir/ledipasir therapy) is highly effective and results in minimal side effects (Fierer et al., 2014). DAA therapy appears to be equally effective in HIV-infected patients and recommended treatment regimens are the same (barring drug-drug interactions) (2016). Reinfection with HCV in HIV patients is common, especially with those who have high levels of sexual activity and in patients who use recreational drugs (Martin et al., 2013) although reinfection in such patients may be over-estimated due to emerging dominance of treatment-resistant strains (Abdelrahman et al., 2015).

1.5 Molecular features of HCV

1.5.1 Virion morphology

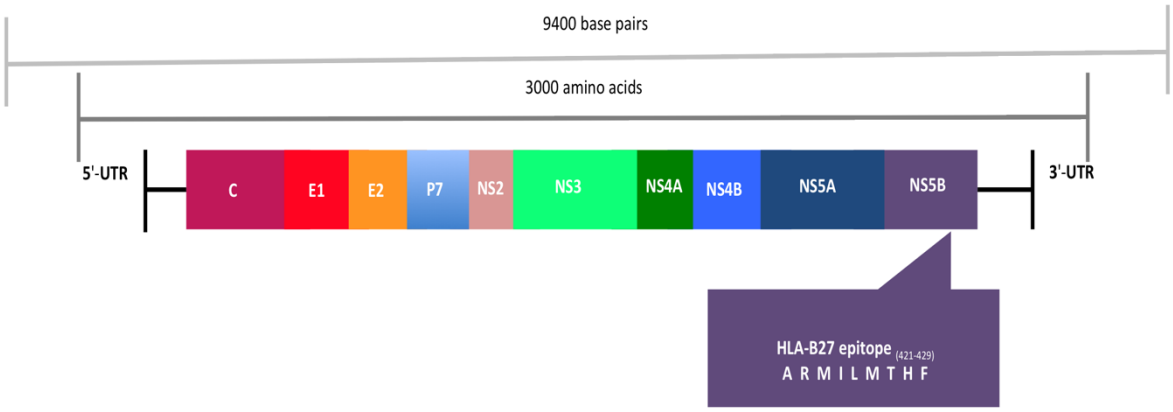
HCV particle structure consists of an envelope glycoprotein surrounding a viral core (C) protein that associates with the HCV viral genome. HCV particles derived from infectious serum have a diameter of between 30-80 nm (Bradley et al., 1985, He et al., 1987, Yuasa et al., 1991) whereas HCV particles derived from purified HCVcc have a different diameter of about 60 and 75 nm. HCVcc particles have more than one distinct form, contain electron-dense cores and lack discernible surface properties when observed using electron microscopy (Wakita et al., 2005, Gastaminza et al., 2010, Merz et al., 2011).

1.5.2 The viral genome

The HCV genome is a positive sense single-stranded RNA molecule and is approximately 9.6kb (9600bp) in length (Choo et al., 1991). The genome encodes

a single open reading-frame (ORF) that is flanked by 5' and 3' UTRs. Translation of the HCV RNA genome yields a single polyprotein of 3010 amino-acids. Both HCV and the host proteases process the polyprotein to produce the structural proteins (core, E1, E2, p7) and the non-structural proteins (NS) (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Figure 1-3), p7 has been previously defined as a protein not assigned to any other group; a recent study has shown that p7 may be part of the virion and may have ion channel activity (Griffin et al., 2008).

Figure 1-3: HCV Genome



The HCV RNA genome contains one long open reading frame (ORF), this is flanked by two untranslated region (5' and 3' UTRs). A well characterised HLA B27-restricted NS5B epitope within NS5B is highlighted ($NS5B_{2841\text{ -}2849}$).

1.5.2.1 **The 5' untranslated region (5'UTR)**

The 5' untranslated region (UTR) has been studied extensively and it has been shown that it has a highly conserved sequence consisting of 341 nucleotides that are involved in RNA replication and in HCV translation (Friebe et al., 2001, Honda et al., 1999, Bukh et al., 1992). It's 5' UTR harbors an internal ribosomal entry site (IRES); the IRES is a structure that is important for starting cap-independent translation of HCV RNA. Secondary structure analyses of the IRES reveal four distinct domains named as (I, II, III and IV) (Honda et al., 1996a).

The first domain (domain I) has a stem-loop shape and a small size; this domain has been classified as being unnecessary overall for IRES activity, but it may play a role in regulating translation efficiency (Honda et al., 1996b).

Domains II and III play an essential role in HCV RNA translation in association (Honda et al., 1996a, Honda et al., 1996b). Domain IV has been found to be non-essential for the ribosomal binding (Honda et al., 1996a).

It has been found that there are several host-encoded factors that play important roles in association with the 5' UTR to regulate HCV genome translation and replication. For instance, the elements EIF, La autoantigen, polypyrimidine tract binding protein (PTB) and poly (rC)-binding protein (PCBP2) bind to specific regions inside the 5' UTR (Ali and Siddiqui, 1995, Ali and Siddiqui, 1997, Fukushi et al., 2001). Recently, it has been discovered that the liver-specific microRNA-122 (miR-122) attaches to two different sites inside the 5'UTR between domains I and II; this binding is important for both HCV translation and replication (Jopling et al., 2005, Jopling et al., 2008, Henke et al., 2008).

1.5.2.2 **Core**

The first structural protein encoded by the 5' end of the genome is the core protein that forms the viral capsid shield (McLauchlan, 2009, Santolini et al., 1994). Following translation, a signal peptide is found between core and E1 that targets the endoplasmic reticulum (ER) membrane, where the polypeptide chain is cleaved by a specific cellular enzyme called signal peptidase (SP) that releases Envelope 1 (E1) resulting in the formation of an undeveloped (21 kDa) form of HCV core (Santolini et al., 1994). Next, core goes through another cleavage that is mediated by the cellular enzyme signal peptide peptidase (SPP), contributing to the mature 19 kDa core ~177 amino acid structure (Hussy et al., 1996, McLauchlan et al., 2002a, Santolini et al., 1994, Moradpour et al., 1996).

The mature form of core (19 kDa) has a uniquely shaped dimeric α-helical protein that involves two different domains known as D1 and D2. The first domain (D1) is involved in RNA binding (Boulant et al., 2005). Domain D2 has a significant impact on core transfer to lipid droplets (LDs); any deletions within this region can stop the association between LDs and core (McLauchlan et al., 2002b). D2 structure consists of two amphipathic elements called α-helices

(termed HI and HII); these elements are divided by an unstructured region known as the hydrophobic loop (Boulant et al., 2006). Hydrophobic residues, located within the helix, interact with the phospholipid layer surrounding LDs, and any defined mutations within these residues terminate the association of core-LDs (Boulant et al., 2006).

1.5.2.3 **E1 and E2**

The Envelope 1 (E1) and Envelope 2 (E2) proteins are 35kDa and 60/70 kDa in size and 160 and 360 amino acids long respectively. They play an important role in HCV entry and immune evasion, forming non-covalent heterodimers on the surface of viral particles (Dubuisson, 2007). Both E1 and E2 contain a single C-terminal transmembrane domain (TMD) of 30 amino acids that attaches the proteins to the endoplasmic reticulum (ER) following which, the N-terminal becomes placed within the ER lumen (Cocquerel et al., 1999, Cocquerel et al., 1998) (Dubuisson et al., 2002).

The structure of TMD has been found to possess two hydrophobic regions and these two hydrophobic areas differ at least by one invariant charge residue (Cocquerel et al., 2000). The transmembrane domain of E1 is formed from cleaved signal sequence that directs attachment of the envelope to the ER membrane. SP-mediated cleavage occurs at the E1/E2 and E2/p7 boundaries (Dubuisson et al., 2002). The TMDs of the E1 and E2 play a role in facilitating envelope heterodimer formation (Op De Beeck et al., 2000) and the fusion properties for both proteins (Ciczora et al., 2007).

E1 and E2 have been shown to possess several conserved glycoproteins motifs: E1 possesses around five whereas E2 has around eleven; these glycoprotein motifs undergo N-linked glycosylation when holding the protein that is located within the ER (Goffard and Dubuisson, 2003, Meunier et al., 1999, Dubuisson et al., 2000). This change has been believed to help the glycoprotein to fold and then to facilitate the entry of HCV (Goffard et al., 2005, Helle et al., 2010).

Several studies from HCV isolates have also shown two highly variable regions within the E2 envelope glycoprotein, named as the hypervariable region 1 (HVR1) and hypervariable region 2 (HVR 2)(Weiner et al., 1991).

The HVR1 is situated at the N-terminus of E2, and the variability within this region has been referred to as (i) the capability of this area to accept the amino acid changes and (ii) a region that undergoes strong selective pressure by the host anti-HCV immune responses (Penin et al., 2001). During the course of infection, antibodies targeting the HVR1 are frequently altered, illustrating the high selective pressure on the HVR1 (Forns et al., 1999).

In spite of considerable variation within this region, analysis from different isolates shows that some physiochemical and conformational regions of HVR1 are conserved, revealing the residues that are essential for example for attachment to attach to other proteins, glycosaminoglycans (GAGs) or lipid (Penin et al., 2001). Both E1 and E2 heterodimers are important for virus entry and E2 attaches to three different cellular receptor proteins, to gain entry to hepatocytes, namely, tetraspanin (CD81), occludin (OCLN) and scavenger receptor class B member I (SR-BI) (Liu et al., 2009, Pileri et al., 1998, Scarselli et al., 2002). Another co-receptor responsible involved in HCV entry, claudin-1 (CLDN1), has been less well characterized with regard to interaction sites with HCV envelope (Evans et al., 2007). Cyanovirin-N (CV-N) can inhibit the entry of HCV by interacting with the N-linked glycans located on the glycoproteins (E1/E2), which inhibits the E2-CD-81 interaction (Helle et al., 2006).

1.5.2.4 **p7**

p7 is a hydrophobic protein that is 7kDa in size. This protein is located between Envelope 2 and the non-structural protein 2 (NS2) of the HCV polyprotein. During HCV translation, p7 is attached to the ER membrane (Lin et al., 1994a). Cleavage at the E2-p7 and p7-NS2 boundaries is often delayed and produces incomplete precursor proteins such E2-p7-NS2 and E2-p7 (Dubuisson et al., 1994, Lin et al., 1994a).

It is not known if these precursor proteins have a significant role in virus replication (Jones et al., 2007). Different studies show that p7 contains 2 transmembrane regions TM1 and TM2 and show a double spanning topology with N- and C termini locating close to the ER lumen with a loop site in the cytoplasm (Carrere-Kremer et al., 2002, Isherwood and Patel, 2005, Cook and Opella, 2011, Khaliq et al., 2011, Patargias et al., 2006, Boson et al., 2011, Steinmann et al., 2007). P7 is not essential for HCV RNA replication (Lohmann et al., 1999).

However, inoculating HCV RNA with a p7 deletion into animal models (chimpanzees) reduces HCV transmission (Sakai et al., 2003). Furthermore, an HCV genome without the p7 gene is incapable of producing active infection virus in a cell culture and is associated with a blockage before the assembly step (Jones et al., 2007, Popescu et al., 2011, Steinmann et al., 2007).

Adaptive mutations in p7 can improve HCV particle production (Russell et al., 2008). A study using chimeric viruses has shown that p7 may play a role as a virulence factor (Steinmann et al., 2007, Khaliq et al., 2011). p7 has been classified as a member of the viroporin protein family and its structure has a unique form, a hexameric structure with cation channel activity; this channel is essential for HCV infection in chimpanzees (Griffin et al., 2003, StGelais et al., 2007, Sakai et al., 2003) and is located on the cytoplasmic loop of the protein (Griffin et al., 2004).

P7 function can be inhibited using *Adamantanes* (*amantadine* and *rimantadine*) that inhibit H+ transport through ion channels. Such therapies also block influenza A infection through inhibition of the activity of the M2 ion channel (Fleming, 2001, Griffin et al., 2003). In HCV, sensitivity to these compounds has been found to vary according to HCV genotype (Griffin et al., 2008).

1.5.2.5 **NS2**

NS2 is a protein of 23kDa (Santolini et al., 1995, Yamaga and Ou, 2002). The NS2 C- terminal end contains a protease domain with a circular structure that is

believed to be the exit in the cytosolic side of the ER membrane, while the N-terminus possesses three TMDs (Lorenz et al., 2006, Wang et al., 2013, Fischer and Kruger, 2009). The first TMD has been characterised structurally and contains flexible helicase elements that are linked to a constant α- helix (Jirasko et al., 2008). NS2 carries out autoproteolytic cleavage at the NS2/NS3 junction (Grakoui et al., 1993, Hijikata et al., 1993, Foster et al., 2010), whereas the N-terminus of NS2 is released by SP (Lin et al., 1994a, De Francesco, 1999). Analysis of HCV NS2/NS3 using mutagenic analysis has shown that the dividing of these proteins also requires the NS3 serine protease domain to be present (Grakoui et al., 1993, Hijikata et al., 1993).

Recent studies using a chimeric viral replicon containing the NS3-NS5B regions based on the HCV strain JFH1 with structural genes from other HCV genotypes, has shown the importance of the intra-/inter-genotypic junction site at NS2 and how it has an effect on HCV particle production (Pietschmann et al., 2006, Steinmann et al., 2013). The whole length of NS2 protein is necessary for the production of viral particles, while the proteolytic role of this protein is not essential during assembly (Jirasko et al., 2008, Jones et al., 2007).

1.5.2.6 **NS3-4A**

NS3-4A consists of the non-structural protein 3 (NS3) and a cofactor, non-structural protein 4A (NS4A). NS3 is a multifunctional protein which is 70 kDa in size and contains a serine protease within the first third of the N-terminal (amino acid 1-180). The remaining two thirds within the C-terminal end has nucleoside triphosphatase NTPase and RNA helicase activity (amino acids 181-631). Several studies have examined the role of both enzymes and their function is well described (Raney et al., 2010, Morikawa et al., 2011, Bartenschlager et al., 1995). NS3 to sits on the ER membrane, where it is held in place by the N-terminal end of NS4A (Wölk et al., 2000).

At the ER membrane, the serine-type protease domain of NS3 is initially connected to NS2 and is then cleaved. Afterwards, the NS3 serine proteinase mediates cleavage between the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and the

NS5A/NS5B junctions (Bartenschlager et al., 1993, Grakoui et al., 1993, Lin et al., 1994b). Excluding the NS3/NS4A junction, all junctions are treated *in trans* (Bartenschlager et al., 1994). NS3 mediates cleavage in a binding site that possesses a catalytic triad. This catalytic triad contains His 57, Asp 81 and Ser 139 residues. Mutations introduced within these amino acids can eradicate the cleavage mediated by NS3 (Bartenschlager et al., 1993, Grakoui et al., 1993, Lin et al., 1994b).

The NS3 contains a zinc atom that binds to four residues, three cysteine residues and one histamine (De Francesco et al., 1996, Stempniak et al., 1997); these residues have been shown to have a significant role in the NS3 activity (Hijikata et al., 1993). The association between NS3 and the co-factor NS4A is important for the cleavage of NS3/NS4A and NS4B/NS5A (Failla et al., 1994, Gallinari et al., 1998, Lin et al., 1994b). NS4A contributes to folding of NS3 (Kim et al., 1996, Love et al., 1996, Kwong et al., 1998).

The two-thirds of the C-terminal region of the HCV NS3 protein that have RNA helicase and NTPase activity and forms a Y-shaped molecule. (Tai et al., 2001, Kim et al., 1998, Yao et al., 1997). While NS3 binds to an RNA substrate, the NS3 dimer is required to untwist the HCV RNA (Serebrov and Pyle, 2004). This helicase process needs to be carried out in a series of organized steps (Serebrov and Pyle, 2004, Appleby et al., 2011).

The NS3 alone has fairly poor RNA helicase activity and supported by NS4A and the protease domain, which improve the efficiency of untwisting RNA (Beran et al., 2009, Frick et al., 2004, Pang et al., 2002). Interestingly, NS3 itself has the ability to untwist DNA duplexes, allowing the protein to act on cellular DNA (Pang et al., 2002, Tai et al., 1996) (Dubuisson, 2007).

NS3 therefore plays crucial roles in RNA replication (Lam and Frick, 2006, Lohmann et al., 1999) (Beran et al., 2009). Cell culture adaptive mutations (CCAMs), which improve RNA replication, have been found in both the protease and the helicase domain of NS3 (Lohmann et al., 2003, Krieger et al., 2001). Recent studies have shown that the NS3 helicase domain is also important for

viral assembly, as mutations located within the coding region can rescue the production of HCV from inter-genotypic chimeras that are found to be unable to produce virions (Ma et al., 2008, Yi et al., 2007, Phan et al., 2011).

1.5.2.7 **NS4A**

NS4A is 6kDa in size (54 amino acids) and is the smallest protein encoded by the HCV genome. NS4A is made up of an N-terminal hydrophobic alpha-helix sequence that presents NS3 and NS4A to the cell membrane (Wolk et al., 2000), a central peptide that builds a beta-strand that is found to stabilize NS3 proteins and a C-terminal region that makes an alpha-helix shape at low pH (Phan et al., 2011) (Lindenbach et al., 2007). NS4A works as a cofactor for both the serine protease and helicase activity of NS3 (Bartenschlager et al., 1994, Failla et al., 1994, Lin et al., 1995, Frick et al., 2004, Pang et al., 2002). NS4A enhances ATP binding to NS3 thereby increasing maximum ATP-hydrolysis activity (Beran et al., 2009).

1.5.2.8 **NS4B**

NS4B is 27 kDa in size and is highly hydrophobic. The structure of NS4B contains a central region with 4 TMDs that help the protein to attach strongly to the ER membrane (Hugle et al., 2001, Lundin et al., 2003, Elazar et al., 2004).

The TMDs are connected by three transmembrane loops. The loop connecting the second and third TMD is located within the cytosolic side of the ER membrane (Lundin et al., 2003). This loop contains a nucleotide binding motif (NBM) that binds GTP (Einav et al., 2004) (Einav et al., 2008b). The last five residues of the C-terminus (aa 257 - 261) undergo palmitoylation and may play a crucial role in protein-protein interactions during HCV replication (Yu et al., 2006). Expressing NS4B can cause rearrangement of cellular ER membranes, resulting in formation of punctate shapes that are called ‘membranous web’ and membrane-associated foci (MAFs) (Gosert et al., 2003, Egger et al., 2002, Gretton et al., 2005).

NS proteins that are involved in RNA replication sit within this punctate area

(Elazar et al., 2004, Hugle et al., 2001) and viral RNA interacts with the non-structural proteins within this structure on the ER membrane.

The membrane changes that are made by NS4B are therefore probably crucial for the HCV RNA replication. In addition to providing a suitable environment for HCV RNA synthesis, NS4B may contribute to HCV RNA replication directly. Two studies published in 2003 showed that cell culture adaptive mutations (CCAMs) within NS4B can improve HCV RNA replication by up to 30 fold when compared with HCV RNA containing wild type NS4B (Lohmann et al., 2003, Lohmann et al., 2001, Krieger et al., 2001, Nakamura et al., 2008).

A single amino acid plays a key role in RNA replication and changes here can either enhance or terminate RNA replication (Lindstrom et al., 2006, Blight, 2007). Making a chimeric replicon by inserting Con1 containing NS4B derived from HCV genotype 1b into the HCV H77 strain (genotype 1a) genome increases HCV RNA synthesis up to 10 fold compared with WT (Blight, 2007, Pietschmann et al., 2009). In addition, a Con1 (genotype 1b) genome containing HCV genotype 1a NS4B results in defective HCV RNA replication (Paredes and Blight, 2008, Pietschmann et al., 2009).

Replication of the defective chimeric genome can be improved by introducing mutations within NS3. Based on these data, it is likely that NS4B has the ability to interact with the replication mechanism in a genotype-specific way and compatibility between NS3 and NS4B is needed for RNA replication (Paredes and Blight, 2008). NS4B can also inhibit HCV RNA replication through binding to the negative-strand (Einav et al., 2008a). Therefore, NS4B has more than one role; it contributes to a suitable environment for replication complex (RC) assembly, and has an impact on controlling RNA synthesis within RCs.

1.5.2.9 **NS5A**

The non-structural protein 5A (NS5A) consists of a 447-aa membrane-associated phosphoprotein. It plays a significant role in controlling HCV RNA replication and in the formation of HCV particles. NS5A exists as two types known as hypo- and

hyper-phosphorylated forms, with a size of 56kDa and 58 kDa respectively. NS5A has three different domains, known as domain I, domain II and domain III, separated by low-complexity sequences I and II (LCS I and LCS II) (Tellinghuisen et al., 2004). The first domain has been well characterized. It is attached to a zinc atom that contributes to RNA replication (Tellinghuisen et al., 2004). The crystallographic structure of domain I reveals a dimer with a groove that binds either ssRNA or dsRNA (Tellinghuisen et al., 2005).

NS5A can bind to both positive and negative strands of RNA (Huang et al., 2005). Domain I also contains an amphipathic α-helix (AH) that is conserved across all isolates and is necessary for membrane localization; disruption of the AH leads to impaired HCV replication (Elazar et al., 2003, Brass et al., 2002). Domains II and III are less well characterised; domain II is unstable in normal conditions (Liang et al., 2006). Domain III is natively unfolded (Hanoulle et al., 2009, Verdegem et al., 2011) and displays a propensity to partially fold into an α-helix (Verdegem et al., 2011).

Domains I and II are essential for RNA replication. The third domain (D-III) is unnecessary for HCV RNA replication (Appel et al., 2008, Tellinghuisen et al., 2008b, Ross-Thriepland et al., 2013) but can be manipulated to allow molecules containing green fluorescent protein (GFP) to be inserted without inhibiting the HCV RNA replication process (Appel et al., 2005, Liu et al., 2006, McCormick et al., 2006, Moradpour et al., 2004), thus illustrating the features of NS5A within live cells. A study using GFP showed that the NS5A fusion protein is associated with HCV RNA undergoing active replication (Targett-Adams et al., 2008).

The phosphorylation of NS5A has an impact on HCV RNA replication; HCV RNA replication can increase when hyperphosphorylation of NS5A is reduced (Appel et al., 2005, Evans et al., 2004, Neddermann et al., 2004). An interaction has been reported between NS5A and the human vesicle-associated-membrane protein A (hVAP-A). This protein is involved in intracellular trafficking and HCV RNA synthesis (Gao et al., 2004, Tu et al., 1999, Zhang et al., 2004).

Experiments have shown that hyperphosphorylated NS5A binds less efficiently to hVAP-A when compared to basally phosphorylated protein (Evans et al., 2004). Phosphorylation may therefore have a specific role in regulating HCV RNA replication.

Domain III of NS5A is necessary for HCV particle formation and is involved in binding to HCV core during viral replication (Appel et al., 2008, Tellinghuisen et al., 2008a). A single mutation within a single serine site in domain III results in impaired HCV particle production without eradicating the association between HCV NS5A and lipid droplets (LDs)(Tellinghuisen et al., 2008a). Deletions within domain III do not affect the ability of NS5A to interact with LDs, but they do disrupt the domain III connection with core (Appel et al., 2008).

NS5A is involved in a number of other interactions (Macdonald and Harris, 2004). Protein kinase R, an INF-induced protein that phosphorylates eIF-2a binds to NS5A (Samuel, 1993, Kim et al., 2011, Gale et al., 1997) and can interact with epidermal growth factor (EGF), activating factor of the Ras-Erk signalling pathway (Macdonald et al., 2003, Macdonald et al., 2005, Mankouri et al., 2008).

1.5.2.10 **NS5B**

HCV replication starts with the synthesis of a complementary negative RNA strand (–RNA), utilizing the positive stranded HCV genome as a template. This negative strand acts as a template for further positive strand synthesis. The HCV RNA dependent RNA polymerase (RdRp) encoded by the NS5B gene carries out both steps. RdRp enzyme is a 68 kDa protein consisting of 591 amino-acids and has been comprehensively characterized (Behrens et al., 1996, Lohmann et al., 1997, Lesburg et al., 1999, Simister et al., 2009). It is a major target for antiviral intervention. The NS5B catalytic domain has a distinct sequence that is shared by other RdRp enzymes - GDD.

The ssRNA template attaches to a groove between the finger and thumb domains. Nucleotide triple phosphates (NTPs) can access the RNA active site within a tunnel within the palm domain.

The HCV RNA-dependent RNA polymerase (RdRb) fits within the membrane proteins known as tail-anchored proteins 20. These proteins have features including 1) a posttranslational membrane targeted by an insertion sequence (hydrophobic C-terminal) mapped to 21 C-terminal amino acid residues within the NS5B region; 2) integral membrane association; and 3) a functional protein domain (Wattenberg and Lithgow, 2001)Two different studies have shown that the RdRp of HCV contains an insertion sequence that penetrates through the phospholipid bilayer of the ER as a transmembrane segment (Ivashkina et al., 2002). Old structure predictions have indicated that this segment is implemented as a helical fold within the hydrophobic membrane (Schmidt-Mende et al., 2001).

The NS5B crystal structure shows a specific catalytic domain that is followed by a C-terminal extension that binds to the transmembrane region by an active site groove. The catalytic domain consists of 530 N-terminal amino acids, showing the three subdomains (fingers, palm and thumb). NS5B has a special feature; the fingers' subdomains have an extension that interacts with the thumb subdomain; this interaction limits the flexibility of one subdomain by the other. This results in a totally surrounded active site into which NTP molecules can attach. The RNA template is enclosed in a groove that leads to the active site; the NTPs access this site via an NTP tunnel. RNA synthesis by NS5B initially happens via a specific mechanism in the absence of primer (known as a *de novo* mechanism). Mapping of the NS5B complex with oligonucleotides has shown that the NTP binding site used for de novo initiation has two locations (catalytic and priming sites) (Bressanelli et al., 2002).

This mechanism suggests that this enzyme is essential for *de novo* RNA synthesis. The current NS5B structural model is based on the conformation of the enzyme that is assumed during the synthesis step. The enzyme may be modified to a different conformation when it is moved to the elongation step of replication. The current NS5B structural model shows no clear exit path for double-stranded RNA. So far, it is not clear whether recently synthesised RNA develops as a double-strand or if it unwinds after new nucleotides leave the

active site area. The NS5B structure has been characterised in association with non-nucleoside inhibitors; these drugs attach to the thumb surface about 30 A° away from the active site. This binding site within the thumb surface is close to the allosteric GTP site within the fingers–thumb interface (Bressanelli et al., 2002). Non-nucleoside inhibitors may work by stopping the initiation step of the enzyme via inhibition of a conformational change that is needed in order to proceed with elongation. Oligomerisation of NS5B from HCV may be crucial for RNA synthesis activity (Wang et al., 2003), similar to RdRp in the poliovirus (Lyle et al., 2002).

1.5.2.11 **The 3' untranslated region (3' UTR).**

The 3'UTR contains three different parts; a variable region of 26-70 nucleotides in length, a poly U tract of variable length sequence (20 to 200 nucleotides) and an X-tail of a 98-100 nucleotide sequence. All of these elements are involved in both replication and translation of HCV (Anjum et al., 2013, Blight and Rice, 1997, Tanaka et al., 1996, Dutkiewicz et al., 2006).

The variable region contains two stem loops (VSL1 and VSL2). These regions are not essential for HCV RNA replication in cell culture (Friebe and Bartenschlager, 2002, Blight and Rice, 1997). The poly U region, highly variable in size and structure is made up of uridine residues in combination with other cytidine residues. The poly U poly UC region must be at least 26 nucleotides to allow HCV RNA replication (Friebe and Bartenschlager, 2002). The third part of the 3' UTR, the 3' X-tail is a highly conserved region that contains three stable structure stem loops (SL1-3). Deletion of any of these stem loops leads to RNA replication in cell culture or in chimpanzees being abolished (Friebe and Bartenschlager, 2002, Yi and Lemon, 2003, Yanagi et al., 1999).

1.6 The immune response to HCV

1.6.1 Innate immunity

Innate immunity represents the first line of defence against RNA viruses including HCV. It is characterised by the recognition of viral RNA by pathogen associated molecular patterns (PAMPs). Foreign RNA inside cells is recognised by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and binding protein receptors of intracellular nucleic acid (Cook et al., 2004, Iwasaki and Medzhitov, 2004) that elicit their effects by upregulating expression of antiviral genes.

In hepatocytes, TLR3, protein kinase R (PKR) and retinoic-acid-inducible gene I (RIG-I) are pattern recognition receptors that bind to HCV. Following adaptor protein signalling, IFN type I is activated and secreted (Horner and Gale, 2013). Binding of type I IFN to the cell receptor activates the JAK-STAT signaling pathway eventually resulting in expression of interferon-stimulated genes in the infected cell and in neighbouring cells (Wieland et al., 2014, Gale and Foy, 2005). Interferon-stimulated genes (ISGs), expressed as a response to infection, encode many different proteins including ISG15 and USP18, that have been known to inhibit HCV replication *in vitro* (Randall et al., 2006, Chen et al., 2010).

HCV has counter-mechanisms that act against the antiviral effect of the innate immune response (Bigger et al., 2001, Su et al., 2002, Thimme et al., 2002, Bigger et al., 2004). Serine protease activity from NS3/4A interferes with the viral detecting pathways at the early stage of infection via degradations of MAVS and TRIF in the innate immune response pathway (Horner and Gale, 2013). Core, E2 and NS5A are also associated with the inhibition of JAK- STAT signalling (Taylor et al., 1999, Taguchi et al., 2004, Foy et al., 2005). Interestingly, the presence of ISGs is associated inversely with interferon therapy success (Chen et al., 2005). This may indicate that in cells where ISGs have been already generated, counter-mechanisms may be already be induced by HCV which cannot be overcome with external interferon.

1.6.2 Natural killer cells (NK).

Natural killer (NK) cells are among the first lines of innate defence against harmful organisms. NK cells kill cells infected with viruses by producing cytotoxic molecules such as perforin and granzyme or by tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated killing. NK cells secrete IFN, TNFα, IL- 10, and IL-21, which are all known already to regulate the innate and adaptive immune system.

The activity of NK cells is balanced by both activating and inhibitory signals produced by the interaction between NK receptors and their ligands that are found on the target cells (Jost and Altfeld, 2013). NK cells are present in high levels in the liver and play an important role controlling hepatotropic infections, including HCV (Doherty and O'Farrelly, 2000, Crispe, 2009).

NK cell hyper-activation is often present during the acute phase of infection; for example, NK cell activity has been detected in high levels in intravenous drug users (IDUs) during early infection (Golden-Mason et al., 2010). In one small study of 12 healthcare workers exposed to HCV, NK cell activation was associated with control of infection in 11/12 individuals (Werner et al., 2013). However other studies have shown NK cell activation regardless of outcome (Alter et al., 2011, Amadei et al., 2010, Pelletier et al., 2010).

NK cells remain highly activated in patients with chronic HCV infection and the level of activity correlates with liver inflammation outcome (Bonorino et al., 2009, Oliviero et al., 2009). Natural killer cells from peripheral blood express markers of activation including NKp30, NKp44, NKp46, NKG2C, and NKG2D and can play a role in increasing the degree of inhibiting the receptor expression in NKG2A in positive HCV patients compared to negative HCV individuals (Shoukry et al., 2011, Golden-Mason and Rosen, 2013, Rehermann, 2013). HCV E2 may bind to CD81 on the NK surface and this may inhibit NK cell function (Crotta et al., 2002, Tseng and Klimpel, 2002).

Study data using the rodent lymphocytic choriomeningitis virus (LCMV) model show that NK cells can control CD4 helper cells that then mediate the function of CD8 T cells in the chronic phase (Waggoner et al., 2012). NK cells also interact with dendritic cells and this may also influence innate and adaptive responses. NK cells generate IFNγ and TNFs, that stimulate mature DCs and improve their ability to target virus T cells. DCs also secrete IL-2 and IL-15, which are known to increase NK cell activation (Marcenaro et al., 2012).

1.6.3 Dendritic cells (DC)

Dendritic cells alongside macrophages are the major antigen-presenting cells (APCs) in humans. They connect the innate and adaptive immune systems and influence the direction of the specific immune response to HCV. DCs promptly mature as a reaction to danger signals including TLR ligands bound to PAMPs and cytokines released from other cells (Steinman and Banchereau, 2007).

They are divided into two different types: myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDC). mDCs contains two subsets: the first subset is the mSC-1 which is a primary T cell stimulator. The second subset is the mDC-2 that have an impact on recognising wound infections. The second type of dendritic cells are pDCs, similar to plasma cells. mDCs are involved in antigen processing and presentation, whereas pDCs, which can recognise virus infection as well, secrete type I and III interferons. Plasmacytoid dendritic cells have been shown to have a strong ability to recognize HCV RNA via toll-like receptor 7 (TLR-7) when it exists as part of the hepatocyte-infected cells (Takahashi et al., 2010). mDCs and pDCs have both been shown to have an association with HCV infection outcome.

Low frequencies of mDCs and pDCs have been shown to correlate with a chronic outcome of infection (Longman et al., 2004, Wertheimer et al., 2004, Longman et al., 2005, Piccioli et al., 2005). Pelletier and colleagues found that hyper-responsiveness of dendritic cells correlated with spontaneous clearance of HCV as a result of better priming of specific T cells (Pelletier et al., 2013). Other groups have linked defective DC activity and chronicity, and the correlation of

DC response to TLR ligand interactions that increase T regulatory cell (Treg) propagation (Bain et al., 2001, Dolganiuc et al., 2003, Sarobe et al., 2003, Rodrigue-Gervais et al., 2007, Dolganiuc et al., 2008).

Dendritic cells may polarise the adaptive immune response during early infection by releasing different quantities of regulatory cytokines. The anti-inflammatory cytokine, IL-10 limits activation of cells from both the innate and the adaptive immune system to maintain a homeostatic state. IL-12, in contrast, activates the adaptive immune response and polarises T- helper type 1 CD4+ T cells towards a Th1 profile (Ma et al., 2015, Steinman and Hemmi, 2006).

1.6.4 Adaptive immune responses to HCV

The adaptive immune system (that includes the specific B and T-cell mediated response) plays an essential role in clearance of HCV. The role of cell-mediated immunity is indicated by the link between class I and II human leukocyte antigens (HLA) and outcome. Studies in chimpanzees have also shown that CD4 and CD8 T cells are essential for the spontaneous clearance of HCV. The role of neutralising antibodies (nAbs) in achieving viral clearance needs to be explored in wider studies. In one study, memory CD4 and CD8 T cells were detected in both blood and peripheral blood cells from patients who had spontaneously cleared HCV 20 years before, while antibodies had declined at that this time (Takaki et al., 2000). Re-infected individuals show a higher rate of clearing HCV spontaneously compared to individuals with primary infection, indicating that adaptive immunity is central to the control of infection (Mehta et al., 2002, Aitken et al., 2008, Osburn et al., 2010).

1.6.4.1 **The CD8+ T cell response during acute HCV infection**

Following primary HCV infection, CD4 and CD8 T cell responses appear after 6-8 weeks. The delay before virus-specific T cells emerge includes the time that is required for the HCV antigen to reach the lymph nodes and be recognised by antigen-presenting cells (APCs) but could also be delayed by the liver's ability to produce immunologic tolerance (Bowen and Walker, 2005a, Crispe, 2009).

An early strong specific CD8 T cell response is associated with spontaneous clearance of HCV. Several different epitopes are recognised in both animal models and humans (Cooper et al., 1999b, Lechner et al., 2000c) and spontaneous clearance has been linked particularly to CD8 T cell responses directed against the non-structural proteins (Smyk-Pearson et al., 2006).

Patients who progress to chronicity often exhibit evidence of immune escape due to mutations within CD8 T cell epitopes. Epitopes containing mutations may lose the ability to bind to MHC class I alleles, are therefore not detected and stimulate responses from CD8 T cells poorly (Erickson et al., 2001, Bowen and Walker, 2005b). HLA alleles from the host exert selective pressure on specific epitopes; these mutations may return to wild type sequences when the HCV is transferred to a new patient who does not carry the same HLA type or when the HCV epitope is not pressured by the immune system any more (Timm et al., 2004a).

The host HLA may also induce selection pressure at a population level, resulting in viral adaptation within a specific genetically-related population (Gaudieri et al., 2006). (Soderholm et al., 2006). Viral fitness limits the variability within some epitopes and the likelihood of those epitopes persisting in a population (Soderholm et al., 2006, Uebelhoer et al., 2008). Several class I HLA alleles such as HLA-B27 play a strong role in HCV outcome. Such HLA-restricted epitopes often cannot withstand mutation due to a high virus fitness cost (Dazert et al., 2009, Neumann-Haefelin et al., 2006, Neumann-Haefelin et al., 2011). Lack of binding to the TCR is another way in which HCV can evade the T cell response. A single mutation within the MHC class I $NS3_{1406}$ epitope, for example, can result in poor responses as this mutant exploits a 'hole' within the T cell repertoire (Wolfl et al., 2008).

A strong CD8 T cell response is associated with the spontaneous clearance of HCV. Studies using MHC class I tetramers have shown that specific T cell responses can increase to as much as 8% of the T cell population recognising one single epitope during spontaneous clearance (Lechner et al., 2000c, Badr et al., 2008). Studies in chimpanzees show even higher levels of tetramer stained cells

in the liver compared to the peripheral blood in association with a high level of the T cell activation marker CD69 (Shoukry et al., 2003b).

MHC class I tetramer may be used to carry out phenotypic characterisation of HCV-specific CD8 T cells *ex vivo*. Early expression of the IL-7 receptor (CD127) on specific CD8 T cells is a predictor of clearance, whereas the loss of specific CD8 T cells correlates with chronicity (Golden-Mason et al., 2006, Shin et al., 2013). Memory T cells produced after primary HCV clearance also express high levels of CD127, in addition to Bcl-2 (CD127hi, Bcl-2hi) (Abdel-Hakeem et al., 2010, Badr et al., 2008).

These data reflect that seen in the LCMV mouse model, where CD127 is a marker that occurs in long-lasting memory T cells (Kaech et al., 2003).

Specific CD8 T cells targeting HCV may commonly express exhaustion markers and this phenotype is associated with progression to chronicity. PD-1 may play a role in activating memory cells as well as exhaustion in patients with HCV (Urbani et al., 2006, Bowen et al., 2008, Kasprowicz et al., 2008). The exhaustion markers cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), killer cell lectin-like receptor G1 (KLRG1), 2B4 (CD244), mucin-domain-3 (TIM-3), CD160 and T cell immunoglobulin are associated with exhaustion and progression to chronicity (Bengsch et al., 2010, Kared et al., 2013). Galectine-9 (Gal-9) is a TIM-3 ligand that is upregulated during acute HCV infection in patient plasma and liver before progression to chronicity (Kared et al., 2013, Mengshol et al., 2010). Blocking TIM-3, CTLA-4 or PD-1 can result in preservation of specific CD8 T cell function and blocking more than one of these results in a synergistic decrease in exhaustion (Penna et al., 2007, Nakamoto et al., 2009b). CD4 T cell mediators such as IL-21 can also help in saving the T cell from TIM-3 and Gal-9 mediated cell death (apoptosis) (Kared et al., 2013).

Analysing virus-specific CD8 T cells that are associated with HCV clearance has resulted in the identification of multiple CD8 cell effector molecules (Badr et al., 2008). The secretion of IFNγ, IL-2 and CD107a (a marker of CD8+ T cell degranulation) are associated with clearance (Alter et al., 2004, Badr et al.,

2008). T cell proliferation capacity is also central to outcome (Einav et al., 2008b, Lauer, 2013). Patients that progress to the chronic phase have impaired CD8 T cell cytokine production and proliferation (Gruener et al., 2001, Appay et al., 2002, Wedemeyer et al., 2002).

Detecting specific CD8 T cells within HCV chronic patents is difficult in the peripheral blood whereas in the liver they can be detected quickly (He et al., 1999, Radziewicz et al., 2007b). Patients in the chronic phase of HCV infection express high levels of 2B4, PD-1 and TIM-3.

HCV specific T cells in the peripheral blood are weaker in cytokine production and proliferation than when they are detected in the blood for the first time, confirming a stunned phenotype (Lechner et al., 2000a).

In conclusion, spontaneous clearance of HCV is associated with strong polyfunctional HCV CD8 T cell responses while progression to chronicity is associated with T cell escape and weak T cell responses in association with the expression of exhaustion markers. During progression to chronicity, mutations within the targeted epitope may lead to immune escape. A loss of specific T cells or a change in the immune regulatory profile that cooperates with specific CD8 T cells may result in progression to chronicity. Finally, a high level of HCV replication may impair HCV-specific T cells via persistent antigenic stimulation that leads to function loss and reduces the CD8 T cell response to HCV. CD8 T cell exhaustion can be worsened by increased expression of ligand that binds to an inhibitory receptor such as (Gal-9) (Abdel-Hakeem and Shoukry, 2014).

1.6.4.2 **CD4+ T cell responses during acute HCV**

A strong and effective CD4 response is an important factor in virus clearance (Missale et al., 1996, Gerlach et al., 1999). The association of proliferation following stimulation with peptides derived from the non-structural proteins was one of the first insights into CD4 T cell function during clearance of acute HCV infection (Diepolder et al., 1995, Missale et al., 1996).

Loss of CD4 responses is associated with progression to chronicity (Thimme et al., 2001b, Lechner et al., 2000c, Gerlach et al., 1999).

One study identified 14 unique CD4 epitopes that were presented by patients in the acute phase in HCV clearance (Day et al., 2002). Out of these 14 epitopes, some were recognised as promiscuous epitopes that are presented by many class II HLA-DR alleles (Shoukry et al., 2004, Schulze zur Wiesch et al., 2005). Unlike CD8 epitopes, escape mutations were found to happen rarely within MHC class II restricted CD4 T cells epitopes (Fleming et al., 2010, Fuller et al., 2010). However, the CD4 response to HCV has been much less well characterized - in spite of increased technical knowledge about MHC class II tetramers, there has been a lack of direct *ex vivo* studies of HCV-specific CD4 T cell responses during acute HCV infection in humans.

While difficult to detect, HCV-specific CD4 T cells have been found in chimpanzees up to 7 years after resolved HCV infection using MHC class II tetramers (*ex vivo*) (Bowen et al., 2008). Proliferation of CD4 T cells was found to be rapidly lost, which has an impact on cytokine production, especially IL-2 (Semmo et al., 2005a, Schulze Zur Wiesch et al., 2012). Blocking PD-1, IL-10 and TGF-β pathways *in vitro* can lead to rescue of CD4 T cell proliferation capacity and the increased production of cytokines in chronically infected patients (Raziorrouh et al., 2011). The central role of CD4 T helper cells to maintain CD8 T cell function has been demonstrated in chimpanzees where declining CD4 function is associated with the accumulation of escape mutations within CD8 T cell epitopes (Grakoui et al., 2003b).

The phenotype of CD4 cells in patients that spontaneously clear HCV has been described in small studies. One such study showed an increase in $CD161^{hi}$ CCR6+ CD26+ CD4+ Th17 T cells in spontaneous clearance. These immune cells secrete high amounts of IL-17A and IL-21, and these two cytokines have also been found in high concentrations in plasma from patients who have spontaneously cleared HCV (Kared et al., 2013). Increased plasma levels of IL-21 in late acute infection is associated with reduced apoptosis of virus-specific CD8 T cells mediated by Tim-3/Gal-9 (Kared et al., 2013).

This highlights the function of IL-21 as one of the main cytokines influencing outcome in acute HCV. Interestingly, similar findings have been found in the HIV and LCMV model (Johnson and Jameson, 2009). More studies are needed to investigate the role of alternative IL-21 sources such as NK cells and follicular helper T cells (Tfh).

As well as classic Th1 and Th2 type CD4 cells, Th17 and T regulatory cells (Tregs) may influence outcome during acute HCV infection (Manigold and Racanelli, 2007, Ebinuma et al., 2008, Rowan et al., 2008). Treg cells may have an impact on the inhibition of HCV-specific CD4 and CD8 T cells. CD39+ CTLA4+ Treg cells were found to be present for longer when patients progressed to the chronic phase (Kared et al., 2013).

As described above, Gal-9 has an effect on CD4 and CD8 T cells via an interaction with Tim-3; Tregs are a source of Gla-9 and therefore may inhibit HCV-specific T cell responses via this mechanism (Kared et al., 2013). In the chimpanzee model, Treg cells can inhibit the specific immune response to HCV in a subsequent infection (Park et al., 2013). Chronic HCV patients have been found to have expanded Treg cell populations; it may be that these expanded Treg populations may limit disease during persistence by reducing inflammation.

In conclusion, during acute HCV infection, specific CD4 T cells responses are primed. Exhaustion of CD4 T cells has a negative impact on IL-2 production, IFNγ and proliferation. Reduced proliferation of CD4 T cells results in a failure to support a strong CD8 T cell responses and leads to chronicity. Early induction of IL-21 can increase CD4 responses and reduce CD8 T cell exhaustion. Treg cells may inhibit CD4 and CD8 effector T cells via producing Tim-3 ligand and the regulatory cytokines IL-10 and TGF-β.

1.6.4.3 **Humoral responses during acute HCV infection**

As HCV reaches high levels in serum following initial transmission, the antibody response (seroconversion) is detected around 2-8 weeks of infection (Rehermann and Nascimbeni, 2005, Dustin and Rice, 2007). Numerous studies have shown

that antibodies classically target the highly variable regions (HVR) of the HCV envelope 2 (E2) glycoprotein both *in vitro* and *in vivo* (Farci et al., 1996, Shimizu et al., 1996). In some chimpanzee studies, spontaneous clearance is not associated with antibodies (Bassett et al., 1999, Major et al., 1999).

In humans, antibodies are associated with spontaneous clearance in some studies (Logvinoff et al., 2004, Netski et al., 2005). However, neutralizing antibodies (nAbs) may only appear after the patient progresses to chronicity in association with rapid generation of B cell HCV escape mutations (von Hahn et al., 2007). A recent study showed that nAbs can have activity against heterologous HCV infection but a major feature of HCV B cell responses is that they are directed against patient-specific isolates.

Methods for investigating B cell responses often require the use of HCV pseudo-particles (HCVpp) that consist of a lentiviral backbone and the envelope genes of HCV. Using HCVpps, HCV clearance correlates with the emergence of high titres of nAbs (Osburn et al., 2014). HCV antibodies may also induce antibody-dependent cellular cytotoxicity (ADCC) (Nattermann et al., 2005). ADCC is based on the attachment of the variable region of Abs to HCV-infected cells and binding of the Fc region to fragment crystalisable (Fc) receptors, expressed on innate immune cells such as natural killer cells.

Targeting of infected cells and lysis occurs as a result of binding the FcγR3a (CD16) that triggers cytokine production and degranulation of NK cells. Several studies have shown that ADCC also plays a role in reducing viral replication in patients infected with HIV (Baum, 2010, Berger and Alter, 2011). More studies are needed to understand the role of ADCC-inducing antibodies during early HCV infection and reinfection.

In HCV humoral immunity protection is limited by several factors. First, it is known that envelope protein immunogenicity if it is not high, can lead to a slowed Ab response especially during first exposure to the virus (Dowd et al., 2009). Second, the E2 protein contains the HVR, characterized by a high mutation rate, and these mutations can help the HCV to be escape Ab

neutralization (Weiner et al., 1992). These mutations often follow a reduction in cellular immunity around 6 months into infection during progression to chronicity (Liu et al., 2010). Lastly, epitopes targeted by nAbs are protected via glycosylation as well as by complex linking to lipoproteins from the host. This mechanism slows or limits Ab efficiency *in vivo* (Helle et al., 2007).

In conclusion, current data shows a link between the appearances of nAbs and how they can play an important role in clearing the HCV spontaneously. However, these findings are still tentative and more studies are needed. A recent study showed the crystal structure of E2 and how it is linked to the neutralizing antibody AR3C (Khan et al., 2014, Kong et al., 2013).

Such studies of the HCV envelope structure will help to provide more information about the interaction between E1/E2 with their receptors as well as with nAbs. More studies of how T cells interact with B cells are still needed.

1.6.5 Summary of immune responses and immune evasion during acute HCV infection

HCV infection initially activates the immune system following triggering of the innate immune system via cellular sensors such as TLR, RIG-I, and PKR. These stimulate the IFN signaling cascade resulting in the production of interferon types I and III and interferon-stimulated genes (ISGs) in infected cells. Natural killer cells in the liver are activated via interferon. These, hepatocytes, and other APCs (Kupffer and dendritic cells) then activate the immune system against HCV by presenting peptides via MHC class I and II receptors to CD8 and CD4 cells, respectively. Migratory DCs transfer the HCV antigens from the infected liver to draining lymph glands.

CD4 T cells including Th1 cells are primed, and support CD8 T cells in inhibiting viral replication through the production of IFNγ and TNFα. CD4 cells also produce IL-2 which increases proliferation of CD8 T cells. Th2 CD4 responses support the production of B cells and IL-4 and IL-6 may help in the generation of nAbs. It is not clear yet what the role of Tfh cells is in developing nAbs within

the acute HCV phase, and in addition it is unclear what the role of ADCC is in maintaining HCV infection. The interaction between NK and DCs and the influence of this interaction on the specific CD8 T cell function also needs to be studied further.

HCV has the ability to evade the immune response by inhibition of the interferon signaling pathway, limiting the function of NK cells, the production of escape mutations within CD8 T cell epitopes and exhaustion of the acquired immune system (both CD4 and CD8 T cells) via upregulation of PD-1, Tim-3 and CTLA-4.

Tregs can reduce the response of the innate immune system against HCV directly via IL-10 and TGF-β production or producing Gal-9 that has been well defined as increasing apoptosis of virus specific CD4 and CD8 T cells (Abdel-Hakeem and Shoukry, 2014).

1.6.6 The HLA-B27 allele and disease

The human leukocyte antigens (HLA) are encoded on chromosome 6 and are the human major histocompatibility complex (MHC). Three MHC class I molecules (A, B and C) are expressed on the cell surface of all nucleated cells and express endogenous (intracellular) peptides to T-cell receptors on cytotoxic T cells (Bowness, 2015). The MHC class I HLA-B27 allele has traditionally been associated with disease, for example, it is strongly associated with arthritides including ankylosing spondylitis, Reiters syndrome and psoriatic arthritis. It has also been shown to have importance in viral infection and acts as a restriction factor for the highly specific CD8+ T-cell response (Gomard et al., 1984).

1.6.6.1 **Peptide Presentation by HLA-B27 to CD8+ Cytotoxic T Cells**

HLA-B27 is synthesised in the endoplasmic reticulum as a multiprotein complex that has peptide-loading capcity. MHC-restriction with relation to was initially described by Zinkernagel and colleagues in mice with lymphocytic choriomeningitis virus (LCMV) infection (Zinkernagel and Doherty, 1973, Zinkernagel and Doherty, 1974). Subsequently, in humans, the internal

nucleoprotein of influenza virus was found to be targeted by cytotoxic T cells (rather than surface hemagglutinin). The targeted region was noted to be short in length – the size of a short peptide (Townsend et al., 1986). The HLA-B27-restricted peptides from both influenza and HIV shared common structural features between them (the HLA binding sites), but differed in amino acids that affected T cell receptor recognition (Huet et al., 1990). The crystal structure of HLA-B27 was subsequently described by Madden and colleagues (the second HLA epitope to be described after HLA-A2) (Madden et al., 1991). The HLA-B27 molecule contains a binding groove with a B pocket (which contained different residues than HLA-A2) and is oriented to attach a positively-charged side chain (arginine) located at the second position of the bound peptide (Madden et al., 1992). Several thousand different peptides with arginine at the second position have been found to be bound to B27 alleles expressed on the cell surface. These may include self-proteins, including HLA molecules as well as foreign antigens (Jardetzky et al., 1991).

HLA-B27-restricted cytotoxic T lymphocyte responses to several viruses have been studied at length and ae associated with immunodominant responses targeting a small number of epitopes. For instance, in HIV infection, a mutation within the gag protein of the virus that is targeted by the CTL response impairs recognition and is associated with clinical progression (Goulder et al., 1997). HLA-B27 has been shown in several studies to be strongly associated with protection against viral infections, including infection with HIV and HCV (den Uyl et al., 2004). Immune escape from CD8 responses is limited by low viral fitness of mutated viruses and by cross-reactivity of T cells (Dazert et al., 2009).

In individuals infected with HIV, HIV-specific CD8+ T-cells recognise an immunodominant HLA-B27-restricted epitope (KK10) and this is associated with partial control of infection and long-term non-progression of HIV infection (Nixon et al., 1988a) (Kaslow et al., 1996) (Goulder and Walker, 2012). The importance of HLA-B27-restricted epitopes in limiting HCV infection and protection of patients from progressing to the chronic phase has also been described (Hraber et al., 2007, McKiernan et al., 2004).

There are more than 100 subtypes of HLA-B27 recognised so far. These subtypes differ in primary amino acid sequence but have common elements between them including the key structure, peptide binding and antigenic features (Bowness, 2015).

1.6.6.1 **HLA-B27 and HCV**

While the immune response defining HCV clearance versus persistence outcome is not very well understood, several studies have shown that the broad, diversity and T cell qualities are essential in controlling HCV infection outcome (Cooper et al., 1999a, Thimme et al., 2001a). Evidence for the role of T-cell function is supported by the presence of specific HLA molecules that impact the outcome of HCV infection (Mina et al.). Some HLA alleles are associated with reducing the disease progression of HIV-1 to AIDS level; HLA-B27 and HLA-B57, for example (Bashirova et al., 2011). These also have an association with the clearance of HCV infection. Protective HLA alleles classically present immunodominant epitopes to cytotoxic lymphocytes (CTL). Mutations within these epitopes can lead to progression to chronicity in patients infected with HCV (Kim et al.).

The protein targeted is often essential for the life cycle of the virus; such proteins are often highly expressed and may be vital for viral replication. Targeted proteins often contain conserved epitopes, within which mutations will result in a high fitness cost, which will lead to the low possibility of escaping from a CTL response. Such epitopes are listed in **Table 3-4**.

1.6.6.2 **The protective role of HLA-B27 in HIV infection**

Early HIV infection is usually associated with a peak HIV viral load followed by the initiation of an immune response that controls the infection. This control coincides with lowering of the HIV viral load and stabalisation of the CD4+ cell count decline. Following an asymptomatic period that lasts from 2-10 years, the HIV viral load starts to increase as the CD4+ T-cell count drops, resulting in AIDS in untreated patients. A small number of patients develop strong CD8 responses to HIV; these patients can control the HIV and have lower HIV viral loads and longer asymptomatic periods before the onset of AIDS (Neumann-Haefelin,

2013). These patients may be infected for decades before symptoms start to appear.

A study carried by Kaslow *et al*. illustrated the key role of HLA class I alleles in HIV-infected patients and associated slow progression to AIDS by describing the protective effect of the class I HLA-B27 and HLA-B57 alleles (Kaslow et al., 1996). A more recent study in a cohort of 2700 HIV-infected patients showed similar outcomes and confirmed the protective role of HLA-B27 and HLA-B57 in HIV-infected patients (Goulder and Walker, 2012). HLA- B27 and B57 alleles are not unique in their association with HIV control, but are extremes in a continuous spectrum of 'protective' to 'hazardous' HLA class I alleles (Goulder and Walker, 2012).

1.6.6.3 **The protective role of HLA-B27 in HCV infection**

Interestingly, HLA B27 and B57 alleles are also associated with control of HCV infection. Unlike patients infected with HIV who always progress to chronicity, around 30% of patients with acute HCV will clear the infection spontaneously. HLA class I has a major role in resolution of HCV infection. Patients with HCV infection infected from a single source (rhesus negative women treated with blood products) were protected if they had the class I alleles HLA-B27 or HLA-A3 and were more likely to exhibit spontaneous clearance. The role of HLA-B27 protection in HCV patients has been supported by another large US cohort study; the strongest protection occurred in those patients with HLA-B27, HLA-B62, and HLA-B39 alleles (McKiernan et al., 2004).

More research shows growing evidence about virus-specific CD8+ T-cell responses in individuals with varying HLA types (Dustin and Rice, 2007). Detecting HCV specific CD8+ T-cells responses in the peripheral blood and liver is associated with HCV control and clearance of infection within the acute phase. This may occur in association with the development of antibodies and both can reduce or delay replication of HCV in chimpanzees (Lechner et al., 2000a) (Shoukry et al., 2003b) (Thimme et al., 2001b).

The significant role of the specific CD8+ T-cell response in HCV is strongly supported by the association of HLA-B27 (HLA class I), dominant HLA-B27-restricted epitopes and spontaneous resolution of HCV. The most well characterised epitope is located within the C-terminal region of the nonstructural 5B protein and is nine amino acids in length (at position 2841-9 of the HCV genome and amino acids 421-429 of NS5B) (Neumann-Haefelin et al., 2006) (McKiernan et al., 2004). In patients with HCV monoinfection, a clear association has been found between this HLA-B27 epitope and protection (Dazert et al., 2009). The situation in HIV-infected patients has not been well characterized and is studied further in this thesis.

1.6.7 HIV and hepatitis C virus co-infection

Around 185 million individuals have been infected with HCV (WHO, 2011) and 37 million individuals have been infected with HIV (WHO, 2015). Recently, AIDS has been described as one of the main causes of death in patients who have received highly active antiretroviral therapy (HAART) (Bica et al., 2001).

Up to 30% of HIV-infected patients have co-infection with HCV, with some populations showing an even higher prevalence rate (Vallet-Pichard and Pol, 2006). A minimum of 4-5 million people have been identified as having co-infection (HCV/HIV) due to the same routes of infection transmission (Alter, 2006). It has also been found that 60-90% of HIV-infected haemophiliacs and 50-70% in HIV-infected persons who inject drugs (PWID) have co-infection with HCV (Rockstroh and Spengler, 2004).

The transmission of HIV has dropped since 2001, due to the advent of antiretroviral therapy (ART). More people are living with HIV than ever before for the same reason. A study carried out by Platt et al. in 2016 looked at HCV prevalence in co-infected HIV patient population samples. The highest prevalence was found in North Africa and the Middle East and the lowest prevalence was found in East Africa, whereas, the midpoint of the prevalence of HCV co-infection in HIV-infected individuals (heterosexual people or pregnant

women) was 4.0% (Platt et al., 2016). In MSM, the midpoint prevalence in 80 samples was 6.4%. The highest percentage was found in North America and the lowest was found in East Asia and South and Southeast Asia. In PWID, the prevalence was 29.0%. In general, there was an increase of about 5.8 times ratio of HCV antibody positive in HIV patients compared to mono-infected patients with HCV (Platt et al., 2016).

There are an estimated 2,278,400 patients with co-infection worldwide, of which 1,362,700 are PWID. This overall global prevalence of HCV co-infection in HIV-infected people is about 6.2% (Platt et al., 2016).

Various countries, including France, Germany, the UK, the Netherlands, the USA and Australia have reported epidemics of sexual transmission of HCV between men who have sex with men (MSM). Globally over 1000 cases of co-infection with acute HCV have been reported as a result of sexual or IDU transmission in HIV-infected MSM (Vogel et al., 2011). Phylogenetic analysis of the HCV sequences from infected patients show worldwide links between HCV-infected patients (van de Laar et al., 2009).

1.6.7.1 **The influence of HCV on HIV infection**

The effect of HCV on HIV progression has been investigated in a number of studies. A study carried out on an HIV cohort in Switzerland suggested that HCV infection may result in faster progression to AIDS (Greub et al., 2000). Mono-infected patients with HIV had higher CD4 counts (more than 50 cells/ml) (Greub et al., 2000). CD4 counts were seen to increase by more than 200 cells/mm^3 in HIV mono-infected patients started on HAART when compared with co-infected patients, with a risk ratio of 1.52 (Stebbing et al., 2005, Sullivan et al., 2006). Other studies have shown there is no development risk of a progression to the advanced phase when co-infection with HCV/HIV occurs (Rockstroh et al., 2005, Sullivan et al., 2006). Co-infection with HCV may complicate HIV treatment as well; there is evidence showing an increase of drug-related hepatoxicity in patients treated with ART.

1.6.7.2 **The influence of HIV on HCV infection**

The impact of HIV on HCV co-infection is clearer and affects both the natural history and transmission of HCV. The transmission of HCV has been found to be increased in co-infected patients with HIV; with parental transmission, the risk is—about two-fold higher in HIV-infected mothers (Sulkowski, 2008, Valle Tovo et al., 2007). Patients who have not been treated for HIV have a lower chance of clearing HCV spontaneously; they show a high level of HCV viral load and experience more rapid progression to HCV-related cirrhosis and cancer compared to patients with HCV infection only (Thomas et al., 2000). Patients treated with ART show improved outcomes in patients with co-infection, with a lower level of HCV-related mortality (Shepard et al., 2005).

In MSM, an eight-time increase in the probability of infection with HCV is present in HIV-infected individuals (van der Helm et al., 2011, Wandeler et al., 2012). It has also been suggested that there is a higher rate of re-infection following the spontaneous clearance or treatment in positive MSM with co-infection (HCV-HIV), confirming the need for repeating HCV testing in this population (Martin et al., 2013).

A study by Thomson *et al*. (2009) showed there was a delay in diagnosing acute HCV in HIV-infected patients; this was due to delayed seroconversion. Late diagnosis and treatment of HCV can impact the outcome of the HCV infection (Thomson et al., 2009).

HCV viral loads are higher in co-infected patients than in patients with mono infection, by around 1.08 log copies/ml in body fluids and blood (Shire and Sherman, 2005, Dionne-Odom et al., 2009, Mohsen et al., 2002). Anti-retroviral therapy reduces HCV viral load during HCV infection; in co-infected patients a decrease can be observed in the HCV viral load of up to 1 log, following a 12-month treatment course of HAART; this decline is associated with an increase in the immune response against HCV (Rockstroh and Spengler, 2004).

Progression to the chronic phase is also more common in co-infected patients; and is likely due to immune response perturbation to the HCV infection (Danta et al., 2008). Patients diagnosed with HIV have a greater chance of proceeding to severe liver disease (Mohsen et al., 2002) and progress more rapidly to liver cirrhosis than patients with mono-infection. Progression to liver cirrhosis in co-infected patients versus mono-infected patients occurs an average of 32 years after diagnosis versus 23 years (Mohsen et al., 2003). Patients with HCV cirrhosis who are HIV-infected also have a higher rate for progression to hepatocellular carcinoma (HCC). This occurs after a shorter time and in younger patients, with an estimated rate of 1-4% annually (Garcia-Samaniego et al., 2001, Singal and Anand, 2009).

1.7 Methods used to study HCV

1.7.1 Cell culture of HCV

Kolykhalov *et al*. and Yanagi *et al*. were the first scientists to create a full consensus genome of HCV from a patient who had been infected with genotype 1a; this isolate was designated as "H77" (Kolykhalov et al., 1997, Yanagi et al., 1997). It proved to be highly high infectious when inoculated into an animal model (chimpanzee). There were many attempts to establish replication of this and other genomes *in vitro,* but they were not immediately effective.

The first *in vitro* system developed successfully was the development of an HCV replicon using the non-structural part of the genome of the genotype 1b isolate Con1. In this model, replication-enhancing mutations (REMs) within NS3, NS4a and NS5A have been introduced that enhance replication within tissue culture but may interfere with the production of HCV infectious particles (Pietschmann et al., 2009). An adaptive mutation within NS4B of the Con1 replicon (K1846T) reduces production of HCV infectious particles (Pietschmann et al., 2009). Recently, a modified H77 genome known as H77S has been created that contains several mutations that allow replication and also permits the production of HCV infectious particles (Yi and Lemon, 2009, Yi et al., 2006).

In 2001, Kato et al. developed a new subgenomic replicon based on genotype 2a called JFH1 that was able to replicate without adaptive mutations in cell culture efficiently (Kato et al., 2003, Kato et al., 2001, Date et al., 2004). Four years later, a full JFH HCV genome was found that efficiently replicates in Huh7 cells and produces HCV infectious particles in both the tissue culture and in animal models (Lindenbach et al., 2005, Wakita et al., 2005, Zhong et al., 2005). This isolate and chimeric variants are now used widely in many research laboratories and are known as culture-derived HCV (HCVcc).

This system can be used to study the HCV life cycle starting from viral entry, HCV replication, genome packaging, HCV virion assembly and release of HCV as the last step of the life cycle. Electron microscopy showed circular particles with a diameter of 50-65 nm and the same density compared to other viruses from serum (Lindenbach et al., 2005, Wakita et al., 2005, Zhong et al., 2005).

The viability of HCV recombinant particles was assessed by measuring the infectiousness of HCVcc in animal models including chimpanzees and mice possessing xenografts of human liver (Lindenbach et al., 2005, Wakita et al., 2005). HCVcc recovered after passage *in vivo* were detected at high levels (Lindenbach et al., 2006). These infectious viral particles derived from infected animal models exhibit lower levels of density in comparison with viral particles derived from cell culture, however. Following the first passage of cell culture, these characteristics (higher infectiousness and low density) disappear. HCVcc have been found to be susceptible to inhibitors that target the polymerase and protease as well as interferon-α (Lindenbach et al., 2005, Wakita et al., 2005, Zhong et al., 2005).

As the current HCVcc is based on genotype 2a, the challenge for the future is to find another genotype with the ability to produce infectious viruses in cell culture. In 2006 Yi et al. report the first infectious clone of a new genotype (1a) called H77S that contains 5 REMs. These 5 mutations were obtained following a long iterative procedure introducing multiple adaptations into the subgenomic replicon (H77); these adaptive mutations were located within non-structural

genes (Yi and Lemon, 2004). Virus recovered from transfection into Huh7.5 cells was lower in titre by about 100-1000 fold in comparison with JFH1 replicon.

1.7.1.1 **Chimeric JFH-1 genomes**

As described above, it is possible to create chimeric genomes by joining JFH1 with heterologous HCV genotypes. So far, the essential elements for generating the membrane-bound replicase complex and non-translated areas are obtained from the JFH1 strain. Core to NS2 genes may be introduced from a different genotype. HCVcc particles were first formed using an intragenotypic chimera utilizing genotype 2a JFH-1 and J6 (Yanagi et al., 1997, Lindenbach et al., 2005). While JFH-1 grows in culture, J6 does not. Further studies were carried out to produce chimeras using JFH-1 and other genotypes.

An initial construct of the genotype 1b Con1 isolate carrying core to NS2 genes was successfully created (Pietschmann, 2006). However, despite the production of infectious HCV, titres of HCV were low. Adaptations were introduced by creating different chimeras with varying crossover regions starting from the C-terminus of E2 up to NS2-NS3. Intensive analysis showed that a crossover position situated after the initial trans-membrane domain of NS2 is the best place to build new chimeras including JFH1-Con1, JFH-J6 and JFH1-H77 (Pietschmann, 2006).

Several studies have shown similar findings about generating chimeric genomes of all seven different genotypes of HCV (Pietschmann, 2006, Gottwein et al., 2007, McMullan et al., 2007, Yi et al., 2007, Jensen et al., 2008, Scheel et al., 2008, Gottwein et al., 2009, Gottwein et al., 2011a, Scheel et al., 2011a)

In vivo, liver-chimeric mice have been used to assess titres of infectious particles following inoculation with different HCV genotypes (genotypes 1-6) (Bukh et al., 2010). The highest virus titre was obtained with a chimeric J6-JFH1, called Jc1, that resulted in 10^6 of infectious HCV virus particles per ml of blood (Pietschmann et al., 2006).

A further advance has been the ability to construct a reporter genome from several HCV chimera genomes that contains luciferase or GFP in order to increase the detection of HCV infection (Koutsoudakis et al., 2006, Tscherne et al., 2006, Schaller et al., 2007). Chimera construction has now been carried out to include the non-structural genes. Viable JFH1 chimeras have now been made that contain the non-structural proteins NS3/NS4A or NS5A was changed from other HCV genotypes (Gottwein et al., 2011b, Scheel et al., 2011a, Scheel et al., 2011b), allowing researchers to study the activity of antiviral compounds against NS3/NS4A and NS5A.

1.7.2 The HCV replicon system

The propagation of HCV within cell culture (JFH-1 or chimeras of JFH-1) remains imperfect due to low levels of replication within cell culture and the need to carry out experiments in containment level 3 facilities.

However, it is possible to increase levels of replication *in vitro* using HCV complementary DNA (cDNA) that contains the sequence from the NS3-NS5B genes (Bartenschlager and Lohmann, 2001). The first HCV subgenomic replicons were derived from the HCV genotype 1b Con1 isolate. As well as the non-structural genes, this sub-genomic replicon contained the 5' UTR and the beginning of core protein (12 codons) linked with a marker gene (neomycin phosphotransferase (Neo)) allowing for G418 (geneticin) selection, an internal ribosome entry site (IRES) derived from the encephalomyocarditis virus (EMCV) and the 3' UTR from HCV (Lohmann et al., 1999).

The original sub-genomic Con1 replicon tends to result in a low frequency of G418 resistance of 1 colony per one million transfected cells (Lohmann et al., 1999, Blight et al., 2000). This is due to adaptive mutations that are required to allow efficient replication within permissive cell lines. Analysis of Con1 replicons passaged in culture show several adaptive mutations within the non-structural protein region, particularly within NS5A (Blight et al., 2000) (Blight et al., 2000, Krieger et al., 2001, Lohmann et al., 2003, Lanford et al., 2003, Enomoto et al., 1995, Enomoto et al., 1996).

A replicon derived from the Hutchinson genotype 1a strain (H77) has also been successfully created and requires a minimum of two adaptive mutations to be able to replicate efficiently within the cell culture system (Blight et al., 2003, Grobler et al., 2003, Gu et al., 2003, Yi and Lemon, 2004). Such H77 replicons contain a mutation within the NS5A region (S2204I) that allows the replicon to replicate efficiently within human hepatoma cells 7.5 (Huh 7.5) (Blight et al., 2002a, Blight et al., 2003).

Analysis of such replicons following passage has revealed that high replication efficiency of sub-genomic H77 is associated with an additional adaptive mutation (P1496L) within the nonstructural protein 3 (NS3) (Blight et al., 2003). A more robust H77 replicon was described by Voitenleitner *et al*. in 2012 following the introduction of adaptive mutations within two other regions.

The first adaptive mutation was introduced within NS4A (K1691R) and the second within NS4B (E1726G). These two adaptive mutations have been found to substantially improve replication *in vitro* (Voitenleitner et al., 2012).

Following the development of new replicons, transient RNA replication assays were developed to measure the efficiency of replicon replication. Different reporter genes were used to measure replication following the RNA transfection into Huh 7.5 cells; these reporter gene include lactamase and luciferase (Blight and Norgard, 2006).

The luciferase gene is used as a reliable indicator for RNA replication, as production of each single luciferase gene indicates RNA synthesis. Firefly luciferase was introduced into a bicistronic sub-genomic replicons; replication can thus be measured and compared with a polymerase defective replicon that is designed to carry a mutation in the RNA (changing the GDD to GND). Generally, the adapted sub-genomic replicon (Con1) can replicate up to 100-fold higher than a defective replicon containing GND after 48 to 72 hours (Krieger et al., 2001).

1.7.3 Permissive cell lines for HCV replication

HCV targets hepatocyte cells as the main site for replication, while other cells, such as dendritic cells, lymphocytes and monocytes, may be targeted by HCV although there is conflicting evidence regarding replication within these cells (Laskus et al., 2000, Goutagny et al., 2003). Huh 7.5 cells, a hepatoma cell line, were derived from Huh 7 cells following treatment with a high concentration of human interferon- α (IFNα). This allowed them to have a high tolerance to IFN, which assists the sub-genomic replication.

These cells are currently the most important permissive cell lines available at the time of writing. Their permissive behaviour is in part related to defective the retinoic acid inducible gene-I (RIG-I) production, a protein known to stimulate interferon production (IFN -type I) (Blight et al., 2002a, Sumpter et al., 2005).

In culture, Huh 7 and 7.5 cells are highly variable and permissiveness for infection is variable and relates to the number of passages carried out over time. This can make large differences, as much as 100-fold. These differences are not related to other factors such as adaptive mutations that were introduced to enhance the quality of sub-genomic replicon replication (Blight et al., 2002a, Blight et al., 2000).

The replicon system has been used to characterize HCV protein function, to study host-virus interactions and *in vitro* replication and can be readily altered using site-directed mutagenesis. In this thesis, a genotype 1a replicon system was used to investigate the impact of mutations that emerged in patients as a result of pressure from the T cell mediated immune response.

1.7.4 Next-generation sequencing (NGS)

Next-generation sequencing has dramatically changed the possibilities for viral population analysis within infected hosts. NGS can be used to examine HCV strains present as majority and minority variants and is far more reliable for

producing whole genome coverage than classical sequencing methods (Sanger sequencing).

As NGS is such a powerful technique, generating millions of sequence reads in a single run, a metagenomic approach can be used to identify whole viral genomes and as the technique is unselected (and does not require specific PCR primers) can be used to discover new viruses. NGS has recently been used to identify the aetiology of Merkel cell cancer (Feng et al., 2008) and to identify a novel bunyavirus in patient with "severe fever with thrombocytopenia syndrome" (Xu et al., 2011).

This metagenomic approach recently aided the characterisation of the virome in children with acute diarrhea and fever (Wylie et al., 2012). While this technique is powerful, it is inefficient as the majority of sequence reads produced is not viral in origin. It can be improved, as described later in this thesis by the use of target enrichment (magnetic beads attached to virus-specific oligonucleotides) in order to purify the virus of interest.

This thesis describes the use of a metagenomic and target enrichment approach for sequencing HCV (and HIV in co-infected patients).

1.7.4.1 **NGS using the Illumina platform**

The Illumina platform is currently used more widely than other available NGS platforms due to efficiency and cost. The principle is based on a sequencing-by-synthesis approach, meaning that the four nucleotides are added with DNA polymerase into a flow cell channel. These are detected as each nucleotide is associated with a separate fluorescent label (Zhang et al., 2011). The NGS process involves several steps 1) the addition of nucleotides to elongating strands of DNA; 2) the simultaneous detection of nucleotides to multiple DNA fragments; 3) A wash step removing fluorescent labels and allowing the next reaction to be detected (Mardis, 2011). This process is described further in Chapter 2.

The main error found when using the Illumina platform is a substitution error, meaning that an incorrect nucleotide is identified due to the de-blocking step not being well performed causing a cluster to fall out of phase or due to interface noise as a result of the incomplete cleavage of fluorescence label prior to DNA cycles (Mardis, 2013). However, Illumina currently produces data of higher quality than other platforms, with low error rates, making it the first choice for many genome-sequencing projects (Zhang et al., 2011).

Next-generation sequencing creates a library of millions of DNA fragments. These DNA fragments may be read from both sides; this is called paired-end sequencing and allows for greater fragment lengths to be detected. This also enhances the analysis of the NGS data by providing overlap areas that are duplicated. Alignment scripts used to align sequences of interest to reference genomes take into account the length of the synthesized DNA fragments in the sequence library to reach the most accurate alignments (Korbel et al., 2007).

1.7.4.2 **Errors and limitations of NGS**

NGS requires a small number of non-specific PCR steps, and this may result in PCR-based error (Poh et al., 2013). The use of high fidelity polymerase enzymes and limiting the number of PCR cycles reduces this error to lower than that seen with traditional PCR-based methods. It is a highly sensitive technique and is also highly prone to cross-contamination error.

Sequence reads tend to be shorter using the Illumina platform than those generated using other methods e.g. Sanger sequencing. This is due to the signal-to-noise ratio that limits the NGS read length (Mardis, 2013). Sequence read length is increasing over time with advances in Illumina-based technology.

Control samples may be included in each run to assess error rates (Hillier et al., 2008b). This can be used to give a detailed picture about 1) the type of error that has occurred within each sequence, for instance, deletion, insertion or substitution; 2) the kind of error mode that should be used to correct sequence reads and 3) missing regions from the sample sequenced (Mardis, 2013).

Many tools have been designed to align large amounts of data from short fragment reads created by NGS instruments against a reference genome. Defining variants or single nucleotide polymorphisms (SNP) or spotting an over or under-enriched region can be achieved using a variety of different methods.

1.7.4.3 **Mapping**

The most important step in NGS data analysis is the assembly and mapping of sequence reads to a reference sequence. As NGS produces a huge amount of data, two fundamental issues need to be considered: one is that the required usage of the data needed as NGS produces a huge amount of it and the other is the error profile. Traditional methods require several days to map the data to original reference genomes or computationally intensive software such as Smith-Waterman dynamic programming, BLAT or BLAST. To overcome these problems, new methods have been developed.

Two bioinformatics researchers from Ohio State University initially introduced six different programs to overcome this problem and improved hash/index-based short sequence alignment to reference genomes. These six parallel methods include dividing the reads, dividing the genome, dividing reads and genome, suffix-based assignment (SBA), SBA after partitioning reads and SBA after partitioning genome. Another method called CloudBurst was introduced by Schatz *et al*. and used to read single-end reads.

Another two complementary algorithm methods were introduced by BreakDancer (BreakDancerMax and BreakDancerMini), which allow analysis of more than one pool and more than one library in more than one sample. New algorithm analysis software was introduced using quality scoring to obtain the maximum accurate analysis from fewer sequences; this software is called GNUMAP (Bao et al., 2011). Recently, more software has been developed and introduced to the market; examples of this are PASS (a program to align short sequences)(Campagna et al., 2009), SOAP (short oligonucleotide alignment program) (Li et al., 2008b), Bowtie, an ultrafast, memory-efficient short read aligner (Langmead et al., 2009), CloudBurst (Schatz, 2009), MAQ (mapping

quality) (Li et al., 2008a), ZOOM (Zillions of oligos mapped) (Lin et al., 2008), SHRIMP (accurate mapping of short colour-space reads) (Rumble et al., 2009), and PERM (efficient mapping of short sequencing reads with periodic full sensitive spaced seeds (Chen et al., 2009).

In this thesis, an in-house mapping programme called Tanoti (manuscript under review) designed by Dr Vattipally Sreenu was used to overcome errors related to the presence of highly divergent viral genomes.

1.7.5 Enzyme-Linked ImmunoSpot (ELISPOT) assays

The ELISPOT technique was established 1983 in Sweden by Cecil Czerkinsky's group who were interested in defining antigen-specific Antibody Secreting B Cells (ASCs). Since then, ELISPOT has been modified for use in different applications and is used widely to monitor cellular immune responses in both humans and animals; this application has been found to be a clinically helpful tool in diagnosing tuberculosis and graft tolerance or rejection in transplant patients. The technique has been verified as one of the most effective methodologies used in monitoring the cells mediating immunity due to its accuracy and sensitivity in detecting low levels of antigen-specific T or B cells. It is capable of detecting one positive cell from a PBMC population; each spot represents a single reactive T or B immune cell.

The test gives both qualitative and quantitative measurement, as it qualifies specific cytokines such as IFN or other secreted molecules and quantifies the number of cells responding to stimuli. The high sensitivity of the ELISPOT test allows the detection of rare antigen-specific frequencies, which was difficult or impossible by other methodologies in the past.Aims and hypotheses

Patients with the HLA B27 allele were studied from a larger cohort of patients with acute HCV infection. These included individuals with and without HIV co-infection.

1.8 Aims and hypothesis

We aimed to study the impact of the HLA B27 allele in defining the immune response in spontaneous clearance versus progression to chronicity in patients with and without HIV co-infection during the acute phase.

In order to achieve the above aim, we used the following methods:

Next-generation sequencing to detect variation in the HCV genome over time in HLA B27+ patients with acute infection.

2. Viral fitness of variants detected using NGS, quantified in the HCV genotype 1a replicon system.

3. T cell assays (ELISpot and flow cytometry) to characterize the adaptive immune response in HLA B27+ patients.

Chapter 2: Materials and Methods

2.1 Materials

Table 2-1 DNA Manipulation & Purification

Solution	Components
Equilibration buffer (EQ1)	0.1 M sodium acetate (pH 5.0), 0.6M NaCl, 0.15% (v/v) Triton X-100
Cell Resuspension buffer (R3)	50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 μg/ml RNase A
Lysis buffer (L7)	0.2 M NaOH, 1% (w/v) SDS
Precipitation buffer (N3)	3.1 M potassium acetate (pH5.5)
Wash buffer (W8)	0.1 M sodium acetate (pH 5.0), 825 mM NaCl
Elution buffer (E4)	100 mM Tris-HCl (pH 8.5), 1.25 M NaCl
Buffer	
TBE (10x)	0.9 M Tris-HCl, 0.9 M boric acid, 0.02 M EDTA
TE buffer	10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA
Agarose gel loading	5x TBE, 50% sucrose, 1μg/ml BPB

Table 2-2: Cell lines

Cells	Description	Source	References
Huh-7 cells	Human hepatoma cell line	Dr John McLauchlan University of Glasgow Centre for Virus Research [CVR]	(Lohmann et al., 1999)
Huh-7.5 cells	Human hepatoma clonal cell line derived from Huh-7 cells	Prof. Charles Rice [Rockefeller University]	(Blight et al., 2002b)
Huh-7/SGR-JFH1 cells	Human hepatoma cell line harbouring the JFH1 subgenomic replicon	Dr John McLauchlan [CVR]	(Lohmann et al., 1999)

Table 2-3: Enzymes

Restriction enzymes	Source
Mung bean nuclease	New England Biolabs
Taq polymerase	Applied Biosciences
Multiscribe RT	Applied Biosciences

Table 2-4 Cell culture medium

Component	Source
Dulbecco's Modified Eagles Medium (DMEM)	Gibco (Invitrogen Life Technologies)
10% Fetal calf serum (FCS)	Gibco (Invitrogen Life Technologies)
100 units/ml Penicillin/streptomycin	Gibco (Invitrogen Life Technologies)
1x Trypsin (10x Stock)	Sigma
Phosphate buffer solution (PBS)	Gibco (Invitrogen Life Technologies)

Table 2-5 Commonly Used Chemicals

Chemical	Abbreviation	Source
Agarose	-	Melford
Ampicillin	Amp	Melford
Ethanol	EtOH	Fischer Scientific
Ethidium bromide	EtBr	Sigma
Methanol	MeOH	BDH Chemicals
Sodium chloride	NaCl	BDH Chemicals

Table 2-6 Bacterial Expression

Solution	Component
L-Broth	170mM NaCl, 10g/l Bactopeptone, 5g/l yeast extract
L-Agar	L-Broth plus 1.5% (w/v) agar

Methods

2.1.1 Patient cohort

In this study, patients were selected based on HLA type from two acute HCV cohorts recruited in St Mary's Hospital, Imperial College London NHS Trust and Gartnavel Hospital, Greater Glasgow and Clyde. In this study, plasma and peripheral blood mononuclear cells (PBMC) were collected from patients prospectively at 1–3 monthly intervals and stored residual samples from the clinical virus laboratory were also available for analysis. Where appropriate, patients were offered treatment for 24-48 weeks with pegylated interferon alpha (pegINFα) and weight-based ribavirin following the diagnosis of HCV infection, according to local guidelines. Patients with spontaneous clearance did not receive treatment. Differences in study sampling times were due to differences in the availability of stored plasma samples obtained retrospectively after diagnosis. In some cases, infection had been present but remained undiagnosed for several months prior to recruitment into the study.

Written consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. This work was a part of the study that was approved by the Riverside Research Ethics Committee and the West of Scotland Research Local Ethics Committee.

2.1.2 Diagnosis of acute HCV

Diagnosis of acute HCV was defined as a positive HCV RT-PCR test result within six months of a preceding negative HCV RT-PCR or antibody test. The infection date was calculated as the midpoint between the last negative and first positive test. Spontaneous clearance was defined as two sequential negative RT-PCR tests (<12 IU/ml) separated by a minimum of three months.

HCV viral load and liver function (measurement of ALT, bilirubin, and albumin levels) were assessed every month for a period of three months following initial diagnosis, and every three months subsequently. Immunology

tests such as CD4, CD8, and HIV quantification were assessed every three months.

2.1.3 Patient treatment outcome

Patients were classified as having had a sustained virological response (SVR) if they remained negative using HCV RT-PCR for 24 weeks after the last treatment dose (2014). Failure to respond to treatment was defined as relapse (detectable viral load after a negative viral load at the end of treatment), null response (<2$\log_{10}$ drop in viral load during treatment) or partial response (>2 $\log_{10}$ drop in viral load during treatment but failure to become PCR negative).

2.1.4 Storage of blood samples:

Blood samples were collected in a Vacutainer® blood collection tube containing EDTA. Plasma and PBMCs were separated by desity-gradient centrifugation. Plasma was stored in 2 ml tubes at –80 °C and PBMCs in liquid nitrogen at -140°C.

2.1.5 RNA Extraction

Plasma was thawed on ice prior to ribonucleic acid (RNA) extraction. Two different methods were used during the study: initially, manual extraction was carried out using a QIAamp® Viral RNA kit (Qiagen) following the manufacturers' instructions; subsequently, extraction was automated using an automated platform; EasyMAG® (Biomerieux).

2.1.5.1 **QIAamp® Viral RNA kit (Qiagen)**

Sample processing was carried out by mixing 140 μL of plasma and 560 μL cell lysis buffer (buffer AVL) containing 5.6 μL carrier RNA. The mixture was incubated for 10 min at room temperature before adding 560 μL of ethanol. The solution was then transferred into columns and centrifuged at 8,000 g.

Two washing steps were then carried out: 500 μL of wash buffer AW1 was added; the sample was centrifuged at 8,000g, 500 μL of wash buffer AW2 was added; centrifugation at 8,000g was repeated.

A further step was carried out to remove any remaining ethanol from the washing buffer, since this can affect the RNA yield. Finally, the RNA was eluted using 60 μL of AVE buffer (RNase-free water containing 0.04% sodium azide).

2.1.5.2 **EasyMAG® NucliSENS Extractor**

Extraction with the EasyMAG® was performed according the manufacturers' instructions. Briefly, 400 μL of each sample was loaded on to a disposable sample vessel, which was then loaded onto the EasyMAG®. Following the initial lysis incubation, 100 μL of magnetic silica was added to each sample. Samples were eluted in 60 μL of elution buffer and before being transferred to a 1.5 ml microcentrifuge tube and stored at –80°C.

2.1.6 cDNA Synthesis

Complementry DNA (cDNA) synthesis was carried out using either Superscript III (Invitrogen) or Maxima H (Thermo Scientific) according to the manufacturers' instructions.

2.1.6.1 **Superscript III ® reverse transcriptase kit**

The Superscript III RT-PCR kit (Invitrogen) was used to reverse transcribe-extracted RNA into complementary DNA (cDNA) using random hexamers. A solution containing 15μL extracted RNA, 1μL random hexamers, and 9μL of 10 mM dNTPs was heated at 70°C for 5 minutes. The mixture was then placed on ice for 5 minutes. Reverse transcription was carried out by adding 9 μL of 5X First-stand Buffer, 2 μL of 0.1 M DTT, 4 μL of RNAse OUT (20 u/μL), and 1 μL of Superscript III enzyme, and incubating the reaction mixture at 50°C for 90 minutes.

Following this, a further 1 µL of Superscript III was added and the temperature was increased to 55°C for a further 90 minutes. Superscript III was inactivated at 70°C for 15 min, then 1 µL of RNAse H was added and the mixture was incubated for 20 min at 37°C.

2.1.6.2 **Maxima H Minus Reverse Transcriptase®**

Complementary DNA (cDNA) was prepared using Maxima H minus reverse Transcriptase® (Thermo Scientific) by adding 1 µl of random hexamer primers and 1 µl NTPs to 13 µl extracted RNA. The mixture was incubated at 65°C for 5 min before being placed on ice to chill. Subsequently, 4 µl of 5X reverse transcriptase buffer and 1 µl of reverse transcriptase were added and the sample was incubated for 10 min at 25°C followed by 60 min at 65°C. The reaction was terminated by incubation at 85°C for 5 min.

2.1.7 DNA second strand synthesis

The Second Strand Synthesis kit from New England Biolabs was used to make double stranded cDNA. The cDNA products described in Section 2.1.6 were mixed with 8 µL of 10X second strand synthesis reaction buffer, 4 µL of second strand synthesis enzyme, and 48 µL of nuclease-free water. The reaction mixture was incubated at 16°C for 2.5 hours.

2.1.8 Quantitative RT-PCR assays

Real-time PCR reactions were amplified using fast universal conditions on a 7500 Fast Real-Time PCR machine (Applied Biosystems). The total volume of Real-Time PCR Reaction Mix was 18 µL prepared as follows: 1 µL of 18µm Forward Primer, TCTGCGGAACCGGTGAGTAC (Final 900nM), 1 µL of 18µm Reverse Primer, GCACTCGCAAGCACCCTATC (Final 900nM), 1 µL of 5µm FAM Probe, FAM-AAAGGCCTTGTGGTACTG-MGB (250nM), 10 µL of TaqMan Fast Universal Mix® (2x), and 5 µL of nuclease-free water, with 2 µL cDNA added to each well of 96 well plates.

After covering the plate with an adhesive seal, it was centrifuged for 1 min on 1000 RPM before performing the Thermal Cycler Protocol, which involves initially holding at 95°C for 20 sec, before moving on to the second stage of 40 cycles of the following steps: 1) hold at 95°C for 3 sec, and 2) hold at 60°C for 30 sec.

2.1.9 Gel electrophoresis

PCR products were run on a 1% or 2% agarose gel containing ethidium bromide (Life Technologies). PCR products were mixed with 10X BlueJuice® gel loading buffer (1:10) (Invitrogen). DNA ladders of 100 bp and 1 Kbp (Promega) were used to determine sizes of DNA fragments, with choice of ladder based on the DNA fragment size that needed to be excised. DNA bands were visualised under ultraviolet light and appropriate bands were cut out using a clean scalpel blade.

2.1.10 DNA purification

2.1.10.1 **Purification of DNA from agarose gels**

The DNA fragment of interest was excised from an agarose gel, placed in a microcentrifuge tube and weighed. Binding buffer was added at a ratio of 1:1 weight:volume. The chaotropic agent in the binding buffer dissolves agarose, denatures proteins, and promotes DNA binding to the silica membrane in the column. The mixture was incubated at 60°C for 10 minutes, or until the gel slice was completely dissolved and the colour of the solution had changed to yellow (indicating optimal pH for DNA binding). Subsequently, 800 μL of the solubilised gel solution was transferred to a purification column and centrifuged for 1 minute at 13,000 rpm.

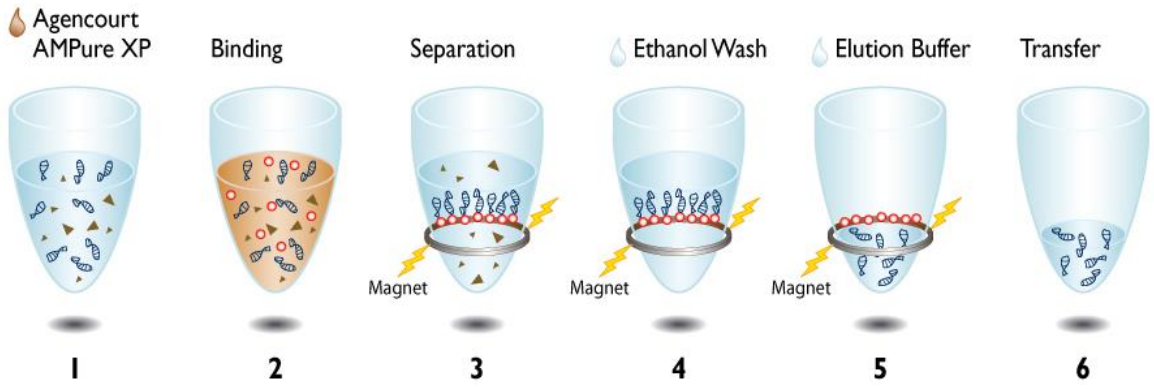
The flow-through was discarded, the column was placed back into the same collection tube, and 100 μL of binding buffer was added to the purification column. The column was centrifuged for one minute, the flow-through was discarded, and the column was returned to the same collection tube. Next, 700 μL of wash buffer was added to the purification column and centrifuged for one minute. The flow-through was discarded, and the column was returned to the

same collection tube. The empty purification column was centrifuged for an additional minute to completely remove residual wash buffer. For elution, 50 μL of elution buffer was added to the centre of the purification column membrane, and the purification column was transferred into a clean 1.5 ml microcentrifuge tube and centrifuged for one minute. Purified DNA was stored at -20°C.

2.1.10.2 **Agencourt AMPure XP® beads**

Magnetic beads were warmed to room temperature before use. DNA was added to the beads, mixed thoroughly, and incubated for 3-5 minutes at room temperature. The reaction was placed on a magnetic plate for 5-10 minutes to separate the beads from the solution. Once separation was complete, the liquid was aspirated from the reaction tube and discarded. Beads were washed twice by adding 200 μL of freshly made 80% ethanol. DNA was eluted in 40 μL of nuclease-free water (Figure 2-1).

Figure 2-1: Agencourt AMPure XP beads workflow



1.Addition of AMPure XP. 2. DNA fragments binding to specific paramagnetic beads. 3. Magnets applied to seperate beads with attached DNA fragments from the solution containing contaminants. 4. Beads with attached DNA fragments were washed twice with 80% Ethanol to remove contaminants. 5. DNA fragments were eluted from magnetic beads. 6. Transfer of eluate to fresh tube (Source: Beckman Coulter user guide).

2.1.11 Measuring DNA concentration

2.1.11.1 **Measurement of nucleic acid concentration using Qubit®**

A Qubit working solution was prepared by mixing 199µL of buffer and 1µL of dye for every sample to be tested and vortexing. Standards were prepared by adding 190 µl of working solution to 10 µl of Standard 1 (representing 0 ng/ µl) and Standard 2 (10 ng/µl) and vortexing. Samples were measured by adding 198 µl of working solution to 2 µl of each sample and vortexing.

All standards and samples were contained in tubes provided by manufacturer during Qubit analysis. DNA concentration was measured using a Qubit® 2.0 Fluorometer (Invitrogen). To measure DNA quantity, a Qubit® ds DNA High Sensitivity Assay kit was used. During RNA quantification, the Qubit® RNA Assay Kit was used. The fluorometer calculates the nucleic acid concentration in the original suspension and displays the result in ng/µL.

2.1.11.2 **Nucleic acid QC using the 2200 TapeStation**

The Agilent 2200 TapeStation® system is an automated electrophoresis platform that has flexible throughput capabilities, which can be used from one sample up to a 96-well plate. D1K ScreenTape analysis on the 2200 TapeStation allows the analysis of DNA fragments of sizes ranging from 35 bp to 1000 bp. It is therefore suitable for analysing library preparations. Analysis was carried out following the manufacturers' instructions. Libraries were prepared for TapeStation analysis by mixing 1 µL of sample with 3 µL of D1K Sample Buffer Mixtures are loaded on to the TapeStation.

For the R6K, protocols are similar with the exceptions that 1 µL High Sensitivity R6K Sample Buffer is mixed with 2 µL of RNA sample, and that samples are denatured by incubation at 72°C for three minutes before being placed on ice for two minutes. We then place the samples on ice for 2 minutes before briefly centrifuging the samples to collect the contents in the base of the tubes.

2.1.12 Bacterial cloning

2.1.12.1 **TOPO-TA® Cloning kit**

A mixture was made containing 4 μL of PCR product, 1 μL of salt solution containing 1.2M NaCl and 0.06M $MgCl_2$, and 1 μL of TOPO® vector. The mixture was incubated at room temperature for 30 minutes. The PCR product disrupts the expression of the lethal *E.coli* suicide gene *ccd*B, therefore only colonies containing the PCR product will grow when the mixture is transformed into One Shot® TOP 10 cells (Invitrogen).

Transformation was carried out as follows: 2 μL of the PCR product/TOPO vector mixture was added to a vial of chemically competent One Shot® TOP10 *E.coli* cells and incubated on ice for 30-60 minutes. Cells were then heat-shocked for 30 sec at 42°C before being transferred back to ice immediately and incubated for a further 5 minutes before adding 250 μL of SOC medium (2% tryptone, 0.5% yeast extract 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose).

The resulting mixture was shaken horizontally (300 rpm) at 37°C for 50-90 minutes. An aliquot of 100 μL from each transformation vial was spread on a pre-warmed agar plate containing 100 μg/ml ampicillin or 30 μg/ml tetracycline depending on the plasmid antibiotic selection gene used. Plates were incubated overnight at 37°C. Separate colonies were then picked and processed to clonal analysis.

2.1.12.2 **CloneJET® PCR cloning kit**

The CloneJET® PCR Cloning Kit from (Thermo Scientific) was used to clone PCR products generated by proofreading DNA polymerases, such as Pfu DNA polymerase. The first step is a ligation reaction containing the following components: 1 μL of 2X Reaction Buffer, 100 ng of PCR product, 1 μL of plasmid vector, and nuclease-free water to a final volume of 20μL. Samples were incubated at room temperature for 30 minutes.

2.1.13 High efficiency transformation protocol

2.1.13.1 **One shot® Top 10 cells**

One shot® Top 10 cells from Invitrogen® were used following the manufacturer's protocol. First, the vial containing the cells was thawed on ice, using one vial of One Shot® TOP10 chemically competent cells for each transformation. Next, approximately 5 μL (depending on the DNA concentration) of DNA (10pg to 100 ng) was added into a vial of One Shot® cells and mixed gently. We avoided mixing the vial containing the DNA and the cells by pipetting up and down. The vial(s) were incubated on ice for 30 min. They were next heat-shocked for exactly 30 sec at 42°C without shaking, removed from the bath, and placed on ice for 2 min.

Aseptically 250 μL of pre-warmed SOC Medium was added to each vial. The vial(s) was capped tightly and shaken horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator. Afterwards, 150 μL from each transformation were spread on a pre-warmed selective plate and incubated for 24-36 hours at 30°C. next, the remaining transformation mix stored at 4°C, before continuing to a downstream step.

2.1.13.2 **NEB 10-beta Competent *E. coli***

One tube of NEB 10-beta Competent *E. coli* cells was thawed on ice. Once thawed, 50ng of plasmid DNA was added in a volume of 1-5 μL. Mixing was done by flicking the tube 4-5 times. The mixture placed on ice for 30 minutes before being heat-shocked at 42°C for 30 sec. Following heat shock, the mixture was returned to ice for five minutes before 950 μL of it was pipetted into SOC medium and the resulting mixture was placed on a shaker (250 rpm) at 37°C for 60 min. Once this incubation was complete, 200ul of the mixture was spread on an agarose plate containing antibiotics for colony selection and incubated at 30°C for 24-36 hours.

2.1.14 Preparation of DNA following bacterial cloning

2.1.14.1 **Small scale plasmid preparation from transformed bacteria**

The GeneJET Plasmid Miniprep Kit (Thermo Scientific) was used to extract DNA from colonies. To purify high-copy plasmids, 5 ml of E. coli culture was used. Bacterial cultures were harvested by centrifugation at 8000 rpm in a microcentrifuge for 2 min at room temperature. The supernatant was decanted and pelleted cells were resuspended in 250 μL of resuspension solution. The cell suspension was transferred to a microcentrifuge tube: the bacteria were resuspended completely by pipetting up and down until no cell clumps remained.

Once this was done 250 μL of the lysis solution was added and mixed thoroughly by inverting the tube 4-6 times until the solution became viscous and slightly clear, before subsequently adding 350 μL of the neutralisation solution and mixing by inverting the tube 4-6 times.

The suspension was centrifuged for 5 min to pellet cell debris and chromosomal DNA. The supernatant was transferred to a GeneJET spin column. The column was centrifuged for 1 min. The flow through was discarded and the column was returned to the same collection tube. The column was washed twice by adding 500 μL of wash solution, centrifuging for 30-60 sec, and discarding the flow-through.

We returned the column to the same collection tube and centrifuged for an additional minute to remove residual wash solution. The GeneJET spin column was transferred to a into a fresh microcentrifuge tube and 50 μL of the Elution Buffer was added to the centre of the column membrane to elute the plasmid DNA. It was incubated for two minutes at room temperature and then centrifuged for two minutes. The eluate was collected for downstream applications.

2.1.14.2 **Large scale plasmid preparation from transformed bacteria**

The GeneJET Plasmid Midiprep Kit (Thermo Scientific) was used to extract DNA from LB broth culture. After growing up to 50 mL of bacterial culture to an OD600 of 2-3, cells were harvested by centrifugation for 10 min at 5,000 x g, with the supernatant being discarded. The pelleted cells were resuspended in 2 ml of resuspension solution. Lysis was carried out by adding 2 ml of lysis solution and mixed by repeatedly inverting the tube until the solution became viscous and slightly clear. (Vortexing should be avoided as it results in the shearing of chromosomal DNA.)

The tube was incubated for 3 min at room temperature (longer incubations should be avoided as it can cause denaturation of supercoiled plasmid DNA). Following this incubation, 2 ml of neutralisation solution was added and mixed immediately by inverting the tube 5-8 times before adding 500 μL of the Endotoxin Binding Reagent and again mixing by repeated inversion.

The mixture was incubated for five minutes at room temperature, before adding 3 mL of 96% ethanol and mixing by repeated inversion. The mixture was centrifuged for 40 minutes at 4,000-5,000 × g to pellet cell debris and chromosomal DNA. The supernatant was then transferred to a 15 mL tube by pipetting, while avoiding disturbing the white precipitate. Subsequently, 3 mL of 96% ethanol was added and mixed by repeated inversion. Approximately 5.5 ml of the sample was transferred to the supplied column pre-assembled with a 15 ml collection tube. The column was centrifuged for three minutes at 2,000 ×g.

The flow-through was discarded and the column was returned to the same collection tube. Centrifugation was repeated to process any remaining lysate through the purification column. The sample was washed by adding 4 ml of Wash Solution I to the purification column and centrifuging for 2 min at 3,000 × g. Flow-through was discarded and the column was returned to the collection tube before adding 4 mL of Wash Solution II and repeating centrifugation as for Wash Solution I.

Flow-through was discarded and the column was returned to the collection tube. Washing with Wash Solution II was repeated with a centrifugation for 5 min at 3,000 × g to remove residual wash solution. Once this was completed, the column was transferred into a fresh 15 mL collection tube and 350 μL of elution buffer was added to the centre of the purification column membrane.

The column was incubated for 2 min at room temperature before centrifuging for 5 min at 3,000 × g to elute plasmid DNA.

2.1.14.3 **Alignment of NGS Data**

Sequences obtained from NGS were mapped to the H77 reference genome sequence of HCV genotype 1a and 64 other reference (Smith et al., 2014) genomes using an in-house bioinformatics pipeline; Tanoti which is available at http://www.bioinformatics.cvr.ac.uk/tanoti.php.

- **NGS raw data analysis and quality assessment.**

The quality of NGS raw data was assessed using FastQC® (Babrahman Bioinformatics). Low quality sequence (normally located toward the end of the second read when using paired-end sequencing) was filtered, cleaned, and removed, with the aim of reaching a phred score of Q30 (1 in 1000, 99.9).

2.1.15 Nucleotide sequencing and analysis

2.1.16 Illumina bioinformatic analysis

- **Removal of custom adaptors**

Prior to sequencing, more than one adaptor was used to prepare the NGS library. When analysing sequences bioinformatically, these custom adapters were removed from the Nextera® library using the Trim galore command. This resulted in cleaned sequences being shortened by approximately 15% from 150bp to 131bp DNA fragment length. This step improved the quality of DNA fragment to Phred quality scores of at least Q30.

2.1.17 Human hepatoma cells (Huh7.5)

Human Hepatoma Cells Huh7.5 (derived from Huh7 cells harbouring HCV replicon subjected to interferon treatment) (Blight et al., 2002a) were propagated in an incubator at 37°C and with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Cell lines were typically grown in 175 cm^2 tissue culture flasks (Nunc).

Passage was carried out when cells reached 80% of confluence (typically twice per week) by adding 5 ml of ice-cold phosphate buffer saline (PBS) twice and passing over the cell surface by gently rotating the flask. This wash step was used to remove dead cells and any residual fetal bovine serum. This was followed by adding 3ml of 0.05% Trypsin/EDTA to de-attach the cells from the flask. Cells were re-suspended in 10 ml of complete DMEM before re-seeding or use in experiments.

2.1.18 *In vitro* transcription

2.1.18.1 **Site-Directed Mutagenesis**

Mutagenesis reactions were performed using the Quick Change II XL Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer's instructions. The Quick Change site-directed mutagenesis kit is used to make point mutations. Forward and reverse primers (Table 2-8) were designed according to instructions detailed by SDM primers. The desired mutation(s) were included in the middle of the primer sequence of length between 25 and 45 bases.

A PCR mixture of 50 μL consisted of 50 ng plasmid DNA template, 5 μL of 10x reaction buffer, 1.25 μL each of 10 μM forward and reverse mutagenic primers, 1 μL of dNTPs, 1.5 μL of Quick solution (which improved linear amplification), and 1 μL of *pfu ULtra* high-fidelity (HF) DNA polymerase. The PCR conditions are shown below (Table 2-7). Following amplification, each reaction was chilled on ice for 2 minutes before adding *DpnI* restriction enzyme (10 units) to digest the non-mutated dam-methylated parental DNA.

Table 2-7 PCR cycle for Site-Direct mutagenesis

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	1 minute/kb of plasmid length
3	1	68°C	7 minutes

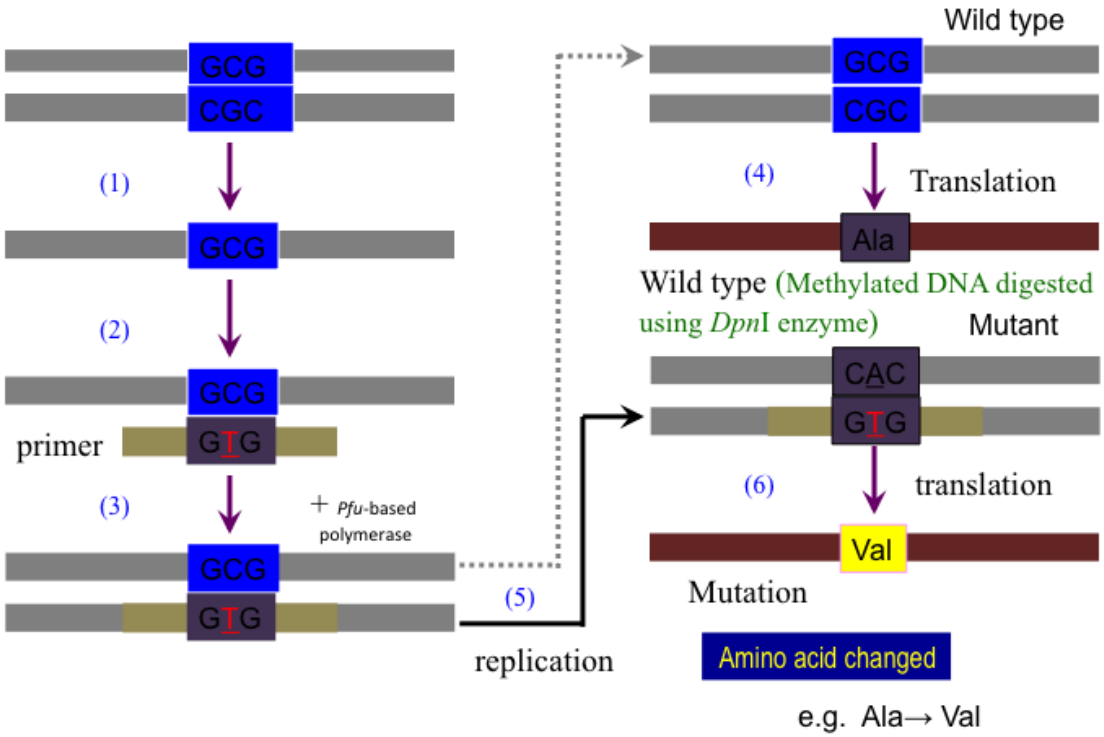
PCR cycling conditions for the site-directed mutagenesis method used to introduce mutations during replicon experiments.

The reactions were gently mixed by pipetting and then centrifuged at 13,000 rpm for one minute, before incubating at 37 ^{o}C for one hour. Following incubation, 2 μL of the DpnI-treated DNA was transformed into NEB 10-beta competent *E. coli* cells as described in Section 2.1.13.2.

2.1.18.1 **Introduction of mutations into the HCV-1a subgenomic replicon**

Several mutations found within and outside an immunodominant HLA B27-restricted epitope within NS5B ($NS5B_{2841-2849}$) were introduced into the SG/Luc/HCV-1a replicon using site-directed mutagenesis. Ten colonies from each agar plate were selected and used to prepare plasmid mini-preps as described in Section 2.1.14.1. Plasmids obtained from the mini-prep were sequenced using Sanger sequencing with appropriate sequencing primers and aligned using CLC Genomics software to assess the successful introduction of mutations.

Figure 2-2: Site-direct mutagensis



An overview of the QuickChange II XL site-direct mutagenesis method (adapted from the QuickChange II XL protocol).

2.1.18.2 **Oligonucleotide Synthesis for Site-Direct Mutagenesis**

Site direct mutagenesis primers was designed using web-based application (http://www.genomics.agilent.com/primerDesignProgram.jsp). Following the addition of HCV sequence, specific nucleotides were changed by identifying alternative nucleotides based on the NGS sequencing analysis.

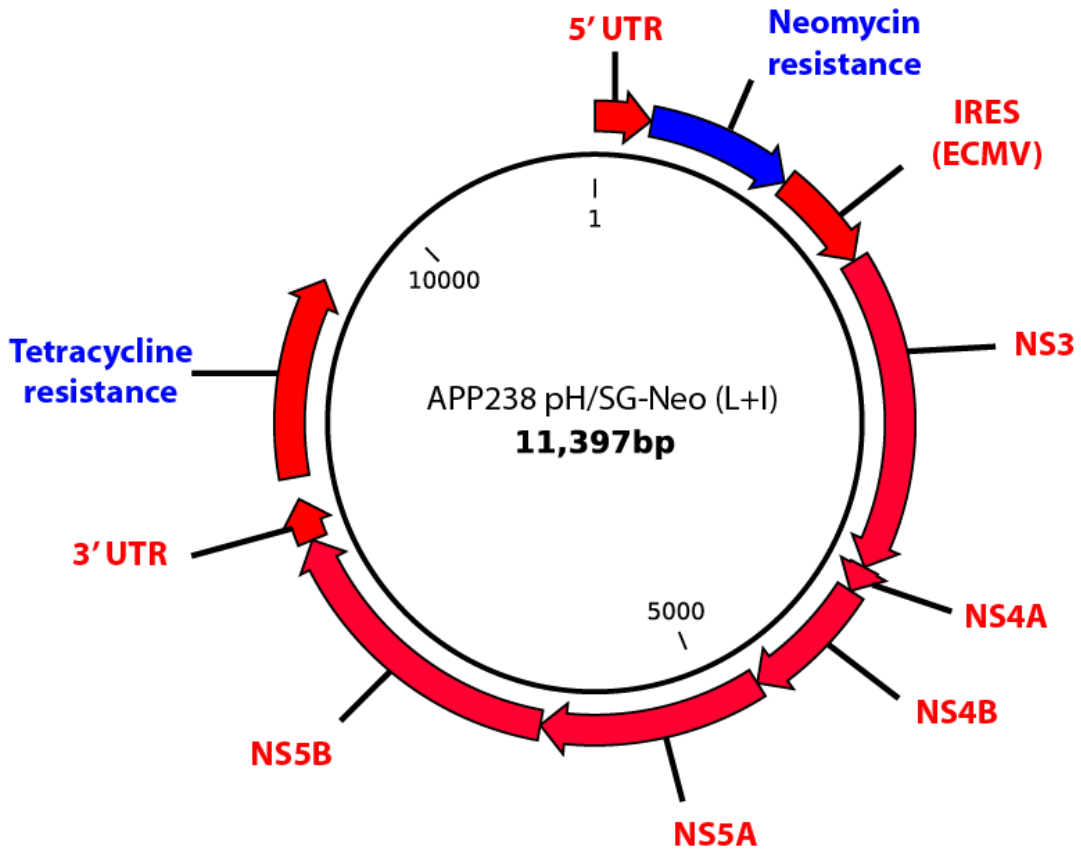
Table 2-8: Oligonucleotide sequences designed for site direct mutagenesis:

Oligo name	Sequence	Bases
A2841V-Fore	CCC-CCA-CAC-TGT-GGG-TGA-GGA-TGA-TAC-TGA-T	31
A2841V-Rev	ATC-AGT-ATC-ATC-CTC-ACC-CAC-AGT-GTG-GGG-G	31
R2842V-Fore	CCC-CAC-ACT-GTG-GGC-GGT-GAT-GAT-ACT-GAT-GAC-C	34
R2842V-Rev	GGT-CAT-CAG-TAT-CAT-CAC-CGC-CCA-CAG-TGT-GGG-G	34
M2843V-Fore	CAC-ACT-GTG-GGT-GAG-GGT-GAT-ACT-GAT-GAC-CCA	33
M2843V-Rev	TGG-GTC-ATC-AGT-ATC-ACC-CTC-ACC-CAC-AGT-GTG	33
I2844V-Fore	CAC-TGT-GGG-TGA-GGA-TGG-TAC-TGA-TGA-CCC-ATT-TC	35
I2844V-Rev	GAA-ATG-GGT-CAT-CAG-TAC-CAT-CCT-CAC-CCA-CAG-TG	35

2.1.19 DNA digestion

DNA digestion was carried out using NEB restriction enzymes and buffers at 37°C for two hours unless otherwise specified. In each case, the total reaction volume was 50 μL, which included 10 ul units of restriction enzyme for 10μg DNA. Reactions utilised the supplier cut smart buffer and BSA, unless stated otherwise.

Figure 2-3 Subgenomic replicon (APP 238 pH/SG-Neo (L+I)



Plasmid encoding a genotype 1a HCV subgenomic replicon was supplied by Apath. (Figure provided by Tamer Abdelrahman).

2.1.20 DNA ligation

A DNA Ligation kit from Thermo Scientific was used for DNA ligation reactions. The aim of ligation was to introduce the intermediate insert into the plasmid vector. The inserts containing the specific mutations were introduced and then analysed *in vitro*. In all cases, the SG/Luc/HCV-1a replicon was used as a vector.

Material	Volume (µL)
Linearised vector DNA	100 ng
Insert DNA (*at 3:1 molar excess over vector*)	Variable
5X Rapid Ligation Buffer	4 µL
T4 DNA Ligase, 5 u/µL	1 µL
Water, nuclease-free	Up to 20 µL
Total reaction volume	20 µL

2.1.20.1 **Linearisation of HCV genomic plasmid DNA and sub-genomic replicon (SGR) DNA**

SGR plasmids were linearised using the restriction enzyme *XbaI*. 10 µg of plasmid DNA was digested in a total volume of 50 µL. The reaction was incubated at 37 ^{o}C for two hours. Linearised DNA was purified using magnetic beads (Section 2.1.10.2) before adding mung bean nuclease (2 units). The reaction was incubated for 30 minutes at 30 ^{o}C. This step enables the sticky end (generated during *XbaI* digestion) to be removed completely.

Linearised DNA was purified using magnetic beads (Beckman Coulter, USA). HCV genomic plasmids were processed using the same protocols but with different restriction enzymes: two single cutter enzymes (*Bsu36I and ClaI*) were used to separate the region of interest containing the desired mutation which had been already introduced using site-direct mutagenesis from the intermediate replicon.

2.1.20.2 **Transcription procedure**

In vitro transcription was performed using the T7 RiboMAX Express Large Scale RNA Production System (Promega). The reaction was carried out in a volume of 20 μL, which consisted of a RiboMAX™ Express T7 2X Buffer, a linear DNA template (1μg total), an enzyme Mix, a T7 Express and nuclease-free water. The reaction was mixed gently and incubated at 37°C for 60 minutes. Following incubation, 1-2 μL of transcribed RNA was examined on an agarose gel to check the RNA quality before electroporation of the RNA into Huh 7.5 cells.

2.1.20.3 **Electroporation of RNA into Huh 7.5 mammalian cells**

Cells were trypsinised and transferred into the appropriate volume of DMEM medium. Cells were centrifuged at 1500 rpm for five minutes at room temperature. Supernatant was removed, and cells were re-suspended in 5 ml of PBS before centrifugation for 5 minutes at 1500 rpm. Supernatant was discarded and the cells were re-suspended into a volume of PBS that was appropriate to produce a cell density of approximately $1x10^6$ cells/ml.

For electroporation, 1 ml of re-suspended cells was transferred into a cuvette (4 mm gap, Apollo) and 10 μg of transcribed RNA was added to the cuvette. Electroporation was done using a Bio-Rad Gene Pulser. Electroporated cells were mixed with DMEM complete medium seeded into 24 well plates at $2x10^5$ cells/well. Seeding was carried out in duplicate wells. Plates were incubated at 37°C in a humidified incubator with 5% CO_2. Plates were checked for bioluminscence at 4 hours, 24 hours, 48 hours, 72 hours, 96 hours and 120 hours post transfection.

2.1.20.4 **Firefly luciferase activity assay**

Following the electroporation of Huh 7.5 cells with sub-genomic viral RNA carrying the luciferase reporter enzyme, the media wasremoved and the wells were washed twice with PBS (200 μL/well). Cells were lysed using a 1X cell culture lysis buffer (Promega) by adding 100 μL and then incubating for five

minutes at room temperature, before transferring 50 μL of lysate to a microcentrifuge tube containing 100 μL of luciferase assay substrate (Promega). The mixture was vortexed and luciferase activity was measured using a GLOMAX luminometer (Turner Biosystems). Luciferase assays were performed in duplicate.

2.1.21 Enzyme-linked immunospot (ELISPOT) assay

Based on HCV Genotype 1a (H77), overlapping peptides were used as described below (supplied by BEI Resources), covering the whole HCV genome (Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B). Peptides were pooled together in ten different pools. The specific HCV T-cell response was measured following overnight incubation with peptide pools. Thawed PBMCs were tested from spontaneous clearer and progressor patients.

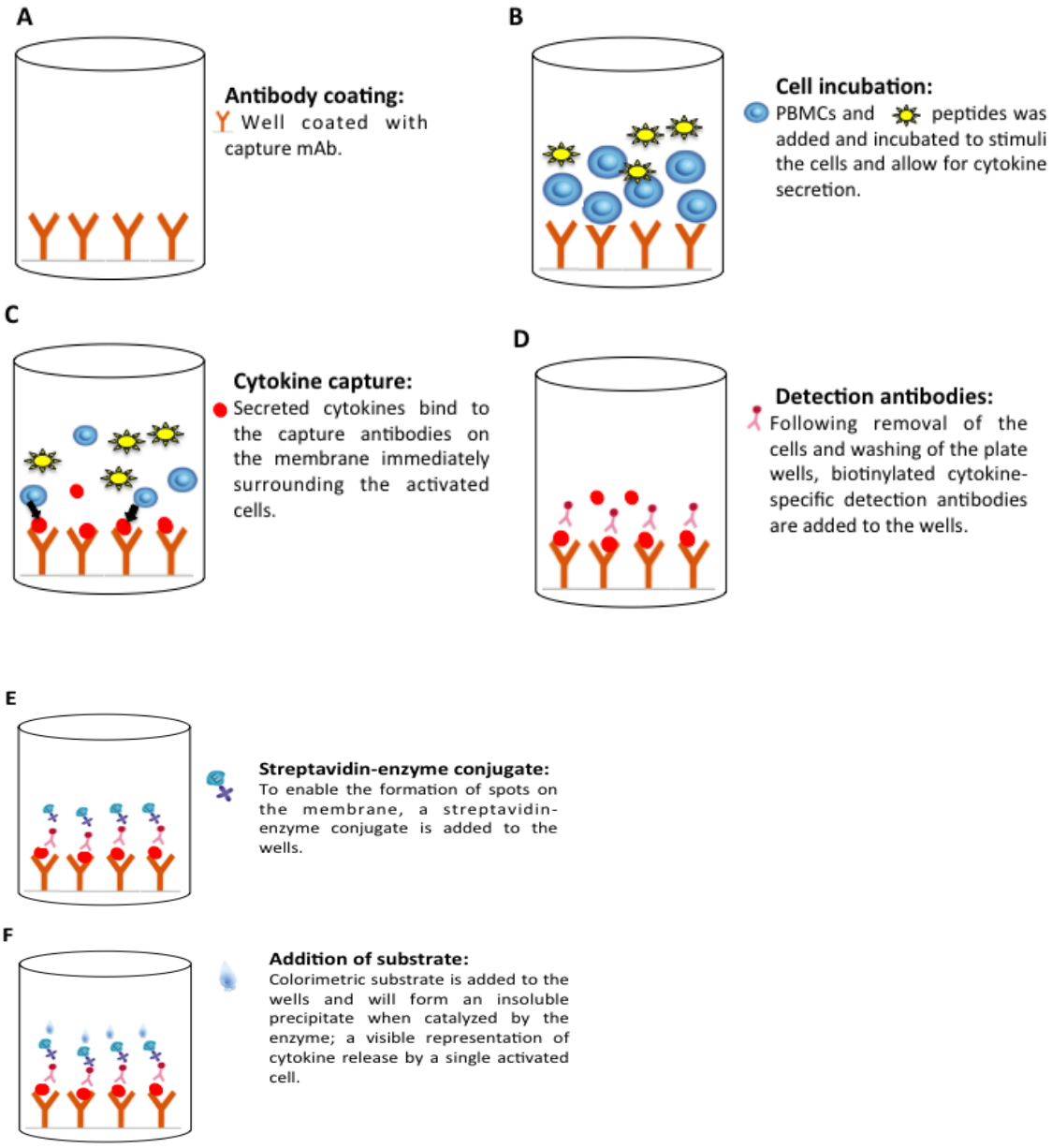
A 96-well plate pre-coated with capture mouse anti-human IFN-γ mAb (1 DIK, Mabtech) was used. Plates were initially washed four times with PBS (200 μL/well) and then blocked with 200 μL/well R10 media (RPMI 1640, Sigma-Aldrich: 10 mol/l Hepes buffer, 2 mmol/l glutamine; and 50 U/ml penicillin-streptomycin, 10% fetal calf serum) and incubated for 30 minutes at room temperature.

Peptides were added at a final concentration of 6 μg/ml with $2.0x10^5$ PBMCs and incubated overnight at 37°C in a humidified incubator with 5% CO_2. Plates were washed five times with PBS (200μL/well). Detection antibody (7-B6-1-–biotinylated secondary mouse anti-human IFN-γ mAb (Mabtech)) was diluted to 1 μg/ml with PBS containing 0.5% fetal calf serum and 100 μL/well of detection antibody was added to plates and incubated for two hours at room temperature.

Plates were rinsed five times with PBS, and 100 μL streptavidin-alkaline phosphate (Mabtech) was added and incubated for 1 hour. Excessive conjugate was removed by washing with PBS. Following this, 100 μL of filtered (BCIP/NBT-plus) substrate solution (Mabtech) was added and plates were incubated until

the spot colour started to be distinct. The reaction was stopped by washing the plates in the sink extensively. Plates were left to dry and spots were calculated using an automated ELISpot reader (AID ELISPOT Reader System, Autoimmune Diagnostika Gmbh). Phytohemagglutinin (PHA 250mg/ml), Influenza, Epstein-Barr virus and Cyromegalovirus (FEC) were added to each plate and used as a positive control to validate the sensitivity of the assay. For the negative controls, cells were added without any peptides (stimuli) and 1 μL of DMSO added. Analysis was carried out using an automated ELISpot reader (AID).

Figure 2-4 The ELISpot procedure



The stages of the ELISpot procedure A) Capture antibody is used to coat the wells. B) Incubation of the PBMC cells and peptides. C) Cytokine is secreted and binds to the capture Ab. D) Addition of specific detection antibodies (biotinylated cytokine). E) Streptavidin-enzyme conjugate added. F) Substrate is added to form a colour as a final step before reading the spots on a reader.

2.1.22 Peptides:

Peptides based on HCV virus H77 (genotype 1a) (genotype 1a; GenPept: AAB67036) were obtained free of charge from BEI Resources. These peptides cover the entire HCV genome (Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B). Each peptide was shipped as lyophilised powder (1 mg per vial). Peptides are supplied as 13 to 18-mers with overlaps of approximately 11 to 12 amino acids.

The core protein was covered by a core peptide pool of 28 peptides, E1- 29 peptides, E2 - 56 peptides, p7 8 peptides and NS2 32 peptides respectively. NS3 was spanned by two pools of 49 and 47 peptides. NS4A and NS4B were covered by an NS4A and NS4B pool (47 peptides). The NS5A peptide pool contained 71 peptides. NS5B was split into 2 pools of 45 peptides. The final concentration of each peptide pool was 6 μg/ml, which were used in the ELISpot experiments to stimulate the PBMCs.

2.1.23 Flow cytometry

PBMCs obtained from HLA-B27 patients were thawed in R10 and 100μl of 1-2x10^6 live cells/well were added to a 96 well plate. Cells were washed with 100μl PBS, spun at 1500g for 5 min and resuspended in 50 μl PBS. Cells were stimulated overnight with peptides (for stimulation flow cytometry) or stained directly with antibody panels 1 and 2. 1ul of live/dead stain was added to the cells, resuspended in PBS (50 ul) and incubated shielded from light for 20 min at room temperature.

Cells were then washed again with 50ul PBS. Next, the cells were re-suspended in FACS fix solution (2%), mixed carefully to ensure no clumping, then incubated at room temperature for 20 min, protected from light. Cells were then washed and resuspended in PBS and incubated overnight.

At the same time, panel 2 were permeablised by adding 100 ul of Perm buffer, spun at 1600 rpm for 5 min and 100 µl was added to panel 1 (Table 2-9). Pentamers was centrifuged at 14000g for 10 min in a chilled centrifuge and 1µl added to the cells and incubated for 20 min at room temperature protected from light. Secondary antibodies were added and incubated shielded from light for 30 min (Table 2-10).

Table 2-9: Flow cytometry panels

Panel 1	Panel 2
1/50 CD3-PO	1/50 CD3-PO
1/200 CD8-PB	1/200 CD8-PB
1/50 CCR7-PE Cy7	1/50 Perforin-FITC
1/50 CD45RA-FITC	1/50 GzB- Alexa700
1/50 CD127-APC	1/100 GzA- PerCpCy5.5
1/50 CD38 – PerCp Cy5.5	1/100 CD161-APC
1/50 HLA-DR Alexa700	1/50 PD-1 PeCy7
PBS	Perm Buffer

Table 2-10: Flow cytometry antibodies

Antibody	FACS: Fluorochrome	FACS: Clone
Live/dead	NIR	Invitrogen
CD3	Pacific Orange	UCHT1 / Invitrogen
CD8	Pacific Blue	RPA-T8 / Beckton Dickinson
CD45RA	FITC	HI100 / Beckton Dickinson
Granzyme B	AlexaFluor700	GB11 / Beckton Dickinson
CD38	PerCpCy5.5	HIT2 / Biolegend
CD127	APC	MB15-18C9 / Miltenyi
CD161	APC	191B8 / Miltenyi
CCR7	PeCy7	3D12 / Beckton Dickinson
HLA-DR	AlexaFluor700	G46-6 / Beckton Dickinson
PD-1	PeCy7	EH12.1 / Beckton Dickinson
Perforin	FITC	dG9 / Beckton Dickinson

2.1.24 Extraction of DNA from whole blood

A mixture of 200 μL of whole blood and 20 μL of proteinase K was incubated at room temperature for ten minutes. Following this incubation, 200 μL of AL buffer was added to the sample mixed thoroughly. The mixture was incubated at 56°C for 10 minutes, before adding 200 μL of 100% ethanol, mixing. The mixture was transferred to a QIAamp Mini spin column and centrifuged for one minute at 8000 rpm.

The filtrate was discarded. The sample was washed using buffers AW1 and AW2 (500 μL for each wash). The Mini spin column was placed in a microcentrifuge tube and 200 μL of elution buffer added before centrifugation for one minute at 8000 rpm.

2.1.25 HLA Typing

Following DNA extraction, HLA typing was carried by the Histocompatility and Immunogenetics service at Gartnavel General Hospital, Glasgow.

2.1.26 Sanger sequencing and analysis

Sanger sequencing was carried out by Eurofins, which uses cycle-sequencing technology (dideoxy chain termination/cycle sequencing) on ABI 3730XL sequencing machines and the corresponding forward or reverse primer were used. Chromatograms were examined for misclassified nucleotides by visualisation using CLC Genomics software.

2.1.27 Nextera XT® DNA Sample Preparation

Using Nextera XT technology, a DNA sample is randomly fragmented into a library of segments of approximately 300 bp by engineered transposomes. Transposomes have free DNA ends and insert randomly into DNA in a 'cut and paste' reaction. This library is then attached to adaptors.

The adptor sequences have binding sites for dual index sequences that are unique to each sample library and allow the fragments to bind to a glass slide or flow cell (Figure 2-5).

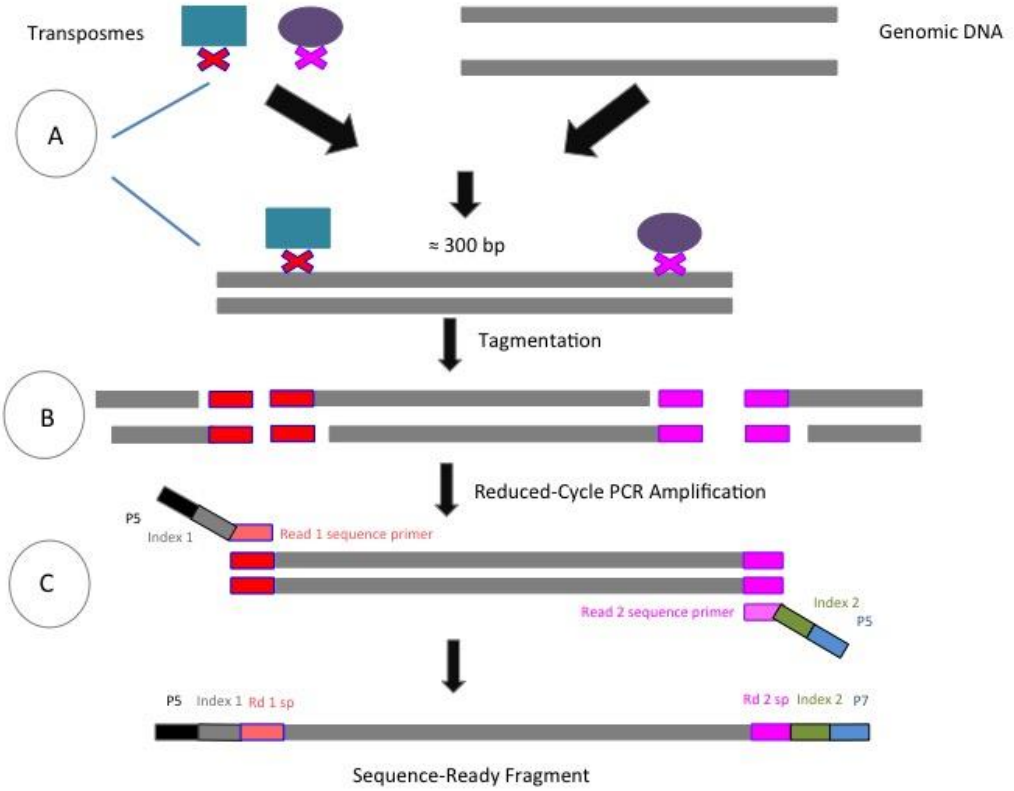
DNA samples are normalised based on their concentration, and measured using a Qubit® as described in Section 2.1.11.1. DNA libraries are then pooled and diluted to a concentration of 12 pM for use on the MiSeq® system. In Illumina instruments, the immobilised templates are clonally amplified to generate millions of molecular clusters, each containing 1,000 copies of the same template. The clustered templates are then sequenced using Illumina's sequencing-by-synthesis technology.

In this process, the addition of fluorescently labelled nucleotides liberates one of four colours that are detected by laser excitation and high-resolution cameras in every run cycle. After 100 to 500 cycles, the instrument will have recorded all or a portion of the sequence of bases in each molecular cluster. The newly identified sequences of bases are then assembled into a new or *de novo* sequence or aligned to a reference genome. A single NGS run may generate paired-end reads of up to a total of 30 million bases.

2.1.28 Illumina sequencing

Illumina sequencing is known as a "sequence-by-synthesis technique". The technique was first described in 2006. During library preparation, template DNA is fragmented and tagmented, and adapters ligated to each end of DNA fragments. Indices are added to label the DNA, thus enabling multiplexing and the attachment of oligonucleotides to the flow cell. The glass in the flow cell contains pre-attached oligonucleotides. Following denaturation, the DNA single strand attaches to the glass via these oligonucleotides and starts to synthesise the complementary strand.

Figure 2-5: Nextera XT workflow



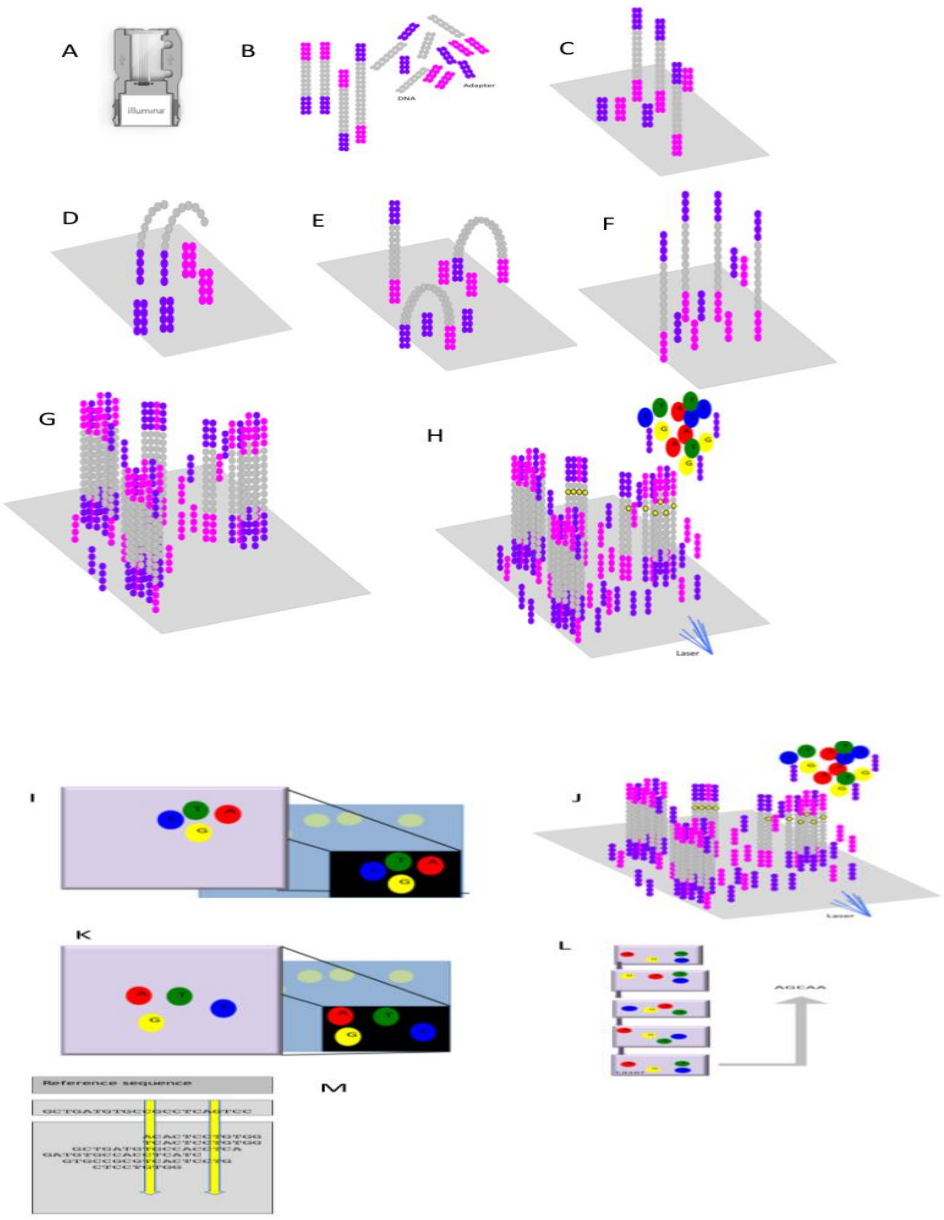
Two tagmentations are required to produce PCR amplifiable library molecules for sequencing. (a) Transposomes integrate into genomic DNA. (b) Tagmentation produces amplifiable and non-amplifiable library molecules until transposomes run out. (c) The library is cleaned to remove Tn5 proteins bound to the ends of DNA fragments, and is then PCR-amplified to add flow cell compatible adapters and dual-indexes for multiplexed sequencing.

Following the synthesis of complementary DNA, denaturation occurs and the original fragment is moved to the washing step. The PCR amplification bridge is then performed: during this step the newly synthesised DNA fragment curves and attaches to the nucleotides on the surface of the flow cell glass. The same process is repeated and, resulting in denaturing of the double stand, which in turn produces two single strands attached to the glass surface. This process is repeated until it produced a high cluster density with multiple copies surrounding the first DNA strand.

Following the generation of cluster density, the first DNA strand is removed and washed away. During this step a fluorescent label is bound to the dNTPs and used for polymerisation. Due to the 3' termination of the incorporation nucleotide, only one nucleotide is added duringeach single cycle. Consequently, the number of cycles in each sample is related to read length.

Two lasers determine the dye attached to each nucleotide: one identifying red fluorescence and detecting nucleotides A and C, while the other identifies green fluorescence and detects nucleotides G and T. Based on the signal intensity of the emission a spectrum is determined and the nucleotide is recognized. Each fragment attaches to the flow cell glass, generates a forming cluster, and each added nucleotide is detected instantaneously. This enables signals from tens of millions of clusters to be read simultaneously. The product is washed away and more nucleotides attach to oligonucleotides on the flow cell surface again (Figure 2-6).

Figure 2-6: Overview of Illumina sequencing



A) Flow cell B) Genomic DNA is fragmented and adaptors ligated to both ends of the fragments. C) DNA attaches to the surface. D) Bridge amplification. E) Fragments become double stranded and double-stranded bridges are formed on the flow cell. F) Denaturation of the double-stranded molecules. G) Amplification: several million dense clusters of double-stranded DNA are generated. H) To determine the first base, the first sequencing cycle begins by adding four labelled reversible terminators. I) Image of the first base after laser excitation. J) The next cycle repeats the incorporation of four labelled reversible terminators, K) Image of the second cycle following laser excitation; this image is taken as earlier, and the second base is recorded. L) Sequencing over multiple chemistry cycles, one base at a time. M) Alignment of data. The data are aligned and compared to a reference and sequencing differences are identified.

- **Phi X control**

Phi X 174 (or ΦX174) was used to assess the quality of each run. Due to the small genome size and the extensive work has been done on it; alignment of Phi X is straightforward. In NGS, Phi X is used at a concentration of 4 nM. Phi X is denatured by adding an equal quantity of 0.2M NaOH to Phi X. The mixture is then incubated at room temperature for five minutes before being diluted again to 20 pM by adding 980 μL of Hybridization Buffer 1 to the Phi X solution.. The solution is then added to the DNA library (100 μL Phi X to 90 μL of DNA library) resulting in a Phi X concentration of 10%.

2.1.29 Statistical analysis

Non-parametric analyses were performed using the two-tailed Mann-Whitney U test or Fisher's exact test as appropriate (parametric testing was not used due to small sample sizes in all comparisons). Data with p values less than or equal to 0.05 were considered to be significant. All statistical analysis was carried out using Graph Pad (Prism) version 6.0.

Chapter 3: Results

3.1.1 Patient cohort

The cohort in this thesis was a study of a larger cohort of patients with acute HCV infection. subsequentely Characteristics of the general cohort are shown in Table 3-1 while the smaller cohort is summarised in Table 3-2. Ten HLA-B27+ patients with acute HCV infection were identified, of whom 9 had samples available for analysis. Patient characteristics including age, route of infection, viral load, HIV status and genotype are shown in Table 3-1. Control patients used in T cell assay experiments are shown in Table 3-2. Overall, 50% (5/10) patients spontaneously cleared HCV; this percentage was 100% (2/2) in HIV uninfected individuals and 37.5% (3/8) in HIV infected individuals. The difference between groups did not reach statistical significance (p=0.44).

Table 3-1 General Cohort description

Clinical measure (units, normal range)	Spontaneous clearance (median, 95% CI)	Progression		Range	HR (95% CI)	p Value
		Plateau viraemia (median, 95% CI)	Fluctuating viraemia (median, 95% CI)			
Number	17 (15%)	53 (47.5%)	42 (37.5%)	–	–	–
Age (Years)	37 (32 to 44)	39 (37 to 42)	39 (37 to 40)	HR represents change in hazard per year of age	1.00 (0.94 to 1.06)	1
Baseline HCV VL log10 (IU/ml)	6.11 (1.79 to 7.35)	6.25 (5.71 to 6.44)	5.89 (5.06 to 6.13)		0.75 (0.55 to 1.01)	0.06
Peak HCV VL log10 (IU/ml)	6.11 (1.79 to 7.35)	6.52 (6.36 to 6.88)	6.21 (5.87 to 6.72)	HR represents change in hazard per log10 change in VL	0.61 (0.46 to 0.80)	<0.0001**
HIV VL (copies/ml)	<50 (<50 to 7044)	<50 (<50 to 1185)	87 (<50 to 6847)		1.00 (1.00 to 1.00)	0.4
Elevated bilirubin (0-17 µmol/l)	30 (12 to 60)	16 (14 to 19)	14 (12 to 17)	<20	1Ħ	
				20-40	1.64 (0.48 to 5.6)	0.43
				≥40	5.04 (1.60 to 15.92)	0.006**
Peak ALT (0-40 IU/l)	347 (128 to 1792)	308 (204 to 420)	500 (316 to 659)	<1000	1Ħ	
				>1000	2.62 (1.01 to 6.80)	0.048*
CD4 count (300-1400 10^6/l)	650 (490 to 829)	510 (439 to 640)	520 (453 to 619)	<650	1Ħ	
				≥650	2.66 (1.02 to 6.91)	
Nadir CD4 (300-1400 10^6/l)	340 (200 to 519)	310 (280 to 350)	315 (270 to 367)	<200	1Ħ	
				≥200	1.51 (0.52 to 4.35)	0.45
Transmission						
IDU§	3/13 (23%)	13/44 (30%)	13/36 (36%)	All patients were MSM and had had recent unprotected sex	1.63 (0.27 to 2.75)	0.36
INDU¶	9/12 (75%)	31/42 (74%)	27/34 (79%)		0.92 (0.25 to 3.46)	0.94
Sexual	14/14 (100%)	46/46 (100%)	39/39 (100%)		-	-
HAART	11/15 (73%)	31/53 (58%)	23/42 (55%)		1.63 (0.57 to 4.70)	0.36

*Significant p value<0.05, **highly significant p value <0.01. †p values shown represent all progressors (fluctuating viraemia and plateau viraemia) versus clearer (spontaneous clearance) groups. ††Indicates baseline. $Intravenous, intramuscular or subcutaneous drug use (crystal methamphetamine, cocaine, anabolic steroids, heroin). ¶Intranasal drug use (ketamine, cocaine). ALT, alanine aminotransferase; HAART, highly active antiretroviral therapy; HCV, hepatitis C virus; MSM, men-who-have-sex-with-men; VL, viral load (Thomson et al., 2011).

Table 3-2 Characteristics of the HLA-B27 patient cohort

Patient number	Genotype	Age	Sex	HCV outcome	HIV status	HAART	CD4 Count	Route of infection
G5	1a	30	M	Clearer	Positive	No	471	MSM
G17	1a	32	F	Clearer	Negative	NA	NA	IDU
G18	1a	35	M	Clearer	Negative	NA	NA	Needlestick
P49	1a	43	M	Clearer	Positive	No	490	MSM
P110	1a	43	M	Clearer	Positive	Yes	500	MSM
P28	1a, 4d	33	M	Progressor	Positive	No	380	MSM
P45	1a	40	M	Progressor	Positive	No	330	MSM
P113	1a	43	M	Progressor	Positive	No	520	MSM
P10	1a	35	M	Progressor	Positive	No	580	MSM
P3	1a	48	M	Progressor	Positive	No	380	MSM

Table 3-3 Patients characteristics – controls

Patient number	Genotype	Age	Sex	HCV stage	HIV status	HLA B27	CD4	Route of infection
P75	1a	31	M	Acute (P)	Positive	-	220	MSM
P96	1a	28	M	Acute (P)	Positive	-	370	MSM
GC1	1a/3a	49	F	Chronic	Negative	-	NA	IDU
GC8	1a	53	M	Chronic	Negative	-	NA	Tattoos /assault
GC10	1a	51	M	Chronic	Negative	-	NA	IDU
P32	1a	27	M	Acute (SC)	Positive	-	340	MSM

3.1.2 DNA library quality

Following AmpureXP® magnetic bead purification, DNA fragment quality was checked and visualized using a 2200 Tapestation (Agilent Technologies), as shown in (Figure 3-1).

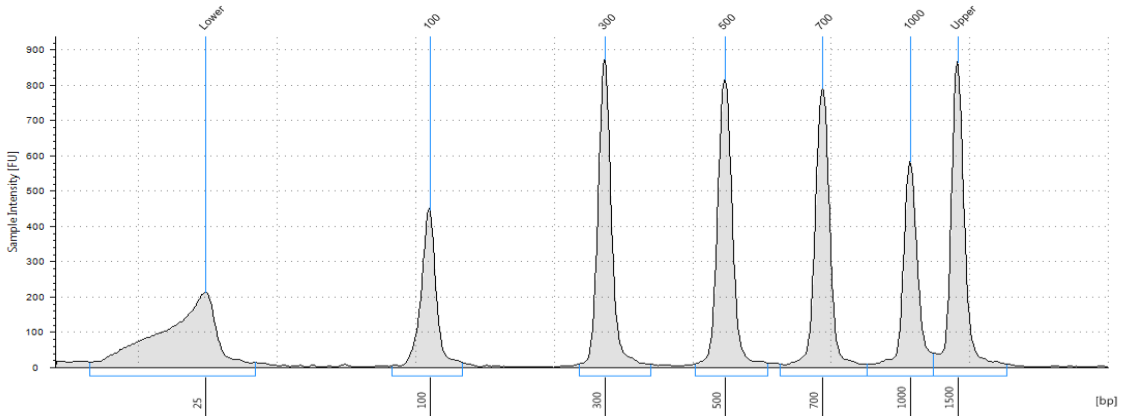
3.1.3 Sequencing quality scores

Next-generation sequencing using the Illumina MiSeq platform is a highly sensitive method but is subject to sequencing error. To assess the quality of NGS sequencing, a metric method called Phred score used to report base-calling accuracy. FastQC® (Babrahman Bioinformatics) was used to measure the Phred score for each raw fastq file.

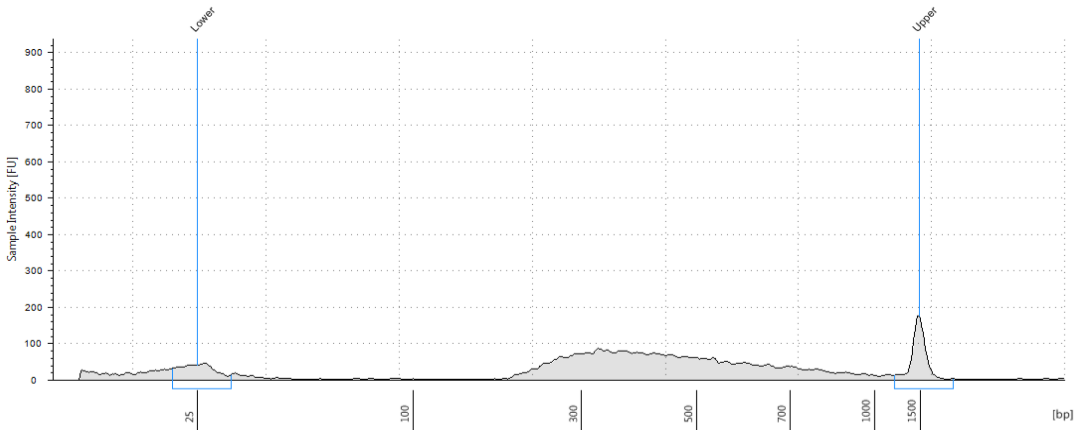
Low quality reads were frequently found at the end of sequences and second paired end sequence reads showed a drop in quality score when compared with first paired end reads (Figure 3-2); these were trimmed and removed to raise the average quality of the Phred score to a minimum of Q30 (1 base call error in 1000 bases or 99.9 accuracy). Additionally, the algorithm designed to calculate the Phred score assesses the probability of a base calling error (Ewing and Green, 1998).

Figure 3-1: DNA fragment size assessed using a 4200 Tapestation® (Agilent)

A)



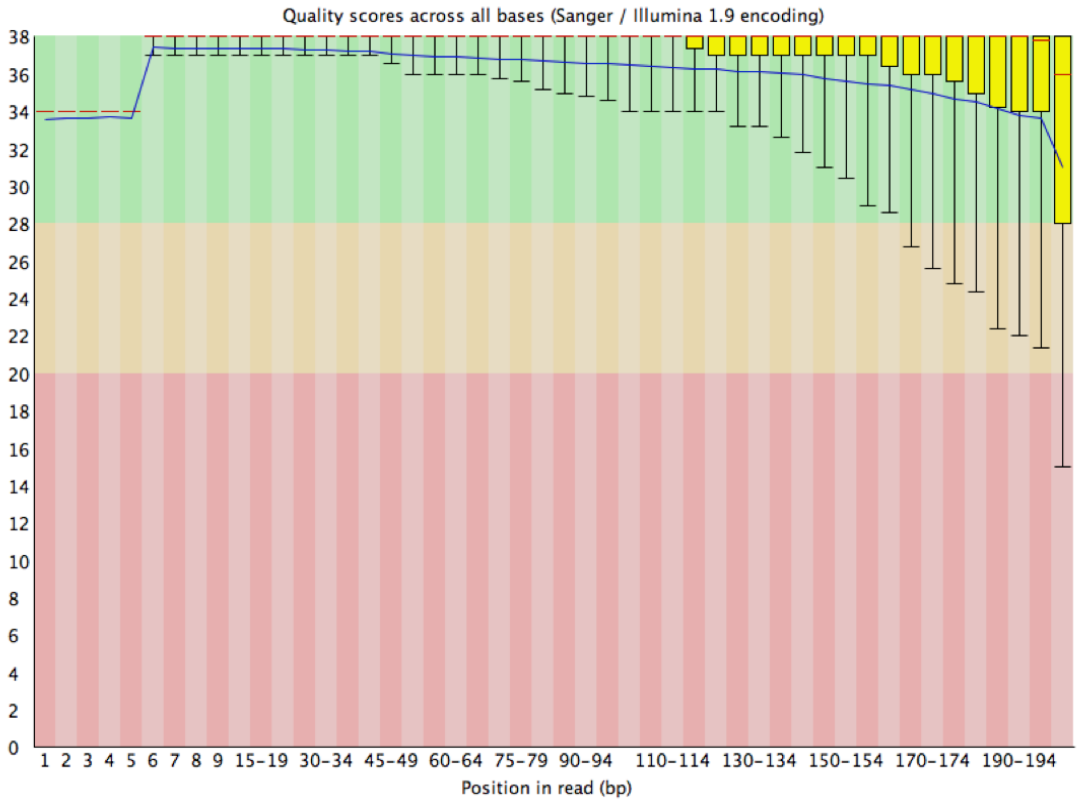
B)



Following Nextera library preparation, DNA fragment size was measured using a D1K DNA screen tape, A) DNA Ladder, B) Nextera library sample with a 300bp peak.

Estimating the accuracy of base calling error can be calculated using the formula $Q = -10 \log_{10} (P)$, where Q is the quality score value and P is the base-calling error probability. Having a Q value of 30 meaning the error probability is 1 in 1000. The amount of data produced by Illumina platform is huge, so the quality score can be used to remove all low quality reads and limit the downstream sequence analysis to the high quality score reads. High quality sequence data is essential for accurate alignments and other downstream analyses.

Figure 3-2: Illustrative example of Phred score data obtained from fastq file



The Average Phred score in this file is Q35 with a reduction in quality towards the end of the sequence reads

3.1.4 Raw data

Sequence data were produced in fastq format (typically 1-2 million reads per fastq file). Fastq files contain sequence data in fasta format with attached quality scores; an example is shown below:

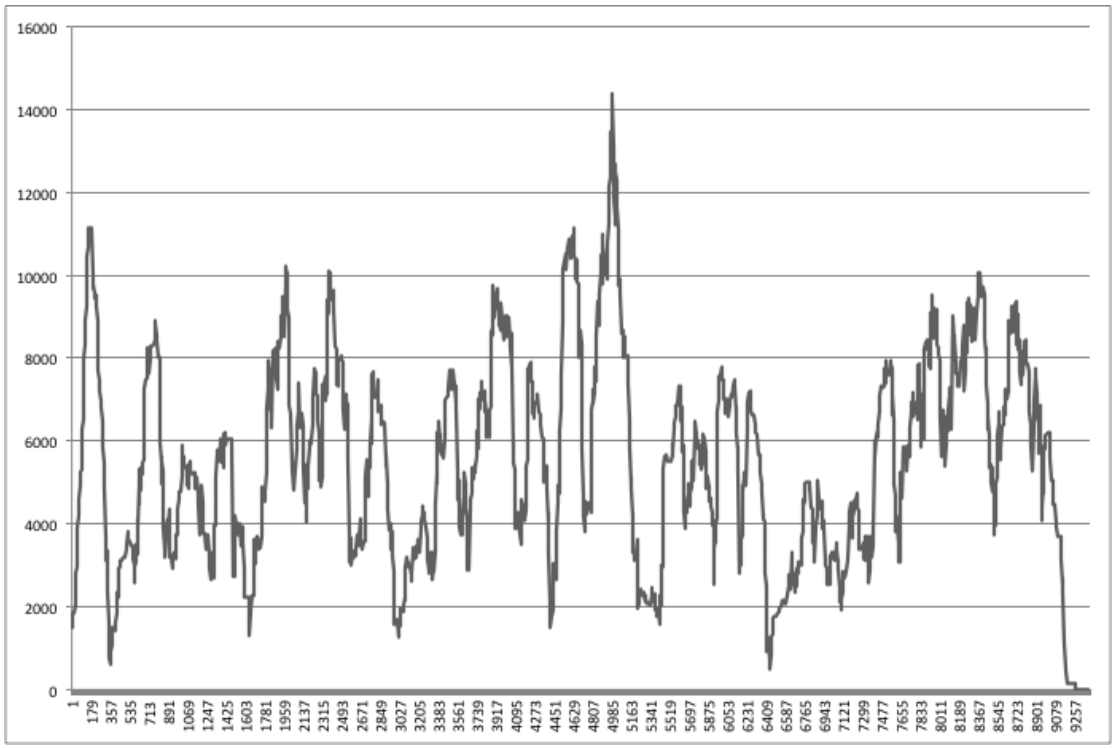
Figure 3-3: An example of Fastq file sequence format

Line 1 (red) begins with an '@' character and is followed by a sequence identifier and a description of the sequence to follow, Line 2 (blue) contains the raw sequence, Line 3 (green) begins with a '+' character, Line 4 (purple) encodes the quality values for the sequence in line 2 and contains the same number of symbols as letters in the sequence

3.1.5 Sequence alignments

Fastq sequences were assembled and aligned to several HCV reference sequences from 64 genotypes and sub-genotypes (Smith et al., 2014) and 60 HIV reference sequences. This was carried out using an in-house mapping program (Tanoti) which is more sensitive than other alignment programs such as BWA and BowTie (personal communication Sreenu Vattipally). Tanoti is based on the BLAST algrithm and maximizes both the alignment depth and coverage when used with NGS reads from highly diverse small genomes such as viruses. Tanoti was compared with other programs to evaluate the speed of performance, reads that mapped to the reference genome and errors, using different viral datasets (real and simulated datasets). Tanoti mapped reads faster and was able to produce more relaible insertion and deletion data. Tanoti employs batch processing and minimizes the usage of memory compared with other programs. It aligns each fastq sequence to reference sequences using BLAST and can increase the number of mapped reads to the reference up to ten fold (as it allows for high diversity; a characteristic feature of viral sequence reads). An example of coverage obtained following mapping with Tanoti is shown in Figure 3-4.

Figure 3-4: Read coverage across the whole HCV genome using Tanoti



Sam coverage carried to show the coverage across the whole HCV genome from a single sample analysed using Tanoti.

Following mapping, variations across the whole HCV genome were identified within unknown and known HLA-B27 restricted epitopes using in-house scripts. Within the HCV genome, previously described B27 restricted epitopes occur in the p7, NS3 and NS5B genes and within the HIV genome; a single gag epitope has previously been well described (Setiawan et al., 2015). The HLA-B27 restricted HCV epitopes we studied are listed in (Table 3-4). HLA-B27-restricted epitope variations within the HIV gag epitope shown in Table 3-5 were also studied.

Table 3-4: HLA-B27 restricted epitopes within the HCV genome.

Epitope	Protein	Position (nucleotide)	Position (aa)	HLA restriction
GRWVPGAAY	P7	2679-2708	780-788	HLA-B*27
GRGKPGIYR	NS3	4815-4844	1492-1501	HLA-B*27
KGGRKP*ARLIVFPDL*	NS5B	8052-8096	2571-2585	HLA-B*27
ARHTPVNSW	NS5B	8799-8825	2820-2828	HLA-B*27
ARMILMTHF	NS5B	8862-8888	2841-2849	HLA-B*27
GRAAICGKY	NS5B	9147-9173	2936-2944	HLA-B*27

HLA-B27 restricted epitopes within the HCV genome are listed. Positions are indicated with respect to the H77 HCV reference sequence (accession number AF009606). Italics indicate the most likely CD8+ epitope within longer sequences.

Table 3-5: HLA-B27 restricted epitope within the HIV genome.

Epitope	Protein	Position (nucleotide)	Position (aa)	HLA
KRWIILGLNK	Gag (p24)	788-817	263-272	HLA-B*27

Positions are indicated with respect to the HXB2CG HIV reference sequence (accession number K03455).

Percentage coverage and depth following alignment to the nearest reference sequence was assessed in HLA B27+ progressors and spontaneous clearers using an in-house programme called Sam Statistics (SAM_ STATS; Table 3-6 and Table 3-7).

Table 3-6: HCV read depth and coverage of samples from HLA-B27+ patients (spontaneous clearers) with acute HCV

Patient ID	Days post infection	Genotype	Viral load	Mapped area	Coverage %	Average depth	Total reads	Method
G5	80 (4.09.2015)	1a	1870000	9387	99.91	9225	454494	TE
G18	108 (17.7.2015)	1a	< 12	9309	99.08	16	799	TE
P49	60 (10.8.2005)	1a	50	9387	99.91	158565	7797018	TE
P49	60 (10.8.2005)	1a	50	9069	96.53	22	1520	MG
P49	60 (10.8.2005)	4d	50	9278	98.48	24852	1349661	TE
P110	86 (25.3.2010)	1a	5325	9357	99.60	5518	268138	TE
P110	450 (24.3.2011)	1a	9548	9179	97.70	4404	209742	TE

Samples sequenced are shown with respect to number of days post-infection, genotype (some patients were infected with more than one genotype) and the average depth and coverage calculated using SAM_STATS. Results from enriched (TE) and unenriched (MG) sequencing are shown. TE–target enrichment MG–metagenomic (unenriched). Viral load data was provided by the clinical diagnostic laboratories.

Table 3-7: HCV read depth and coverage of samples from HLA-B27+ patients (progressors) with acute HCV.

ID	Days post infection	Genotype	Viral load	Mapped area	Coverage %	Average depth	Total reads	Method
P28	406 (6.12.2005)	1a	175004	9378	99.8	2403	135721	TE
P28	406 (6.12.2005)	1a	175004	4428	47.1	6	249	MG
P28	406 (6.12.2005)	4d	175004	9299	100	19179	921986	TE
P28	406 (6.12.2005)	4d	175004	9171	97.3	35	1817	MG
P28	602 (20.6.2006)	4d	407756	9378	99.5	19055	918851	TE
P28	2366 (19.4.2011)	4d	9338	9378	99.5	19055	918851	TE
P28	2366 (19.4.2011)	1a	9338	9386	99.9	10180	503679	TE
P45	108 (30.4.2007)	1a	10012463	9386	99.9	182297	32892823	TE
P45	108 (30.4.2007)	1a	10012463	9209	98	247	18050	MG
P45	377 (24.1.2008)	1a	908557	9365	99.6	9659	474027	TE
P45	391 (07.2.2008)	1a	812612	9386	99.9	83694	4297447	TE
P45	440 (27.3.2008)	1a	996533	9386	99.9	16041	785549	TE
P113	19 (1.10.2008)	1a	5671269	9390	99.9	53392	25541816	TE
P113	19 (1.10.2008)	1a	5671269	9147	97.3	143	9787	MG
P113	19 (1.10.2008)	4d	5671269	9370	99.4	51043	2903676	TE
113	56 (7.11.2008)	1a	1537342	9120	97	56	3809	MG
P10	180 (05/4/2006)	1a	17755	758	8.07	33	178	MG
P10	418 (29.11.2006)	1a	927653	565	6.01	1	5	MG

Samples sequenced are shown with respect to number of days post-infection, genotype and the average depth and coverage calculated using SAM_STATS. Results from enriched and unenriched sequencing are shown. TE-target enrichment MG-metagenomic (unenriched).

Metagenomic sequencing was significantly less sensitive than target enrichment sequencing (this was particularly evident in samples from P28 and P10). Initial batches were carried out using MG sequencing but subsequently, TE was selected as the method of choice.

For each sample, an HCV consensus sequence was calculated (using SAM2CONSENSUS) from sam files with coverage over 90%. Both the consensus sequence and the full alignment sam file were used to identify mutation(s) within and outwith HLA-B27 restricted HCV and HIV epitopes. Mutated nucleotides within the sam alignment were tested using a third in-house program called SamVariation (all in-house scripts provided by Dr Vattipally B Sreenu).

A mapping threshold of 1% was used to report minority variants (10x above the calculated error threshold). Reads calculated at less than 1% were excluded due to the possibility of error during the Illumina clustering process, the possibility of low-level cross-contamination during sample preparation or index sequencing error (Kircher et al., 2011). It has previously been reported that the error rate of the Illumina Miseq platform is < 0.001 indels, ~0.1 substitutions per site (McElroy et al., 2014).

3.1.6 Quality control for NGS data:

3.1.6.1 **Cross-contamination during next-generation sequencing:**

Next generation sequencing is a powerful technique but is limited by a high risk of accidental contamination during RNA extraction, cDNA synthesis and during library preparation. The sensitivity of the technique (millions of sequence reads generated from only 1ng of input DNA) means that it is far more prone to cross-contamination than other less sensitive techniques, including PCR (Naccache et al., 2013, Lysholm et al., 2012, Cheval et al., 2011).

Run-to run contamination may occur on the MiSeq platform (and can be minimised or eradicated using bleach in the wash cycle). Cross-contamination during factory processing is also known to occur within the indexes used to ligate

to samples in order to allow multiplexed sequencing and therefore very low-level cross-contamination is a problem that must be contained and managed rather than completely eradicated (Kircher et al., 2011).

NGS library preparation needs to be carried out with a high level of precautions (including separation of all pre-amplification steps from post-amplification in different preparation rooms).

In order to identify significant cross-contamination, consensus sequences from each sample were mapped to the closest HCV reference(s) and a phylogenetic tree constructed using MEGA 6.6 software in order to identify any evidence of cross-contamination. Negative control samples were included in the sequencing run. One sequencing run generated a consensus sequence in a negative control sample (sample G17) – this came from a very high viral load sample processed in the same batch. It was correctly identified as a contaminant due identity of the full genome to the sample processed next to it following phylogenetic analysis and removed from further analysis.

3.1.6.2 **Alignment bias during mapping to closely related HCV references sequences**

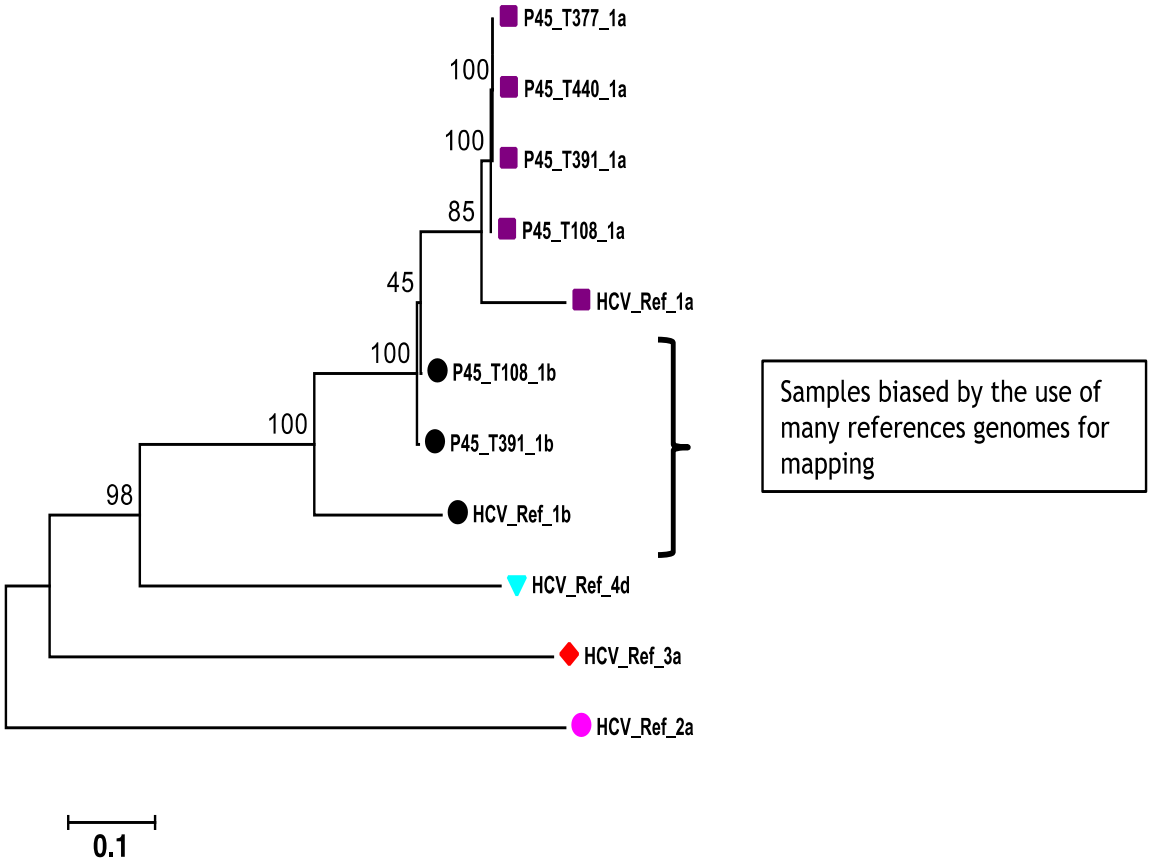
Mapping, the alignment of sequences from raw NGS fastq files to known HCV reference sequences, can be carried out rapidly and is more sensitive than *de novo* assembly so is an attractive technique. However, when the wrong reference sequence is used (for example a different genotype or sub-genotype), bias can be introduced (Bull et al., 2011b).

To overcome this issue, we created an alignment to multiple HCV reference sequences and selected in our analysis only the alignment with the highest level of depth and coverage (using an in-house script called runallReadMapRatio).

An example of bias created by using the wrong reference sequence for mapping is shown in Figure 3-5. Data generated from patient 45 (P45) showed that when NGS sequence reads were mapped to genotype 1b HCV (the patient

was infected with genotype 1a), a bias occurred, resulting in a consensus sequence that was still genotype 1a but subsequently different from the 1a mapped sequence.

Figure 3-5: Phylogenetic tree illustrating bias when the mapping reference sequence used comes from a different HCV subgenotype (P45).



A phylogenetic tree was calculated using maximum likelihood from consensus sequences generated from HCV genomes obtained at different time points in patient 45, using reference sequences from genotype 1a and 1b. Evidence of significant bias introduced during the mapping process is shown. Bootstrap values >70 are shown.

These biases could lead to variation in the phylogenetic tree and in interpretation of epitope data, if variations occurred in these regions (due to minority variants preferentially mapping to the wrong reference sequence). Selection of the correct mapping reference is therefore essential to avoid calculating a biased consensus sequence and wrong conclusions about selection within the genome. In order to identify the correct reference sequence, the ratio of mapped to unmapped reads was calculated and the highest used to identify the correct reference sequence (Table 3-8). For P45 the enrichment was far lower for g1b than 1a indicating the correct genotype was 1a.

Table 3-8: Calculated enrichment in P45 HCV reads mapped to genotype 1a and 1b reference sequences.

Patient No	Days post infection	Genotype	Total reads	Mapped reads	Enrichment (Mapped / total reads)
P45	108	1a	33690336	32892823	97.63
P45	108	1b	33690336	18055468	53.59
P45	391	1a	4478054	4297447	95.96
P45	391	1b	4478054	2469370	55.14

Although a reference sequence with >90 coverage was generated using both genotype 1a and 1b sequences, the enrichment can be seen to be low when the wrong reference sequence was used.

3.1.7 Phylogenetic analysis

16 consensus full genome sequences from seven clinical samples were aligned against HCV references (including genotypes 1, 2, 3 and 4) using the alignment tool MUSCLE, implemented in MEGA software version 6. Bootstrapped trees (1000 data sets) were constructed using the Maximum likelihood (Figure 3-7). The best model was calculated and chosen based on calculation of AIC and BIC using MEGA 6.6 (section 3.1.8). The GTR+G+I had the lowest AIC score and was selected as the best model.

3.1.8 Best model calculation

The GTRGI model was selected as the best fit using AIC and BIC calculations within MEGA 6.6.

Model	#Param	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G
GTR+G+I	59	131565.1618	130953.3109	-65417.64044	0.37924878	1.176578608	2.003189012	0.202540824	0.216385601	0.29844506	0.282594592
GTR+G	58	131718.1372	131116.6562	-65500.31359	n/a	0.335237396	2.009268591	0.202540824	0.216385601	0.29844506	0.282594592
T92+G+I	53	131842.2232	131292.5918	-65593.28376	0.385436508	1.287739357	1.688433932	0.209480174	0.209480174	0.290519826	0.290519826
TN93+G+I	56	131867.8556	131287.1144	-65587.54367	0.385086359	1.243577189	1.773033123	0.202540824	0.216385601	0.29844506	0.282594592
HKY+G+I	55	131939.3788	131369.0075	-65629.4907	0.383492613	1.24035354	1.685755414	0.202540824	0.216385601	0.29844506	0.282594592
T92+G	52	132011.1024	131471.841	-65683.90879	n/a	0.346495695	1.688433932	0.209480174	0.209480174	0.290519826	0.290519826
TN93+G	55	132048.8198	131478.4485	-65684.2112	n/a	0.344607965	1.699863419	0.202540824	0.216385601	0.29844506	0.282594592
HKY+G	54	132099.4428	131539.4414	-65715.7081	n/a	0.34337372	1.685755414	0.202540824	0.216385601	0.29844506	0.282594592

Models with the lowest BIC scores (Bayesian Information Criterion) and AICc values (Akaike Information Criterion, corrected) are considered the best fit. Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) are also presented. Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Assumed or estimated values of transition/transversion bias (R) are shown for each model. They are followed by nucleotide frequencies (f) and rates of base substitutions (r) for each nucleotide pair. For estimating ML values, a tree topology was automatically computed. Codon positions included were 1st+2nd+3rd. All positions

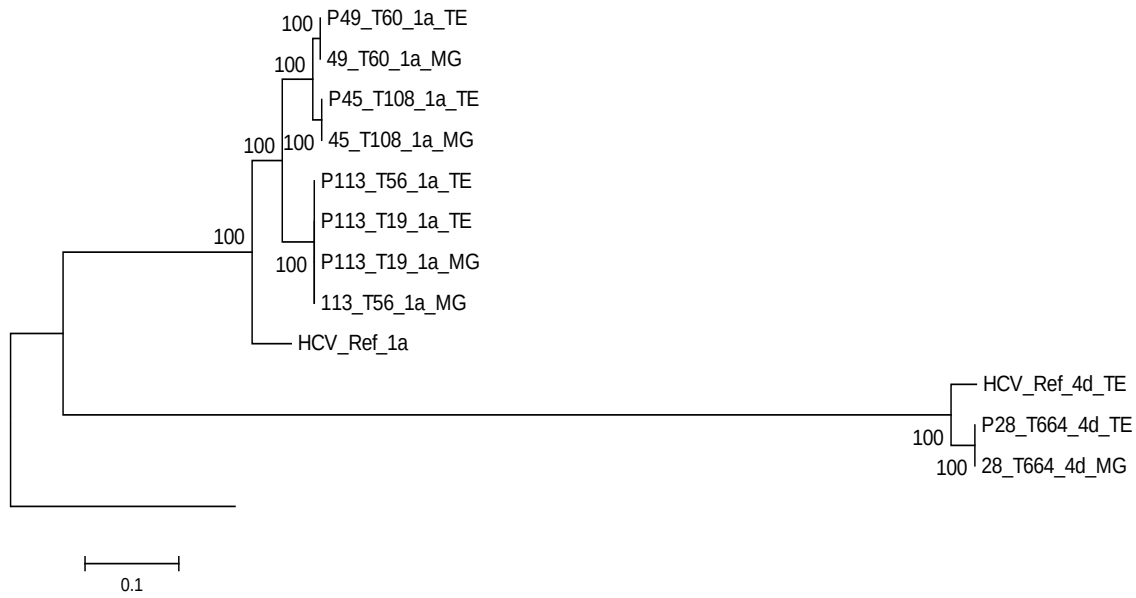
with less than 95 site coverage were eliminated. There were a total of 9103 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [2]. Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

To identify the impact of the CTL escape mutations on HLA-B27 epitope. Next-generation sequence analysed using in-house bioinformatic tools. Data from the NGS run shown in details in Figure 3-7

3.1.9 Target enrichment versus metagenomic (unenriched) sequencing

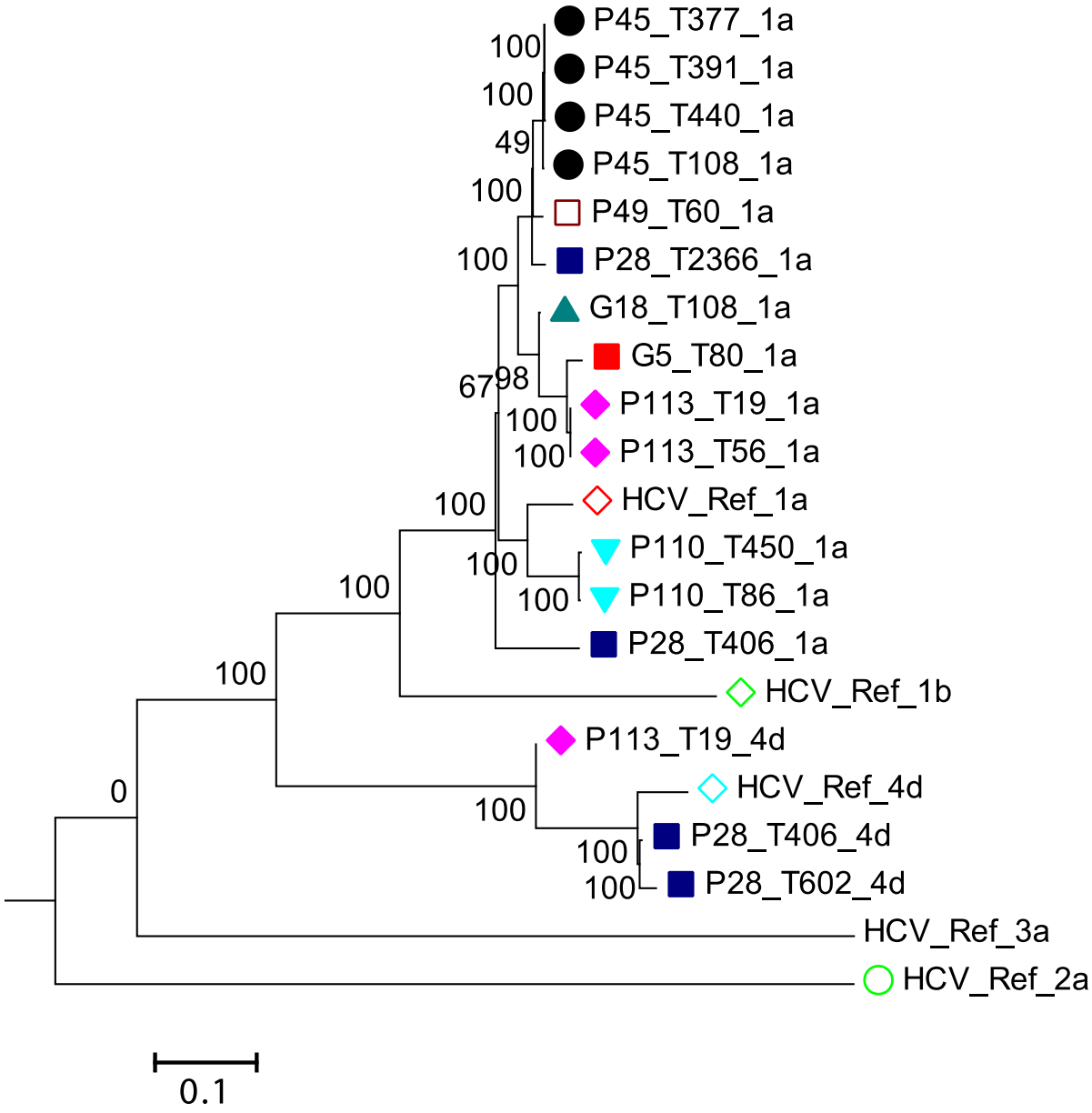
Paired samples where available were compared using both metagenomic and target enrichment sequencing in separate sequencing experiments (target enrichment was developed *in house* around a year after the metagenomic sequencing experiments). A phylogenetic analysis was carried out of paired samples revealing no evidence of bias when using a TE approach (Figure 3-6).

Figure 3-6: Phylogenetic analysis of samples sequenced using metagenomic (MG) or target enrichment (TE).



All MG and TE samples were identical, indicating that bias during target enrichment is minimal or non-existent.

Figure 3-7: Phylogenetic tree calculated using consensus sequences from all samples sequenced.

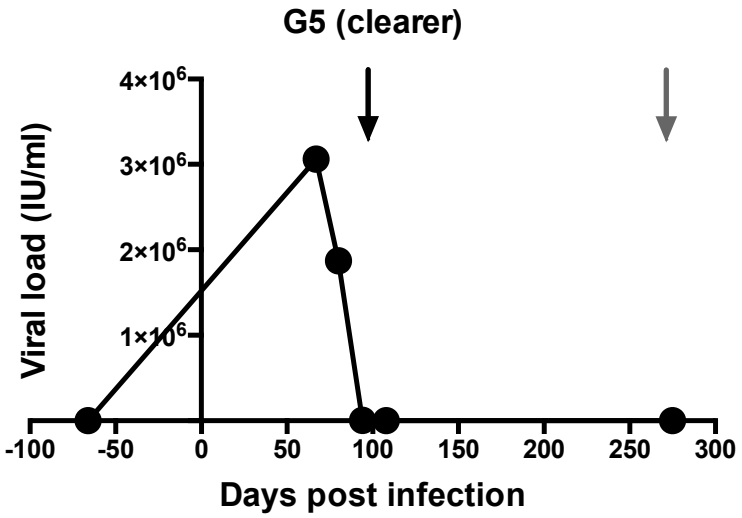


A phylogenetic tree was constructed using maximum likelihood (using consensus sequences from HCV in HLA B27+ patients using MEGA 6.0 and 1000 bootstrap replicates. Bootstrap values >70 are shown.

3.1.10 Viral load dynamics

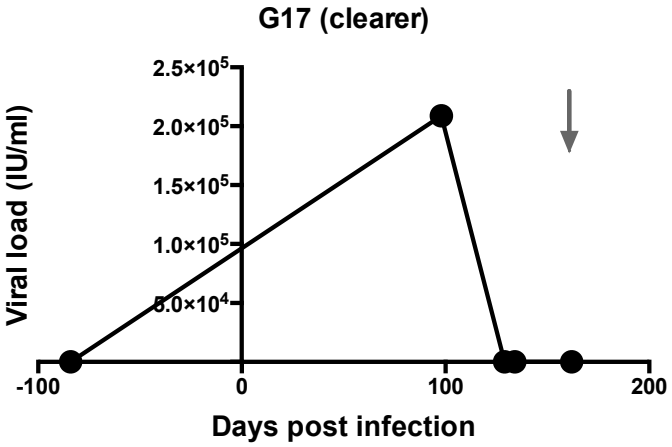
HCV viral loads and sampling times are plotted over time in Figures 3-8 to 3-17. Patients that spontaneously cleared HCV are shown in Figures 3-8 to 3-14.

Figure 3-8: Viral load dynamics and sampling from patient G5



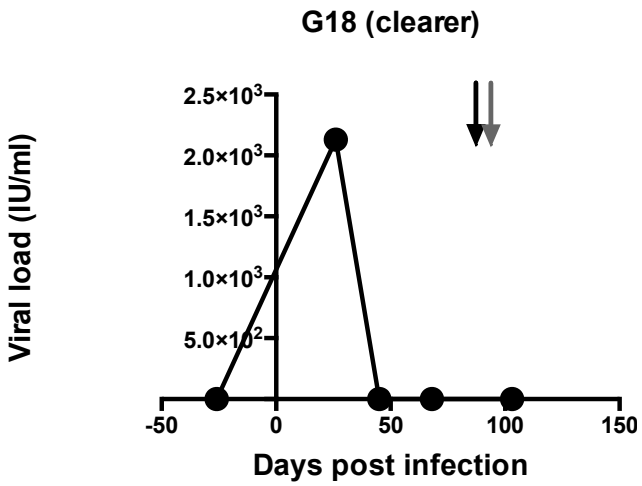
Black arrows indicate samples examined for NGS, gray arrows indicate those available for T cell assays. The estimated date of infection occurs where the line crosses the y axis (midpoint between the last negative and first positive PCR).

Figure 3-9: Viral load dynamics and sampling from patient G17



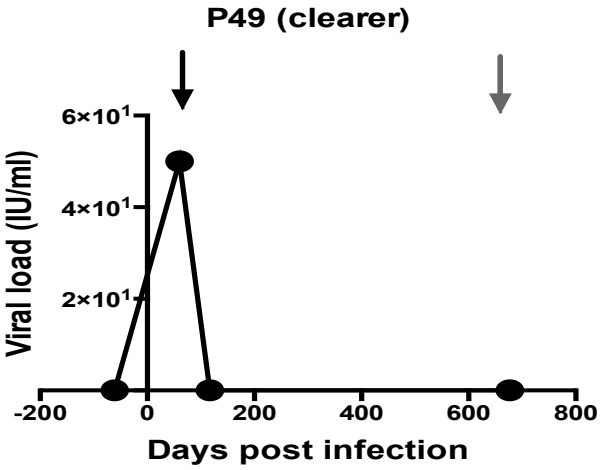
Black arrows indicate samples examined for NGS, gray arrows indicate those available for T cell assays. The estimated date of infection occurs where the line crosses the y axis (midpoint between the last negative and first positive PCR).

Figure 3-10: Viral load dynamics and sampling from patient G18



Black arrows indicate samples examined for NGS, gray arrows indicate those available for T cell assays. The estimated date of infection occurs where the line crosses the y axis (midpoint between the last negative and first positive PCR).

Figure 3-11: Viral load dynamics and sampling from patient P49



Black arrows indicate samples examined for NGS, gray arrows indicate those available for T cell assays. The estimated date of infection occurs where the line crosses the y axis (midpoint between the last negative and first positive PCR).

Figure 3-12: Viral load dynamics and sampling from patient P110 – episode 1 (E1)

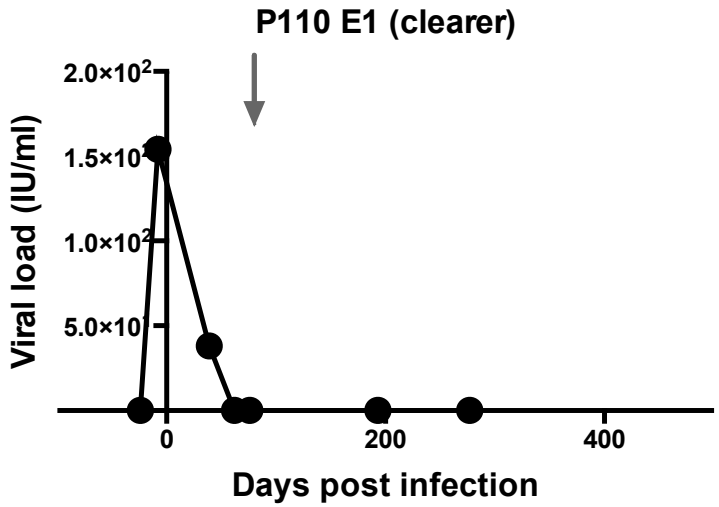
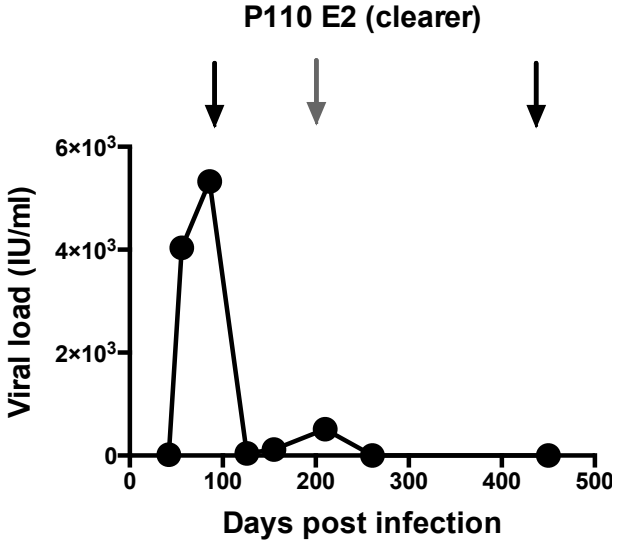


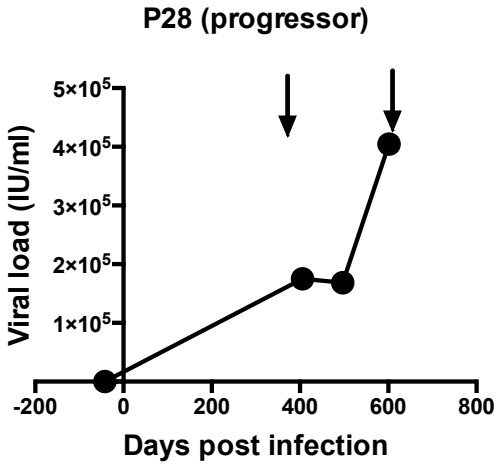
Figure 3-13: Viral load dynamics and sampling from patient P110 – episode 2 (E2)



Black arrows indicate samples examined for NGS, gray arrows indicate those available for T cell assays. The estimated date of infection occurs where the line crosses the y axis (midpoint between the last negative and first positive PCR). Negative viral loads after the onset of viraemia indicate treatment with IFN and RBV.

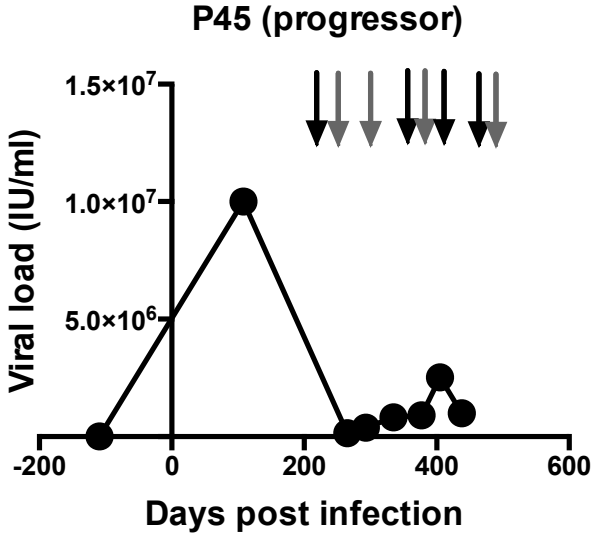
Viral load dynamics and sampling time points for patients that progressed to chronicity are shown in Figures 3-14 to 3-17.

Figure 3-14: Viral load dynamics and sampling from patient P28



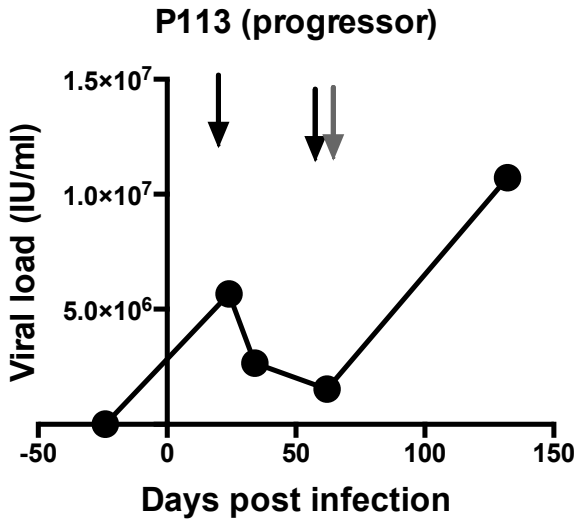
Black arrows indicate samples examined for NGS, gray arrows indicate those available for T cell assays. The estimated date of infection occurs where the line crosses the y axis (midpoint between the last negative and first positive PCR).

Figure 3-15: Viral load dynamics and sampling from patient P45



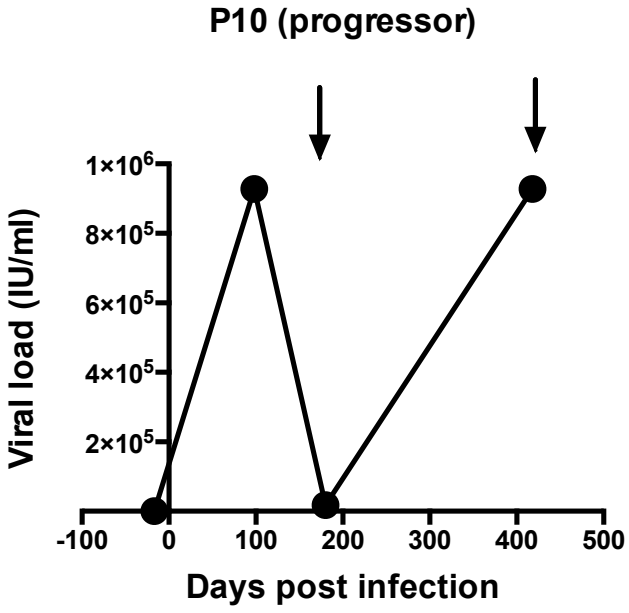
Black arrows indicate samples examined for NGS, gray arrows indicate those available for T cell assays. The estimated date of infection occurs where the line crosses the y axis (midpoint between the last negative and first positive PCR).

Figure 3-16: Viral load dynamics and sampling from patient P113



Black arrows indicate samples examined for NGS, gray arrows indicate those available for T cell assays. The estimated date of infection occurs where the line crosses the y axis (midpoint between the last negative and first positive PCR).

Figure 3-17: Viral load dynamics and sampling from patient P10



Black arrows indicate samples examined for NGS, gray arrows indicate those available for T cell assays. The estimated date of infection occurs where the line crosses the y axis (midpoint between the last negative and first positive PCR).

3.1.11 Mutations located within HLA-B27 restricted HCV epitopes

We carried out NGS on eight patients (seven successfully; one patient had samples only of very low viral load and these were not successfully sequenced), four of whom spontaneously cleared the HCV within the acute phase and another four of whom progressed to chronicity. Two further patients were identified but plasma samples with detectable viraemia were not available. HCV sequence analysis showed varying mutations within the HLA-B27-restricted epitopes.

Both single and multiple clustered mutations were observed among the patients; most patients that progressed to chronicity had two or three mutations within the CD8+ T cell restricted epitope whereas patients who spontaneously cleared HCV had a single mutation or no mutations at all within HLA-B27 epitopes.

3.1.11.1 **Spontaneous clearer patients**

Patient G5 had a single mutation within HLA-B27 restricted epitope within NS3 - G1497T and within the $NS5B_{2841\text{-}2849}$ epitope, single mutations at L2846T/V/P/I and F2849S. A single mutation was also detected within ($NS5B_{2820\text{-}2828}$) at V2825I. Later on, the patient had a negative viral load, following this time-point, and cleared the HCV (Table 3-9).

Patient G18 had several mutations within HLA-B27-restricted epitopes. The previously described p7 epitope showed mutations as follows: W782F, P784S, G785R, A786V and Y788C, as well as at $NS5B_{2820\text{-}2828}$ R2821K, T2823N, P2825L/I/F, V2825K/H, N2826K/H and W2828L. A single mutation was found within $NS5B_{2841\text{-}2849}$ at (1, 4, 5, 6, 8) A2841V, I2844V, L2845M, M2846T/I/L and T2847Y,F (Table 3-10).

Patient 49 (P49) had single mutations within two different epitopes (HLA-B27) p7 A786V and HLA-B27 $NS3_{1492\text{-}1501}$ at K1495R and $NS5B_{2841\text{-}2849}$ at A2841V, I2845V, L2845T/M and M2846L/V/M/I (Table 3-11). HIV gag sequence observed within this patient displayed no mutations (Table 3-17).

Patient P110 (P110) was infected twice with HCV and spontaneously cleared infection twice. Over time, the patient developed three and four mutations within the HLA-B27-restricted $NS5B_{2841-2849}$ epitope at the following sites A2841V, M2843T, I2844V and M2846V/A/T. The HLA-B27 epitope within NS3 showed only a single mutation K1495R. At $NS5B_{2936-2944}$, mutations were present at A2939V and K2943R. A single mutation was observed within the p7 epitope R35W (Table 3-12).

3.1.11.2 **Progressor patients**

Patient 10 (P10) developed multiple clustered mutations within the $NS5B_{2841-2849}$ epitope T418A, A2841V, M2843V, I2844V (Table 3-13).

Patient P28 had no or only one mutation at the first time-point except in one sequence when two mutations occurred within this time-point. Mutations were observed in the first amino acid (A2841V); two mutations were also reported in this first amino acid (A2841V, M2846L and F2849S) (Table 3-14). Multiple mutations occurred within the HLA-B27 restricted epitope in the p7 region; these mutations were observed as R35K, V783A, P784A, A786V and A787T. Two other epitopes $NS5B_{2571-2585}$ and $NS5B_{2571-2585}$ had only one mutation for ($NS5B_{2571-2585}$) mutations observed as K2575R, L2579P and F162Y. The last epitope ($NS5B_{2936-2944}$) contained multiple mutations (R2937K, I2940V and K2843R).

At the second time-point post-infection (day 602) multiple mutations were detected within the ($NS5B_{2936-2944}$) G2936C, A2939P/D, G2942R, and G2943M/R. The last time- point for this patient showed cluster mutations in the ($NS5B_{2841-2849}$) (M2844V and M2846V/L), with single mutations within the ($p7_{780-788}$) and ($NS3_{1492-1501}$). These mutations within different highly conserved epitope regions of the HLA-B27 restricted epitope led to viral escape from the CD8+T cell response.

Co-infected patients were assessed for variation within the HLA-B27-restricted HIV gag epitope and mutations were found including L268M, L270H and N271H (Table 3-18).

Another progressor patient in the cohort Patient 45 (P45) showed multiple mutations within the HLA-B27 epitope at p7 and showed a single mutation across all other HLA-B27 epitopes. This patient exhibited one mutation within two different epitopes at the first time-point ($p7_{780-788}$) and $NS5B_{2820-2828}$ A787V and R2821K mutations also. Other time-points showed multiple "cluster" mutations within $p7_{780-788}$ at V783A and A786V and a single mutation within I1498T K2571R and R2821K. Two subsequent time points showed the same pattern of (Table 3-15).

The HIV sequence from this patient (P45) displayed mutations within the $gag_{263-272}$ at W265C, I266M, L268Q, G269E and N271K (Table 3-19).

Mutations were found mainly within the TCR binding sites and on a few occasions within the anchor binding sites, as has already been described (Stewart-Jones *et al.*, 2005, and Dazert *et al.*, 2009).

Table 3-9: Mutations observed within HLA-B27 restricted epitope within patient G5

Day	HLA-B27 restricted epitope variation in patient G5 (spontaneously cleared + HIV)					
	P7 (780-788) GRWVPGAAY	NS3 (1492-1501) GRGKPGIYRF	NS5B (2571-2585) KGGRKP***ARLIVFPDL***	NS5B(2820-2828) ARHTPVNSW	NS5B (2841-2849) TLW**ARMILMTHF**FSV	NS5B (2936-2944) GRAAICGKY
80	.... 100%(4491)	.... 94%(6655) .....T.... 5%(389)	.... 100%(5157)	.....I... 100%(17853)	.....T..... 35%(6890) .....V..... 58%(11400) .....S... 2%(417) .....P..... 2%(493) .....I..... 1%(330)	..... 100%(9799)

Amino acid sequence of HLA-B27 epitopes in acute HCV patients using NGS technology. Points indicate homologous residues.

Table 3-10: Mutations observed over time within HLA-B27 restricted epitopes in patient G18 (G18)

Day	HLA-B27 restricted epitope variation in patient G18 (spontaneously cleared)					
	P7 (780-788) GRWVPGAAY	NS3(1492-1501) GRGKPGIYRF	NS5B(2571-2585) KGGRKP***ARLIVFPDL***	NS5B(2820-2828) ARHTPVNSW	NS5B(2841-2849) TLW**ARMILMTHF**FSV	NS5B(2936-2944) GRAAICGKY
108	.... 25% (2) ......V.. 50% (4) .....RV.. 12% (1) ..F.S...C 12% (1)	.... 100%(6)	.... 100%(14)	.... 33%(13) .K....... 28%(11) .....L... 2%(1) .....I... 23%(9) ......K.. 2%(1) ...N.I... 2%(1) .....LH.. 2%(1) .....F..L 2%(1) .K..Q.... 2%(1)	.... 66%(30) ....Y.... 2%(1) ....F.... 2%(1) ...V.......... 11%(5) ......V........ 2%(1) ....T...... 4%(2) ....I...... 2%(1) ....M....... 4%(2) ....L...... 4%(2)	.... 62%(17) .K....... 33%(9) ....R. 3%(1)

Amino acid sequence of HLA-B27 epitopes in acute HCV patients using NGS technology. Points indicate homologous residues.

Table 3-11: Mutations observed over time within HLA-B27 restricted epitopes in patient 49 (P49)

Day	HLA-B27 restricted epitope variation in patient 49 (spontaneously cleared + HIV)					
	P7 (2651-2677) GRWVPGAAY	NS3 (4787-4816) GRGKPGIYRF	NS5B (8024-8068) KGGRKPARLIVFPDL	NS5B(2820-2828) ARHTPVNSW	NS5B (2841-2849) TLW**ARMILMTHF**FSV	NS5B(9119-9145) GRAAICGKY
60	......V.. 100%(101856)	...R...... 100%(73302)	.... 100%(145959)	.... 100%(527936)	.... 2%(11987) ....T....... 32%(170276) ....L...... 4%(25382) ......V........ 29%(151530) ....V...... 12%(65359) ....MM...... 6%(31960) ...V........... 10%(53610) ....I...... 2%(12121)	.... 100%(268802)

Amino acid sequence of HLA-B27 epitopes in acute HCV patients using NGS technology. Points indicate homologous residues.

Table 3-12: Mutations observed over time within HLA-B27 epitopes in patient 110 (P110)

Day	HLA-B27 restricted epitope variation in patient 110 (spontaneously cleared + HIV)					
	P7 (780-788) GRWVPGAAY	NS3 (1492-1501) GRGKPGIYRF	NS5B (2571-2585) KGGRKP***ARLIVFPDL***	NS5B (2820-2828) ARHTPVNSW	NS5B (2841-2849) TLW**ARMILMTHF**FSV	NS5B (2936-2944) GRAAICGKY
86	.... 100%(2870)	.... 91%(3350) ...R...... 9%(294)	.... 100%(3379)	.... 100%(7032)	...V..V.V...... 47%(2701) ...V.T..V...... 10%(616) ...V....V...... 8%(479) ...V....A...... 16%(960) ...V..V.T...... 2%(164) ....A...... 29%(795)	.......R. 98%(2430) ...V...R. 2%(26)
450	.... 95%(1760) .W....... 5%(92)	.... 100%(3316)	.... 100%(843234)	.... 100%(7300)	...V.TV.V...... 82%(4300) ...V..V.V...... 13%(714) ...V.T..V...... 5%(219)	.......R. 100%(1144)

Amino acid sequence of HLA-B27 epitopes in acute HCV patients using NGS technology. Points indicate homologous residues

Table 3-13: Mutations observed in HLA-B27+ restricted epitopes in patient 10 (P10)

Day	HLA-B27 restricted epitope variation in patient 10 (progressor + HIV)					
	P7 (780-788) GRWVPGAAY	NS3(1492-1501) GRGKPGIYRF	NS5B (2571-2585) KGGRKP***ARLIVFPDL***	NS5B (2820-2828) ARHTPVNSW	NS5B(2841-2849) TLW**ARMILMTHF**FSV	NS5B (2936-2944) GRAAICGKY
180	ND	ND	ND	.K..... 97%(31) .K..T.... 3%(1)	..... 66%(121) A..V.V..... 31%(57) ....v..... 2%(4)	..... 100%(10)
418	ND	ND	ND	..... 100% (2)	...V.VV..... 100%(3) .....L..... 100%(1)	..... 100%(1)

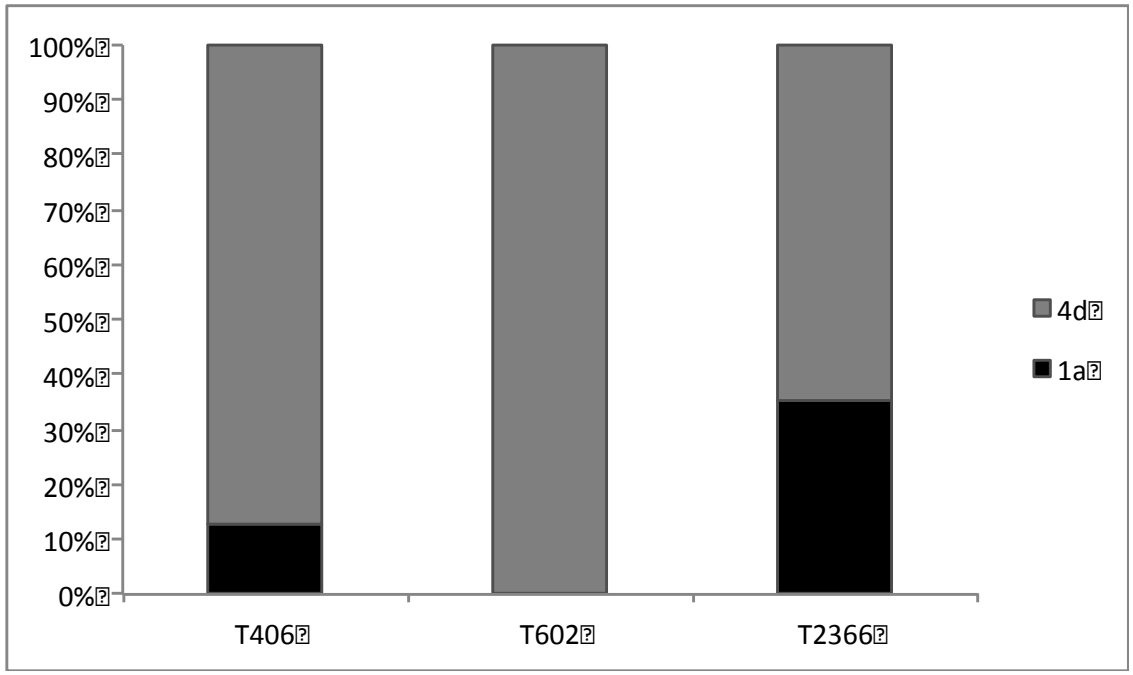
Amino acid sequence of HLA-B27 epitopes in acute HCV patients using NGS technology. Points indicate homologous residues

Table 3-14: Mutations observed over time within HLA-B27 restricted epitopes in patient 28 (P28)

Day	HLA-B27 restricted epitope variation in patient 28 (progressor + HIV)					
	P7 (780-788) GRWVPGAAY	NS3 (1492-1501) GRGKPGIYRF	NS5B (2571-2585) KGGRKP***ARLIVFPDL***	NS5B(2820-2828) ARHTPVNSW	NS5B (2841-2849) TLW***ARMILMTHF***FSV	NS5B (2936-2944) GRAAICGKY
406	...AA.V.. 14%(1) ...A..V.. 43%(3) ...A..VT. 29%(2) .K....V.. 14%(1)	..... 75%(3) .....T.... 25%(1)	..... 67%(8) ....R..... 8%(1) .....P..... 8%(1) .....Y... 17%(2)	..... 100%(9209)	..... 63%(5) ...V..... 13%(1) ...V....L..... 13%(1) .....S... 13%(1)	..... 77%(14) .K..V.... 11%(2) .K..... 5%(1) .....R. 5%(1)
602	ND	ND	..... 100% (2994)	..... 100%(2355)	..... 100%(5569)	..... 25%(1) C..P..RM. 25%(1) .....R. 25%(1) ...D...R. 25%(1)
2366	.....V.. 100%(4467)	...R..... 100%(9501)	..... 100%(6566)	..... 100%(9652)	...V..V.V..... 97%(10757) ...V....L..... 3%(233)	..... 100%(15323)

Amino acid sequence of HLA-B27 epitopes in acute HCV patients using NGS technology. Points indicate homologous residues

Figure 3-18: Number of mapped reads to genotype 1a and 4d referenced at different time points in patient 28



Patient 28 had a dual infection with two different HCV genotypes (1a and 4d). The graph shows the percentage of each genotype present at different timepoints based on NGS reads at the three different time points (T406, T602 and T2366).

Day	HLA-B27 restricted epitope variation in patient P45 (progressor + HIV)					
	P7 (780-788) GRWVPGAAY	NS3 (1492-1501) GRGKPGIYRF	NS5B (2571-2585) KGGRKP***ARLIVFPDL***	NS5B (2820-2828) ARHTPVNSW	NS5B (2841-2849) TLW**ARMILMTHF**FSV	NS5B (2936-2944) GRAAICGKY
108	......V.. 100%(645839)	.... 100%(888356)	.... 100%(355099)	.K....... 100%(1204550)	.... 100%(1293207)	.... 100%(524161)
265					.... 100%(28623)	
377	...A..V.. 86%(4790) ......V.. 14%(752)	.... 98%(8336) ......T... 2% (126)	.... 98%(7211) R............. 2% (86)	.K....... 100%(10441)	.... 100%(11247)	.... 100%(7325)
391	...A..V.. 86%(50139) ......V.. 14%(6360)	.... 97%(74045) ......T... 3%(1611)	.... 100%(58602)	.K....... 100%(137508)	.... 100%(152042)	.... 100%(98327)
440	...A..V.. 91%(8237) ......V.. 9%(752)	.... 98%(11640) ......T... 2%(205)	.... 100%(16366)	.K....... 100%(24126)	.... 100%(26382)	.... 100%(20360)

Amino acid sequence of HLA-B27 epitopes, HCV sequence found in acute HCV patinets using NGS technology. Points indicate homologous residues.

Table 3-15 Mutations observed over time within HLA-B27 restricted epitopes in patient 45

Table 3-16: Mutations observed over time within HLA-B27 restricted epitopes in patient 113

Day	HLA-B27 restricted epitope variation in patient 113 (progressor + HIV)					
	P7 (780-788) GRWVPGAAY	NS3(1492-1501) GRGKPGIYRF	NS5B (2571-2585) KGGRKP***ARLIVFPDL***	NS5B (2820-2828) ARHTPVNSW	NS5B(2841-2849) TLW**ARMILMTHF**FSV	NS5B (2936-2944) GRAAICGKY
19	.... 100%(257044)	.... 100%(326285)	.... 100%(431124)	.....I... 100%(699421)	A..V.V..... 100%(749410)	.... 100%(1384609)
56	.... 98%(25888) ......V.. 2%(528)	.... 100%(61720)	.... 100%(41182)	.....I... 98%(56549) .K....... 2%(969)	A..V.V..... 100%(78300)	.... 93%(34093) ....R. 7%(2436)

Amino acid sequence of HLA-B27 epitopes, HCV sequence found in acute HCV patinets using NGS technology. Points indicate homologous residues.

Table 3-17: Mutations detected within the HLA-B27-restricted HIV gag epitope in patient 49

Day	HLA-B27 restricted HIV epitope in patient 49
	p24 Gag (263-272) KRWIILGLNK
60	.... 100%(4)

Amino acid sequence of HLA-B27 epitopes, sequence found in co-infected patients (HIV/HCV) using NGS technology. Points indicate homologous residues.

Table 3-18: Mutations detected within HLA-B27 HIV epitope in patient 28 (P28)

Day	HLA-B27 restricted HIV epitope variation in patient 28
	p24 Gag (263-272) KRWIILGLNK
406	.....M.... 91%(8929) ....H.. 3%(273) .....M..H. 1%(114)

Amino acid sequence of HLA-B27 epitopes, sequence found in co-infected patients (HIV/HCV) using NGS technology. Points indicate homologous residues.

Table 3-19: Mutations detect within HLA-B27 epitope in patient 45 (P45).

Day	HLA-B27 restricted HIV epitope in patient 45
	p24 Gag (263-272) KRWIILGLNK
108	.... 100%(2264)
391	.... 91%(50) ....M..... 2%(1) ..C..... 2%(1) ...M..... 2%(1) .....QE.K. 2%(1)

Amino acid sequence of HLA-B27 epitopes, sequence found in co-infected patients (HIV/HCV) using NGS technology. Points indicate homologous residues.

Table 3-20 Summary of the median number of mutations within different epitopes in spontaneous clearer and progressor patients.

	No of mutations	
Epitopes	Spontaneous Clearer patients	Progressor patients
P7 (780-788) GRWVPGAAY	0	2
NS3 (1492-1501) GRGKPGIYRF	1	1
NS5B (2571-2585) KGGRKPARLIVFPDL	0	0
NS5B (2820-2828) ARHTPVNSW)	1	1
NS5B (2841-2849) TLWARMILMTHFFSV	1	3
NS5B (2936-2944) GRAAICGKY	0	2

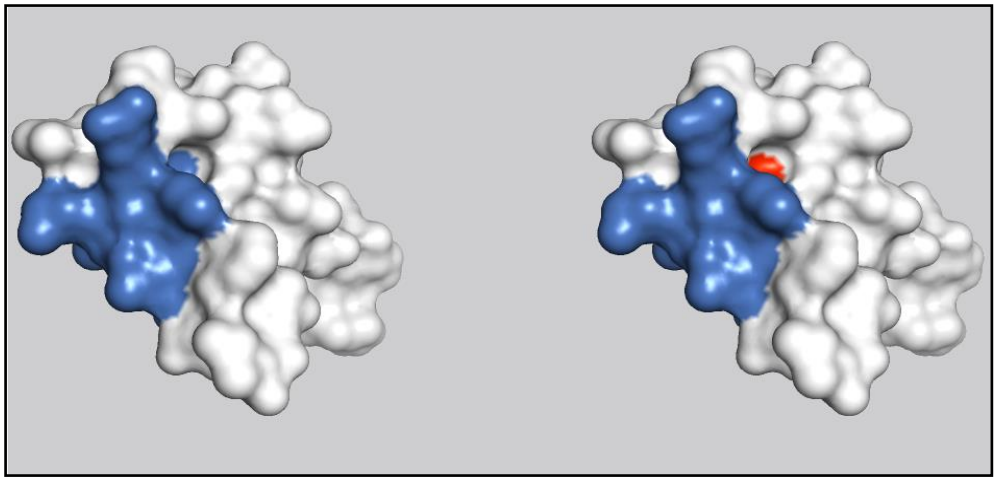
3.1.12 Structural analysis

3.1.12.1 **Predicted structure**

The predicted structure of $NS5B_{2841\text{-}2849}$ was carried out using Phyre 2 software and Pymol software was used to construct the predicted structure of the NS5B epitope, with and without the common mutations obseved in the NGS, using amino acid sequences and the known crystal structure of genotype 1a NS5B from the protein data bank at *www.rcsb.org*.

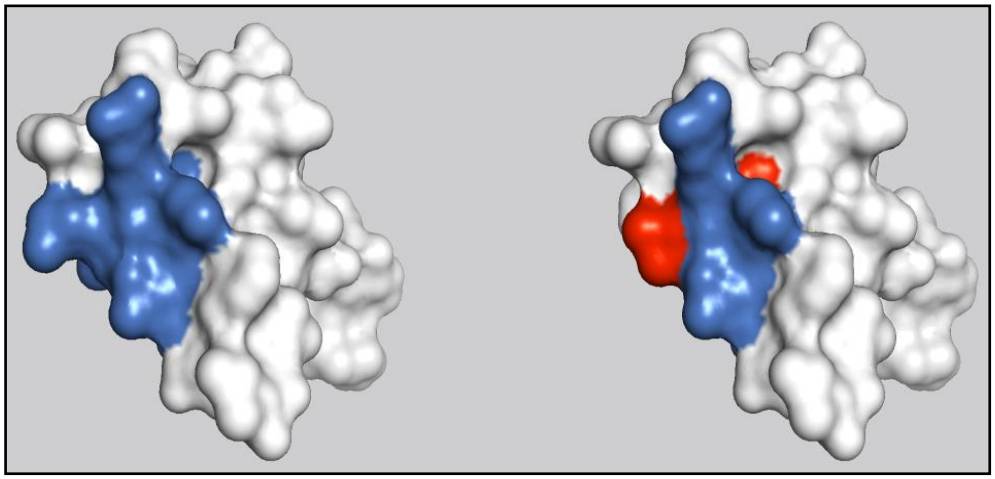
Mutations detected within the immunodominant HLA-B27-restricted 2841 epitope using NGS were used to assess the impact on the predicted structure of NS5B using Pymol software.

Figure 3-19: Single mutation (VRMILMTHF) compared to wild type sequence



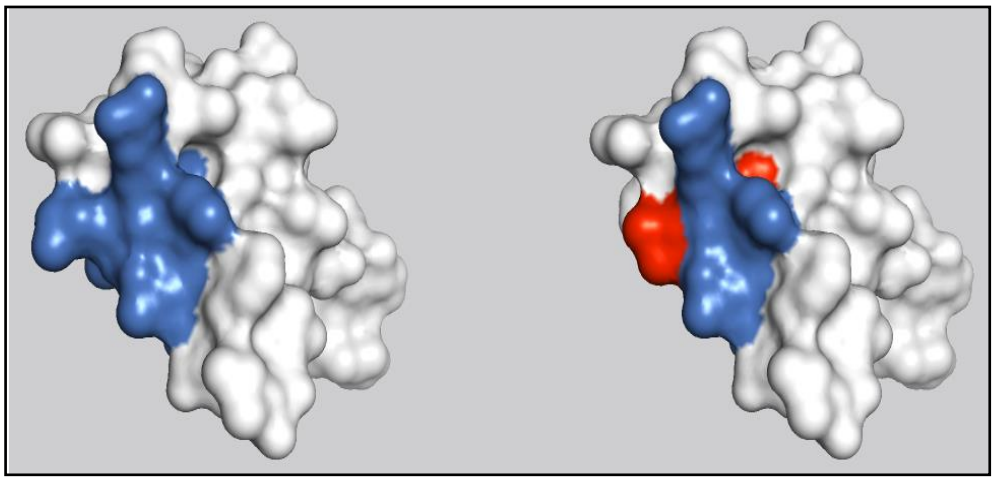
Gray colour shows the NS5B, HLA-B27 restricted epitope highlighted in blue and the red colour indicates the mutation.

Figure 3-20: Two mutations (VRVILMTHF) compared to wild type sequence



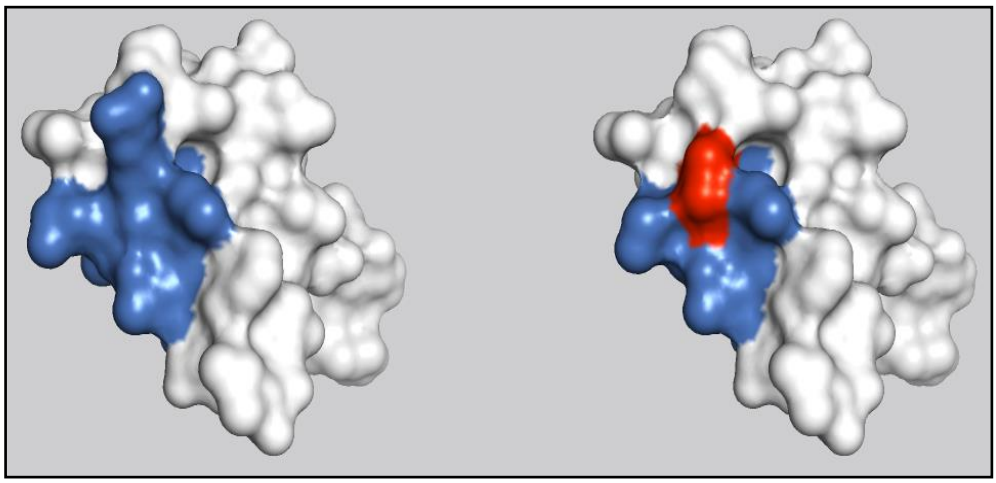
Gray colour shows the NS5B, HLA-B27 restricted epitope highlighted in blue and the red colour indicates the mutations.

Figure 3-21: Three mutations (VRVVLMTHF) compared to wild type sequence



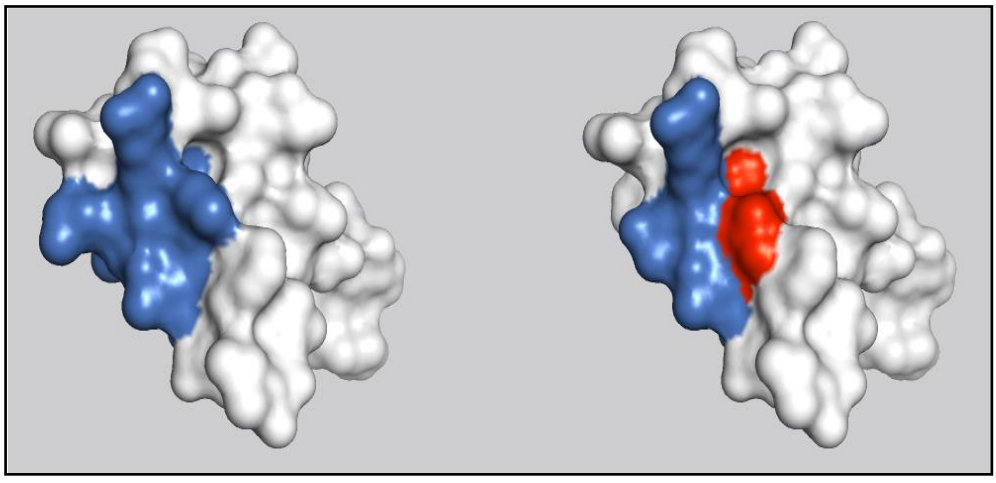
Gray colour shows the NS5B, HLA-B27 restricted epitope highlighted in blue and the red colour indicates the mutations.

Figure 3-22: Single mutation (AVMILMTHF) compared to wild type sequence



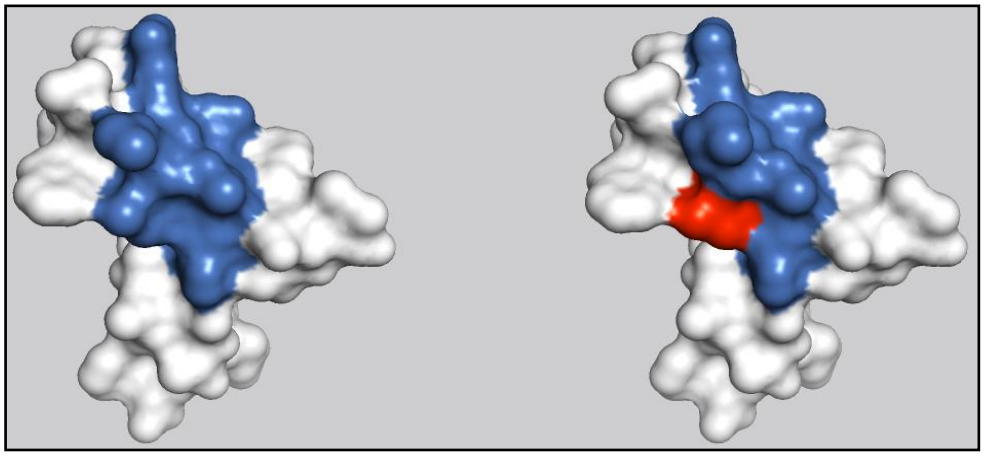
Gray colour show the NS5B, HLA-B27 restricted epitope highlighted in blue and the red colour indicates the mutation.

Figure 3-23: Four mutations (VRTVLVTHF) compared to wild type sequence



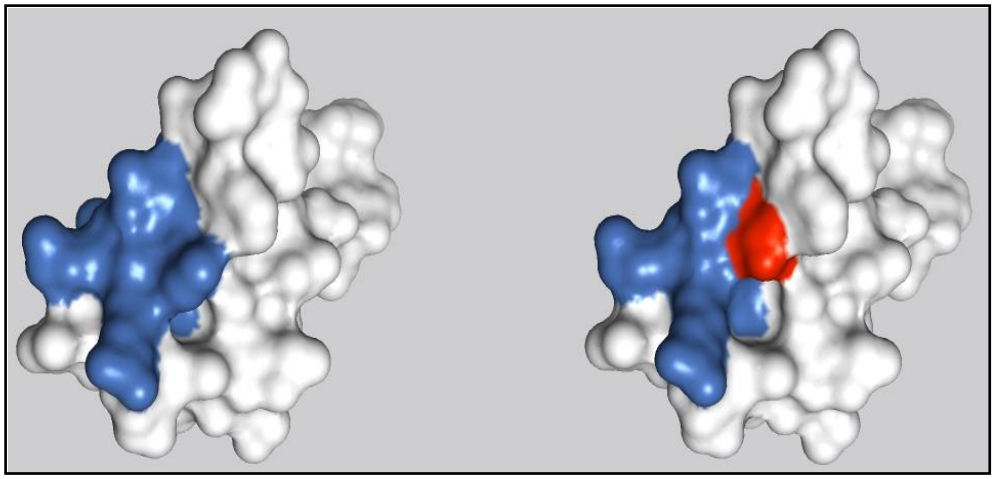
Gray colour shows the NS5B, HLA-B27 restricted epitope highlighted in blue and the red colour indicates the mutations.

Figure 3-24: Single mutation (ARMILTTHF) compared to wild type sequence



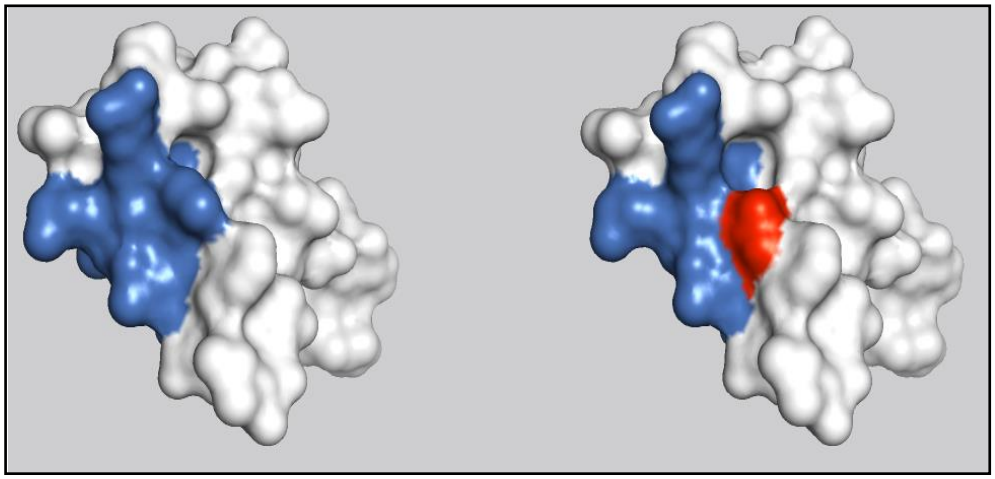
Gray colour shows the NS5B, HLA-B27 restricted epitope highlighted in blue and the red colour indicates the mutation.

Figure 3-25: Single mutation (ARMILVTHF) compared to wild type sequence



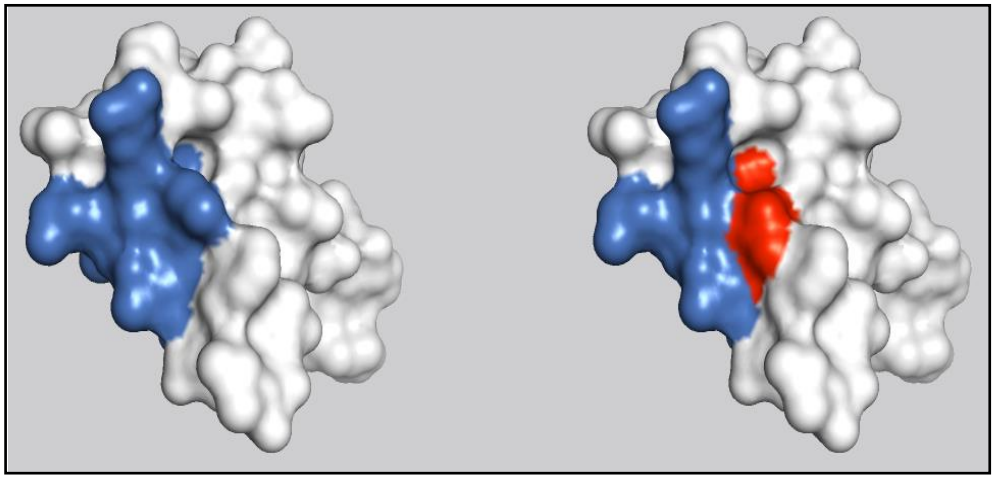
Gray colour shows the NS5B, HLA-B27 restricted epitope highlighted in blue and the red colour indicates the mutation.

Figure 3-26: Three mutations (VRMVLVTHF) compared to wild type sequence



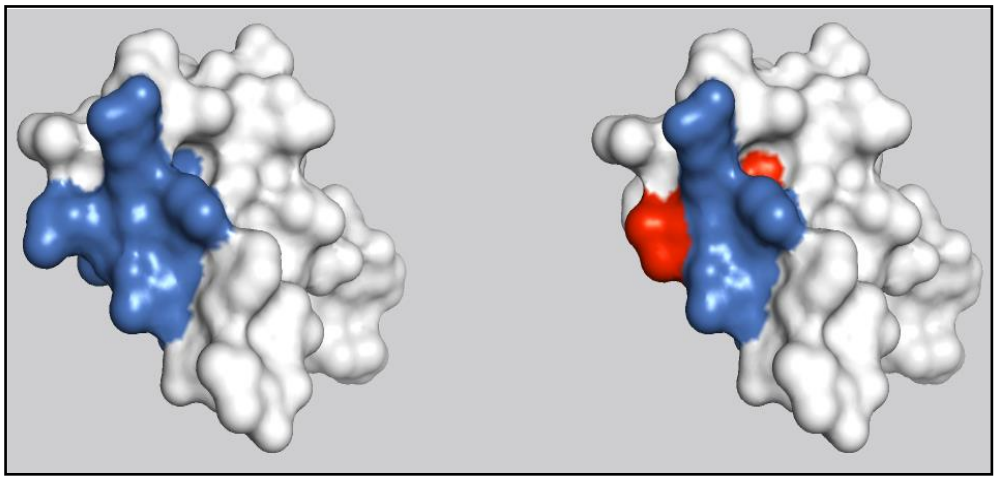
Gray colour shows the NS5B, HLA-B27 restricted epitope highlighted in blue and the red colour indicate the mutation.

Figure 3-27: Single mutation (AVMILMTHF) compared to wild type sequence



Gray colour shows the NS5B, HLA-B27 restricted epitope highlighted in blue and the red colour indicate the mutation.

Figure 3-28: Three mutations (ALWVRVILMTHFFSH) including a substitution outwith the epitope compared to wild type sequence



Gray colour shows the NS5B, HLA-B27 restricted epitope highlighted in blue and the red colour indicate the mutation.

3.1.13 Selection pressure across the HCV genome.

Selection pressure across the entire HCV genome was calculated using dN-dS ratios using Hyphy within MEGA 6.0. The relationship between non-synonymous substitutions per non-synonymous site (dN) and synonymous substitutions per synonymous site (dS) used to measure selective pressure in different regions of the HCV genome. Codon-based selection analysis *dN-dS* was calculated by group.

Initially, selection pressure was calculated between HLA-B27+ and HLA-B27- patients and then in HLA-B27+ spontaneous clearers HCV and progressors. Each region of the HCV genome was assessed for negative or positive selection pressure using Hyphy software. After aligning the sequences, codon-based selection pressure was assessed across the whole HCV genome.

Spontaneous clearer patients displayed largely negative selection pressure with no evidence of significant positive selection (Figure 3-29).This maybe attributed to the absence of a specific strong T cell immune repsonse although

small study numbers may limit the strength of the interpretation of these findings.

Surprisingly, patients who progressed to chronicity also showed no evidence of significant positive selection pressure using dN-dS ratios (Figure 3-30). Two patients (P28 and P45) were assessed individually over time for the likelihood of amino acid changing substitutions across the whole genome (these patients were selected because sequence data was available at multiple timepoints) but no significant positive selection pressure was detected (Figure 3-31 and Figure 3-32 This lack of statistical significance is likely to reflect the small sample size and clearer data may emerge following study of larger numbers of patients.

Finally, HLA-B27+ and HLA-B27- patients were compared. HLA-B27+ patients had evidence of higher positive selection pressure within codon 169 within the core region (labelled according to the H77 reference) (Figure 3-33) than HLA-B27- (negative) patients (Figure 3-34).

In conclusion, positive selection pressure was detected in HLA B27+ patients at a site previously undescribed as being associated with positive selection in HLA B27+ patients. Surprisingly, statistically significantly selected codons were not detected – this may be due to small numbers in the final analysis.

Figure 3-29: dN-dS ratio in HLA-B27+ spontaneous clearer patients

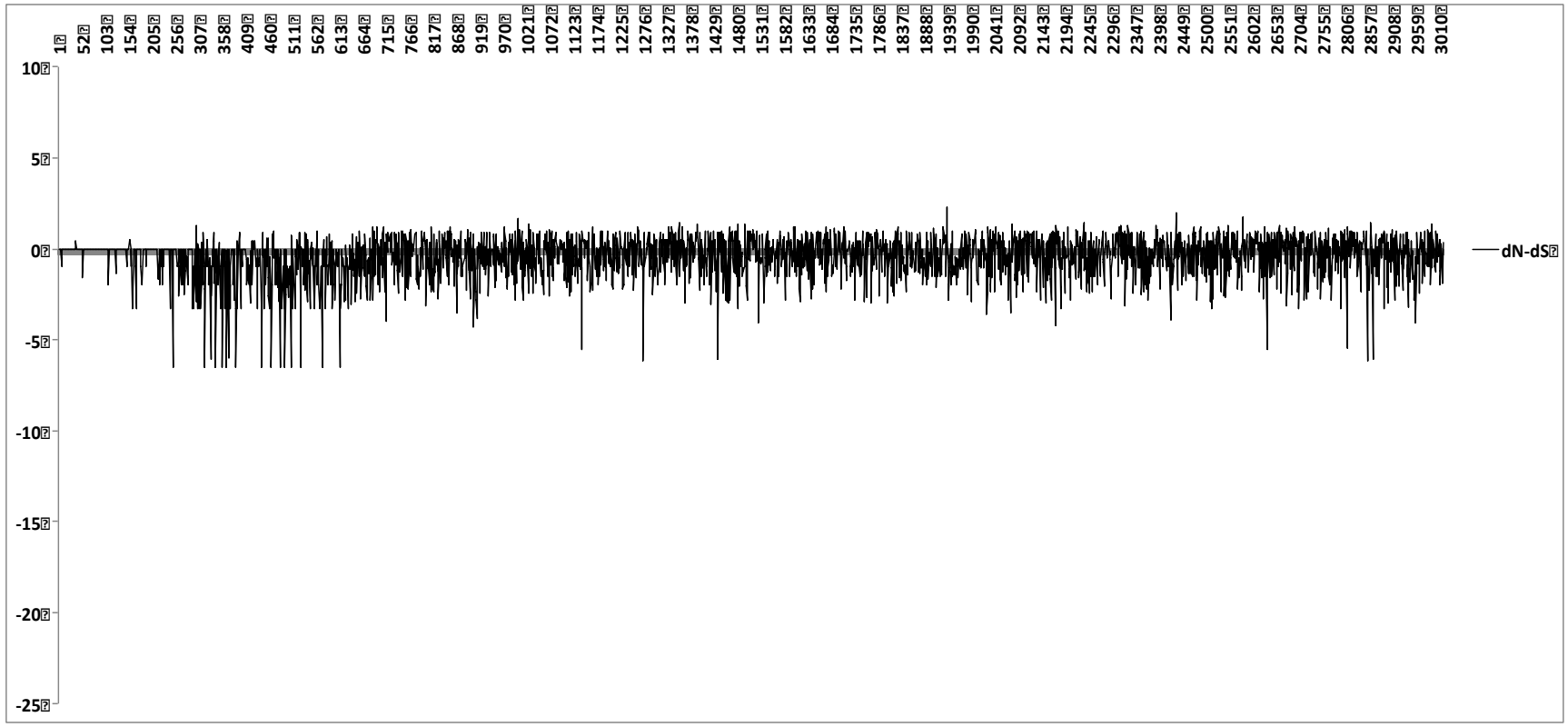


Figure 3-30: dN-dS ratio in HCV progressor patients

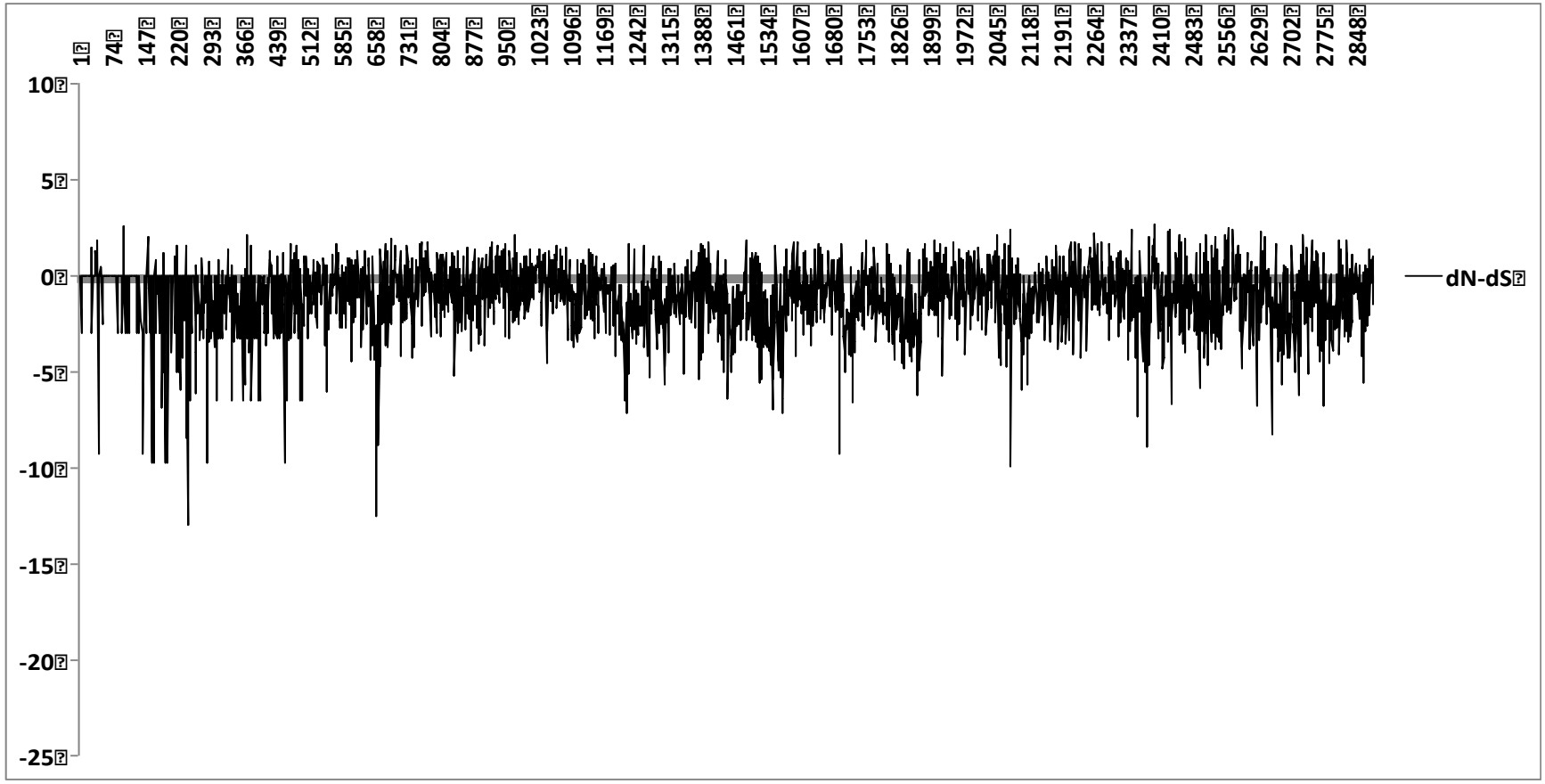


Figure 3-31: dN-dS ratio within progressor patient 28

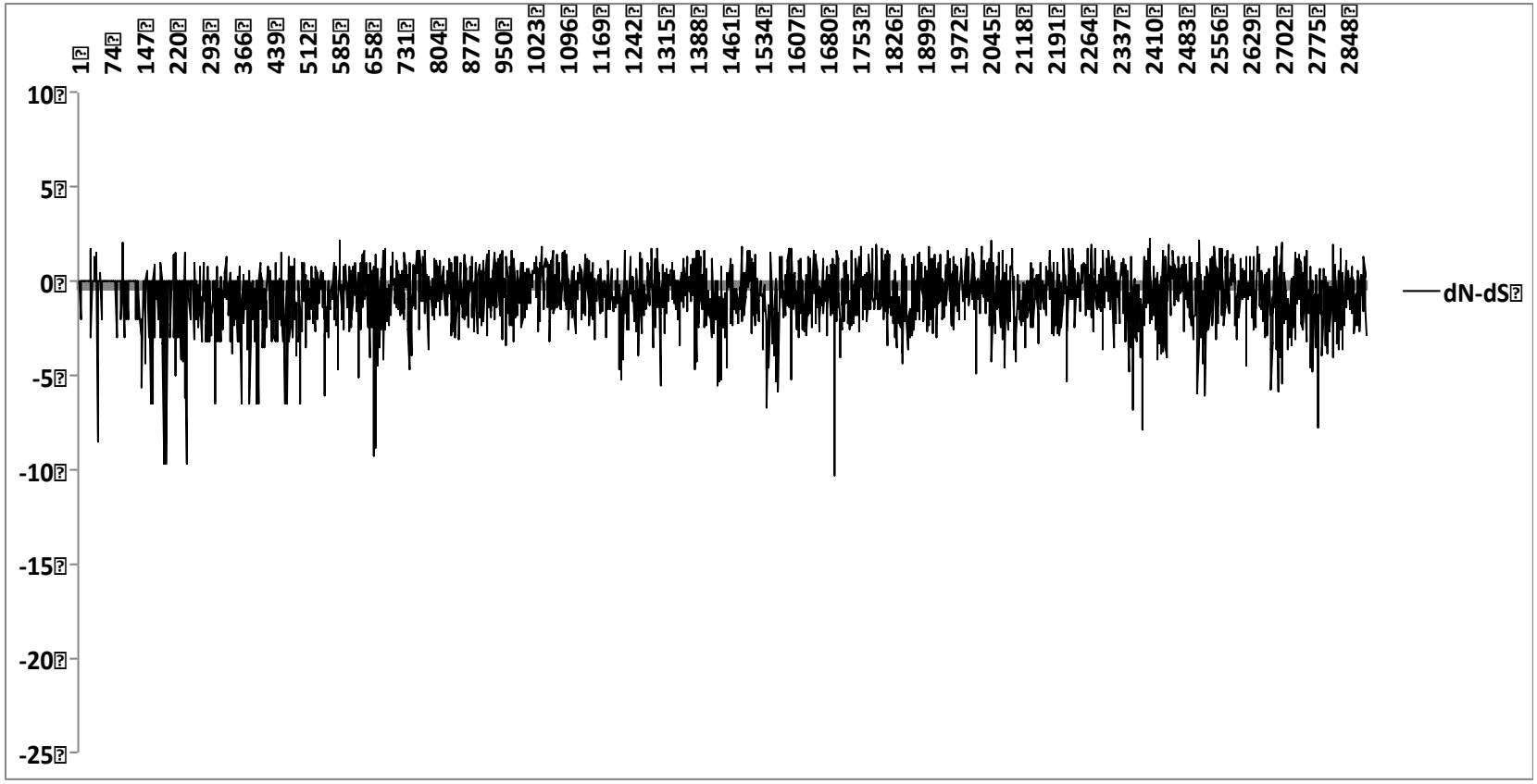


Figure 3-32: dN-dS ratio within the progressor patient 45

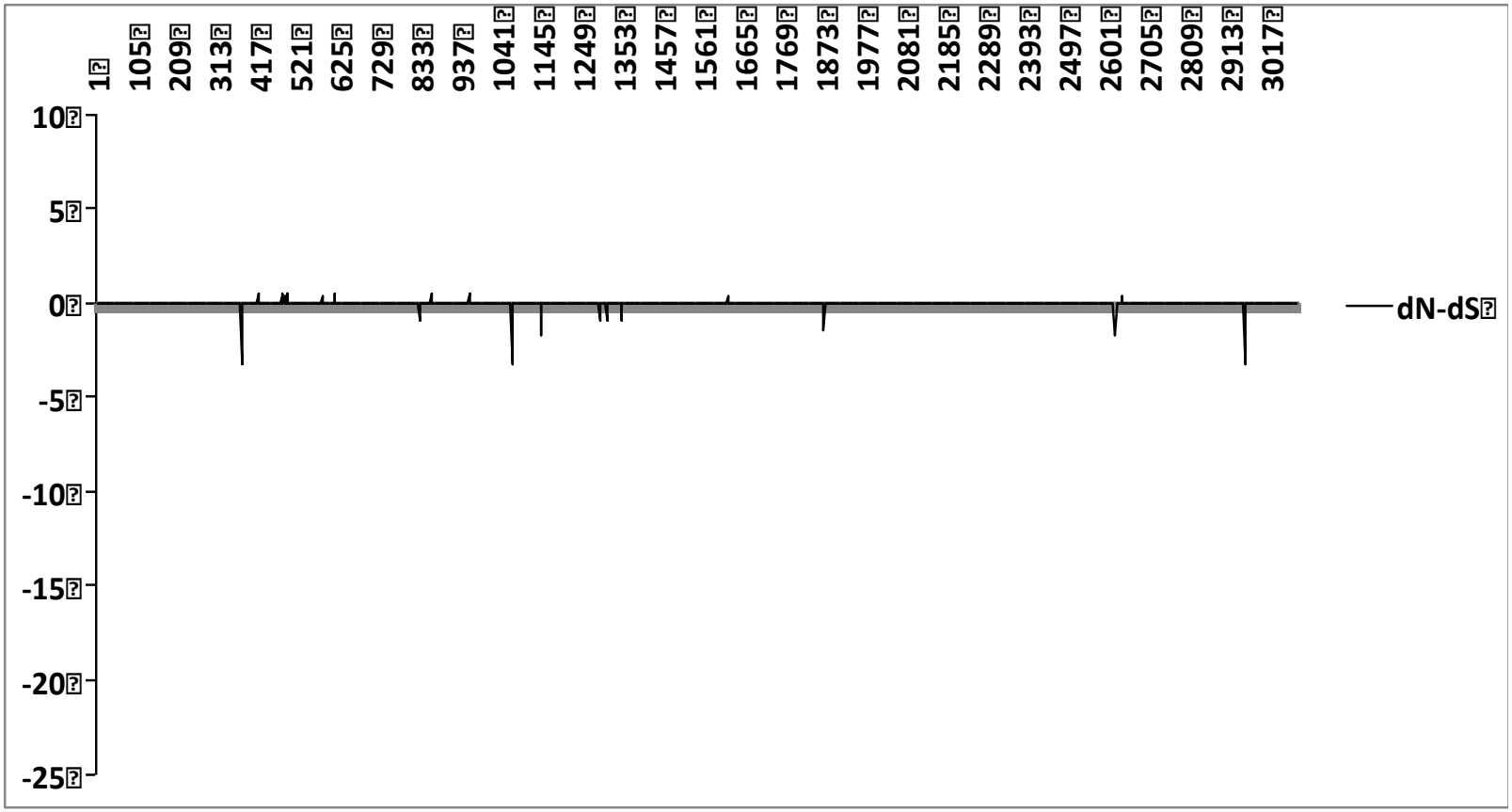
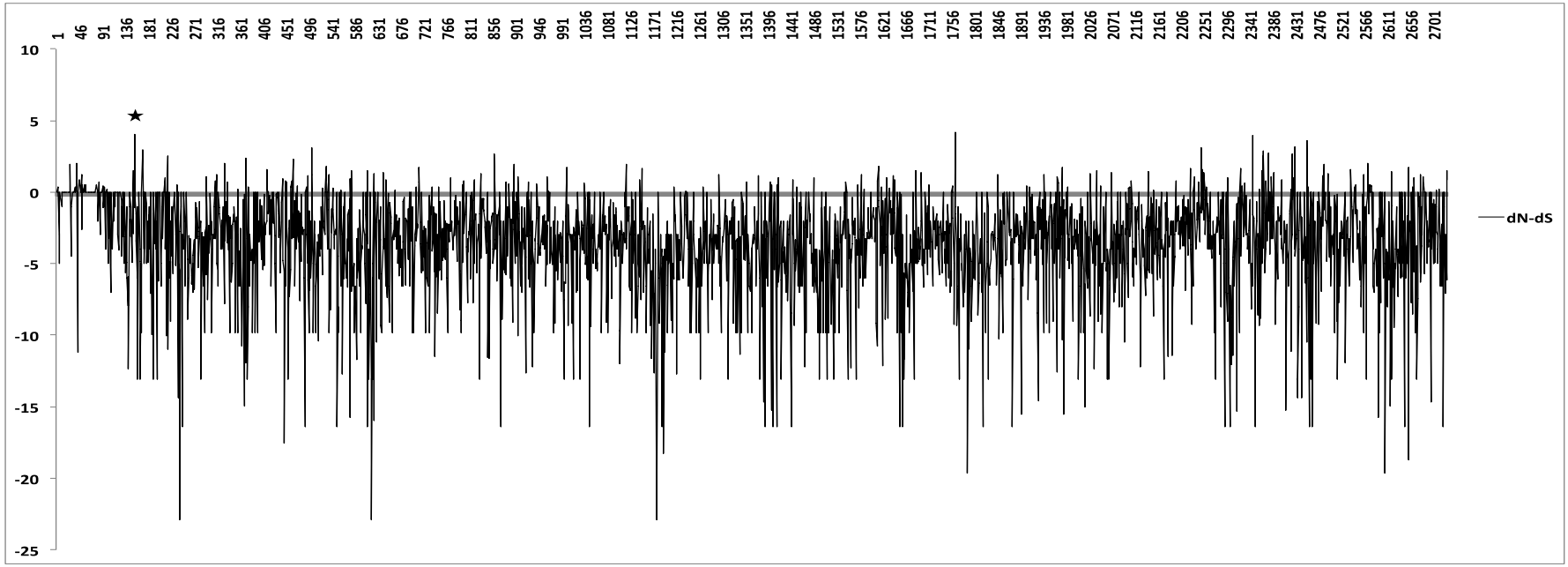
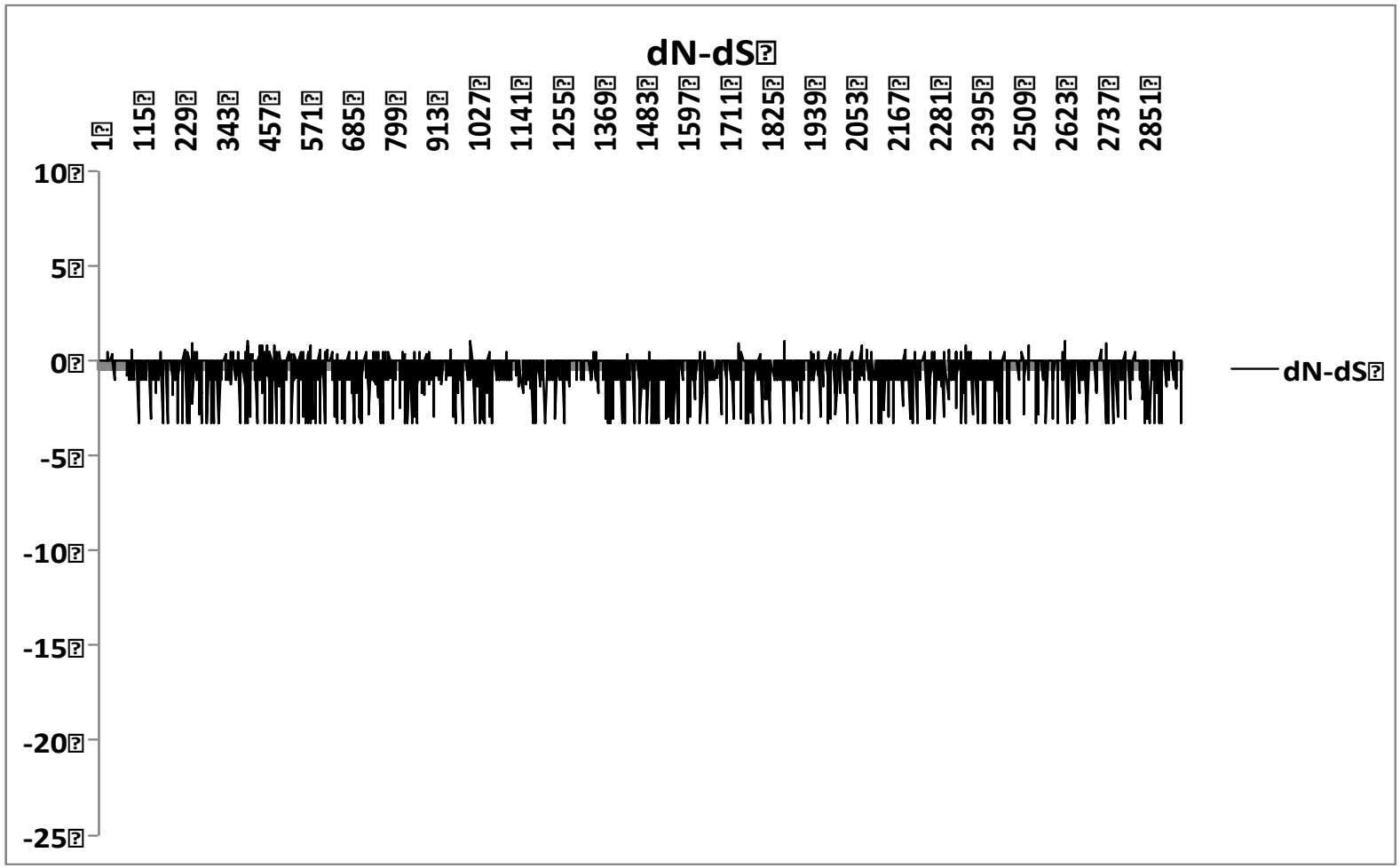


Figure 3-33: dN-dS ratio in all HLA-B27+ patients



dN-dS ratios indicate positive selection on codon 169 (leucine) within core (labelled according to H77 sequence)

Figure 3-34: dN-dS ratio in all HLA-B27 negative patients



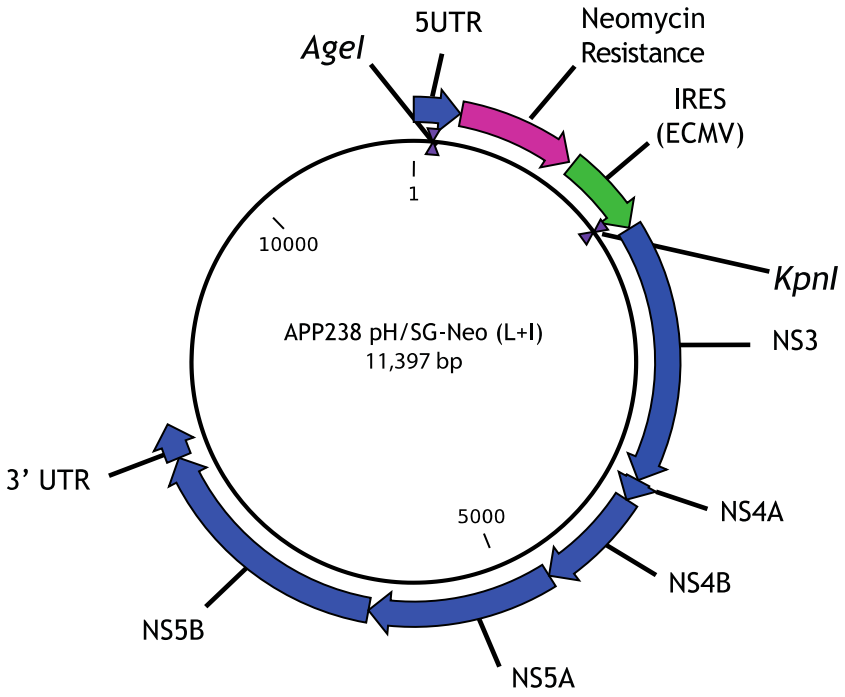
3.2 Functional analyses

3.2.1 Design of a genotype 1a replicon containing a luciferase reporter gene

3.2.1.1 **Introduction of adaptive mutations and a luciferase reporter gene into the genotype 1a replicon**

A genotype 1a sub-genomic replicon (APP238 PH/SG-neo (L-I)), kindly donated by C. Rice (Figure 3-35) was adapted by introducing two mutations within NS4A (K1691R) and NS4B (E1726G) using site directed mutagenesis and cloning. An intermediate universal vector pEGFP-C1 (Figure 3-38) was used as a backbone plasmid to introduce the firefly luciferase gene that was ligated into the genotype 1a sub-genomic replicon.

Figure 3-35: Original sub-genomic replicon HCV-1a



Original genotype 1a sub-genomic replicon (APP238 pH/SG-Neo (L+I) containing the neomycin gene. shows the locations of two single-cutter restriction sites. The restriction enzymes *AgeI* and *KpnI* were used to cut the replicon and introduce the luciferase gene.

3.2.2 Strategy for the construction of a replication-competent genotype 1a sub-genomic replicon.

The original genotype 1a sub-genomic replicon (App238 pH/SG-Neo (L+I)) was modified as follows. New adaptive mutations were introduced into the NS4A and NS4B (K1691R and E1726G) respectively, as well a firefly luciferase gene. This modification was carried out using site-directed mutagenesis and a standard cloning procedure. An intermediate plasmid pEGFP-C1 used to introduce the luciferase gene prior to transfer into the genotype 1a sub-genomic replicon (Figure 3-35). The genotype 1a replicon was used as backbone to introduce the mutations (A2841V, R2842V, M2843V, I2844V and T2838A) within the NS5B region to test the replicative capacity of a replicon containing mutations observed in the HLA B27+ cohort.

3.2.3 Introduction of a luciferase reporter gene into the genotype 1a sub-genomic replicon

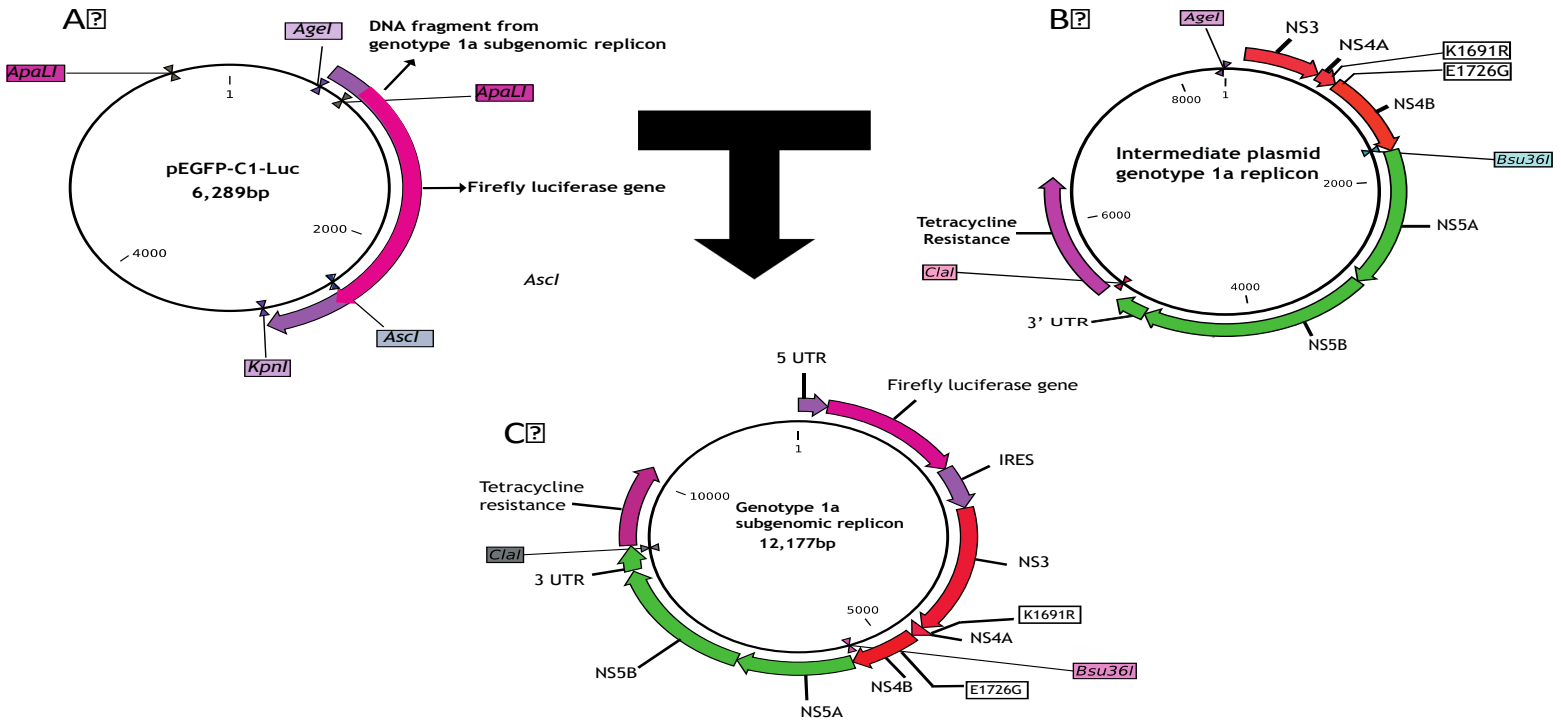
To construct a genotype 1a sub-genomic replicon (APP238 pH/SG-Neo (L+I)) containing the firefly luciferase gene, a fragment composed of the firefly luciferase gene was amplified from pSGR-JFH1 using forward and reverse primers 5`-*ApaLI*-luc : Gtgcac c ATG GAA GAC GCC AAA AAC, 3- luc(stop)-AscI: " GGC GGA AAG ATC GCC GTG TAA gGcgcgcc", to produce a DNA fragment with two restriction sites *ApaLI* and *AscI*, located within the 5` and 3`termini respectively. The length of this DNA fragment about 1.65 KB. These primers was designed using CLC genomic® work-bench version 6.5.1(CLC Bio-Qiagen, Aarhus, Denmark).

The genotype 1a sub-genomic replicon (APP238 pH/SG-Neo (L+I)) was digested using two restriction enzymes *AgeI* and *KpnI*. The new DNA fragment from this diagestion was ligated into intermediate plasmids (pEGFP-C1), this step allow to introduce the firelfly luciferase gene from pSGR-JFH1 into the DNA fragment as the new DNA fragment composed the sites of two restriction enzymes *ApaLI* and *AscI*.

Following introduction of the firelfy luciferase gene into the DNA fragment, the DNA was digested with the *AgeI-KpnI* again, and re-ligated into the original genotype 1a sub-genomic replicon. Next, genotype 1a sub-genomic replicon was used to transform 10-Beta *E.coli* cells. Successfully transformed colonies were then sequenced using Sanger sequencing, to confirm the successful ligation of firefly luciferase gene into the genotype 1a sub-genomic replicon.

The new two adaptive mutations within NS4A and NS4B (K1691R and E1726G) were introduced into the genotype 1a sub-genomic replicon using site-directed mutagenesis. Two restrictions enzymes was used to digest the genotype 1a sub-genomic replicon (*NsiI* and *Bsu36I*) (Figure 3-39), DNA fragment was sequenced using Sanger sequencing to confirm the successful introduction of these two adaptive mutations (Figure 3-40 and Figure 3-41).

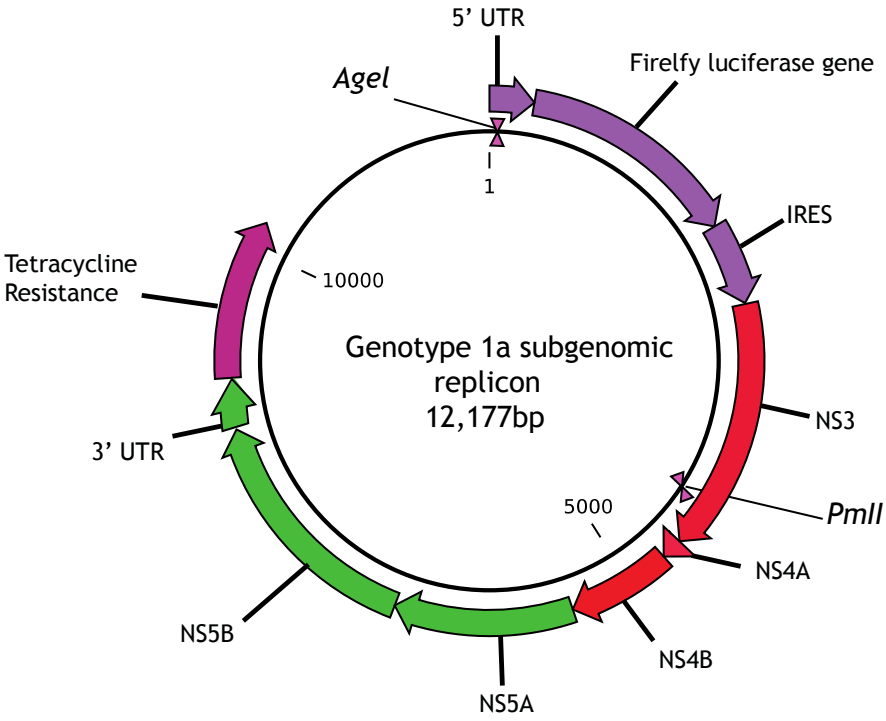
Figure 3-36: Proceedue used to introduce mutations into the genotype 1a replicon



A) pEGFP –C1-Luc used to introduced the lucifersase gene prior to transfer into an intermediate genotype 1a sub-genomic replicon. B) The original genotype 1a sub-genomic replicon C) The final construction of genotype 1a sub-genomic replicon containing the luciferase gene.

Shorter plasmids obtained from genotype 1a sub-genomic replicon were created by digesting the sub-genomic replicon with two restriction enzymes *AgeI* and *PmlI* resulting in a new plasmid (8.4 Kb). This new shorter genotype 1a sub-genomic replicon was used to introduce the mutations within the HLA-B27 restricted epitope within the NS5B region (A2841V, R2842V, M2843V, I2844V and T2838A). The shorter plasmids were used as the chance of error rate is lower during the sub-cloning steps.

Figure 3-37: Final Genotype 1a sub-genomic replicon

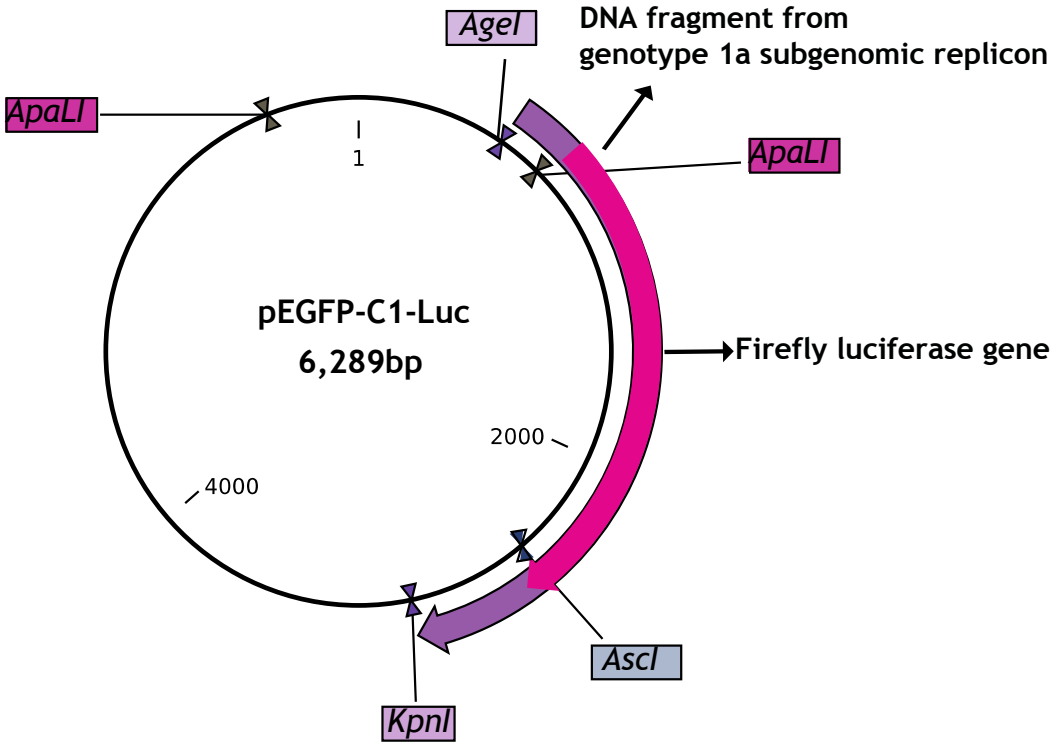


The figure shows the sub-genomic replicon containing the luciferase gene. Two restriction sites are highlighted that were used to cut genotype 1a sub-genomic replicon in order to create a smaller plasmid (8.4 Kb) that was used to introduce required mutations within the $NS5B_{2841-2849}$ restricted epitope. The smaller plasmid was then re-ligated into the main genotype 1a sub-genomic replcion.

3.2.4 Introduction of mutations within the $NS5B_{2841-2849}$ restricted epitope

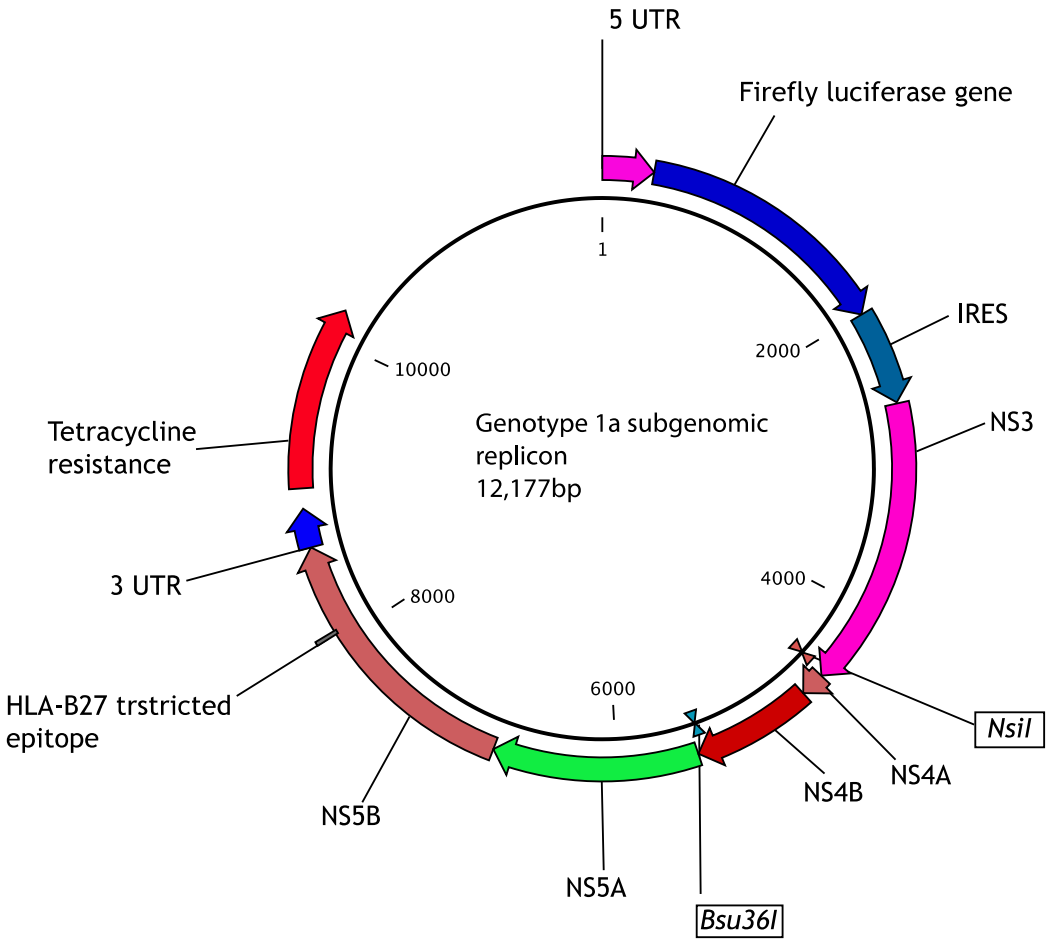
After constructing the genotype 1a sub-genomic replicon, several mutations within the $NS5B_{2841-2849}$ restricted epitope were introduced either single or sequentially, based on NGS detection of mutations from patients using site-direct mutagenesis.

Figure 3-38: Plasmid pGF-C1+Luc containing firefly luciferase gene



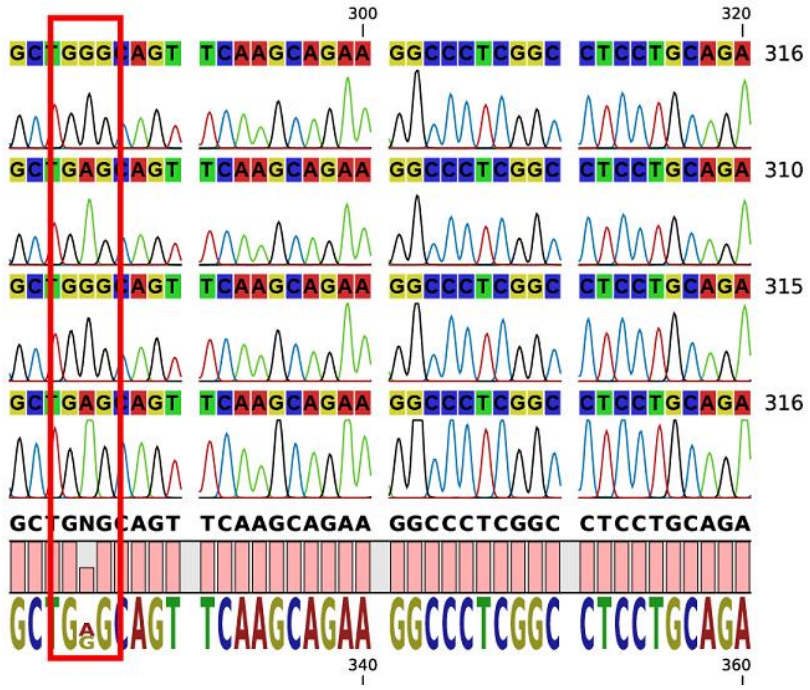
The pEGFP-C1+Luc plasmid (6.2 Kb) was used to introduce the firefly luciferase gene that used to measure luciferase signal to measure replication capacity in the genotype-1a sub-genomic replicon.

Figure 3-39: Genotype 1a sub-genomic replicon digested using *BSu36I* and *NsiI*



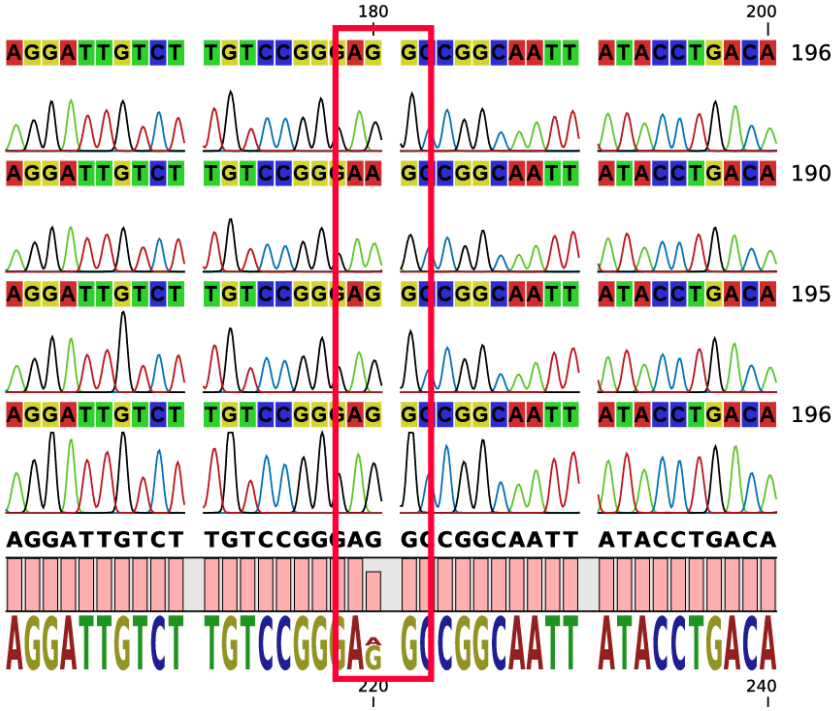
To introduce the two adaptive mutations within the NS4A and NS4B two restriction enzymes used to shorten the plasmid and introduce the mutations successfully.

Figure 3-40: Adaptive mutations within NS4A



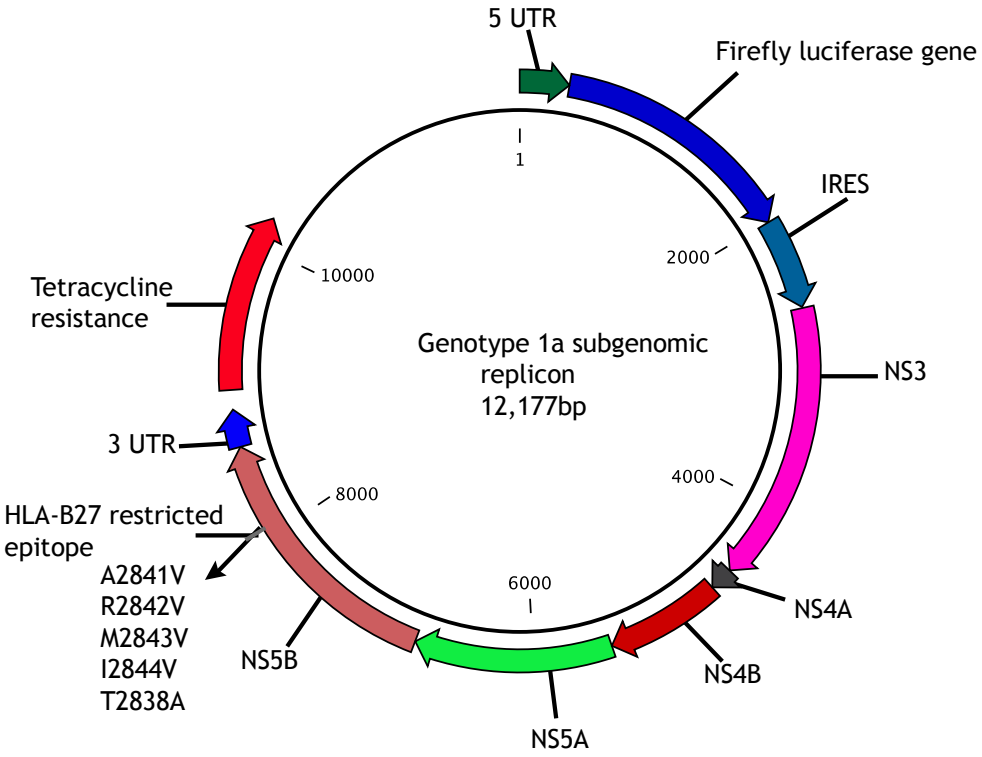
E1726R mutation illustrating the substitution of GAG to GGG

Figure 3-41: Adaptive mutation within NS4B



K1691R mutation illustrating the substitution of GAG to GGG

Figure 3-42: Final construction of the genotype 1a sub-genomic replicon



Genotype 1a sub-genomic replicon (12 Kb) containing mutations within the $NS5B_{2841-2849}$ restricted epitope.

3.2.5 Assessing the replication assay

To assess replication capacity following the introduction of specific mutations within the $NS5B_{2841-2849}$ epitope in the genotype 1a sub-genomic replicon, a transient replication assay was used. In this assay, sub-genomic replicon RNA was introduced into human hepatoma cells 7.5 (Huh-7.5) and replication was evaluated based on luciferase detection.

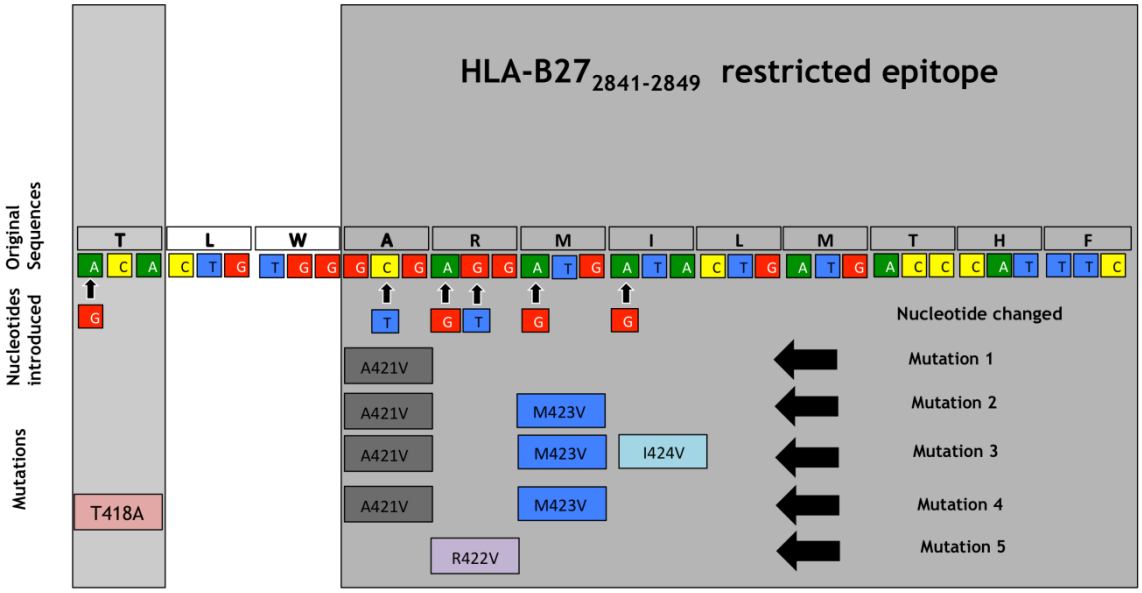
During the transient assay the number of Huh-7.5 cells was adjusted to 150,000 cells in each of the 24 wells. This standardisation was carried out to avoid cell confluence during incubation that could affect the outcome of the replication assay, particularly during later time points. For the genotype -1a sub-genomic replicon, luciferase activity initially decreased after the first time point but increased 48 hours following transfection. The luciferase signal from a genotype-2a pSGR-Luc-JFH1 was higher than the genotype 1a sub-genomic replicon by about 100 fold, as expected from previous reports in the literature. A pSGR-Luc-JFH1/GND replicon was used as a negative control.

In order to optimise the assay using the genotype 1a sub-genomic replicon, Huh 7.5 cells were transfected with varying concentrations of sub-genomic replicon-1a RNA (1, 2.5, 5 and 10 ug). Post-transfection, luciferase activity was measured within 72 hours to study the correlation between the amount of sub-genomic replicon-1a RNA inoculated and luciferase signal levels. Generally, within the first time point (4 h) the luciferase activity was higher as the amount of RNA inoculated increased. However, luciferase activity was the same at the end of the experiment regardless of the amount of RNA initially inoculated into the Huh-7.5 cells. Luciferase activity of the original sub-genomic replicon APP 238 pH/SG-Neo (L+I) was low and identical to the sub-genomic negative control encoding the GND mutation (SG-Luc-1a-GND).

3.2.6 Replicative fitness of mutations within the NS5B $_{2841\text{-}2849}$ restricted epitope

Mutations were introduced into the new sub-genomic replicon containing the luciferase gene in order to measure replication and to assess the effect of mutations that occur *in vivo* within patients from the acute HCV cohort. These mutations were introduced either as a single mutation such as A2841V and R2842V or sequentially such as A2841V/M2843V, A2841V/M2843V/I2844V and A2841V/M2843V/T2838A (Figure 3-43). During all the experiments undertaken, new controls for sub-genomic replicon luc-JFH1 and luc-JFH1-GND were included to validate the experiments.

Figure 3-43: Figures illustrating mutations introduced using site-directed mutagenesis.



Mutations within the ARMILMTHF NS5B$_{2841\text{-}2849}$ epitope selected during progression to chroncity were introduced into genotype 1a replicon system.

3.2.7 The effect of single and multiple clustered mutations on replication

Using results from next generation sequencing, mutations observed *in vivo* from patients who either spontaneously cleared HCV or progressed to chronicity within the highly conserved HLA-B27 epitope (NS5B $_{2841\text{-}2849}$) were introduced to the replicon system.

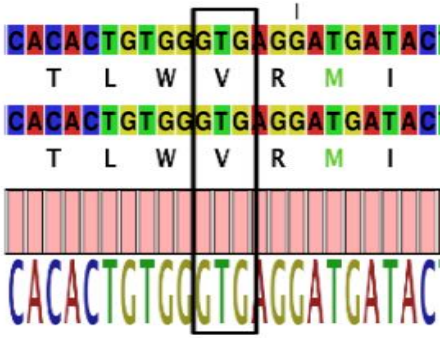
The impact of each mutation on viral replication (fitness cost) was assessed using the genotype 1a sub-genomic replicon as described above. The introduction of the commonly observed A2841V mutation resulted in a 7-fold reduction in replication (Figure 3-44). The R2842V mutation (an HLA B27 anchor site) also showed a 7-fold decline in luciferase activity compared to the sub-genomic replicon (Figure 3-45).

Next, double mutations were tested; the A2841V/M2843V replicon resulted in nine fold lower replication compared to the wild-type (WT) sub-genomic replicon (Figure 3-46).

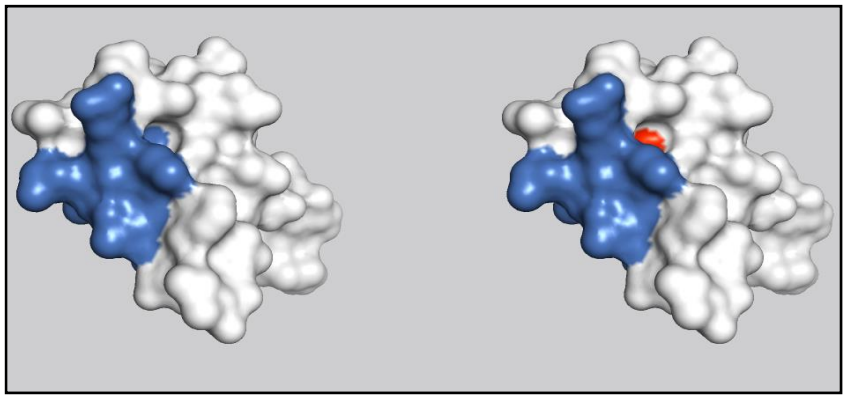
Patients with triple mutations at position I2844V or outside the HLA-B27 epitope T2838A showed a different response based on the mutation location. Triple mutations were introduced sequentially in addition to the other two mutations, for example, A2841V/M2843V/I2844V. Luciferase activity was measured and the replication capacity was increased compared with single or double mutation variants – at around 3-fold lower than WT replicon (Figure 3-47). A mutation (T2838A) commonly found outside the HLA-B27 epitope, was found to restore replication capacity to a similar level compared to the genotype 1a sub-genomic replicon (Figure 3-48).

Figure 3-44: A2841V mutation introduced into the genotype 1a sub-genomic replicon.

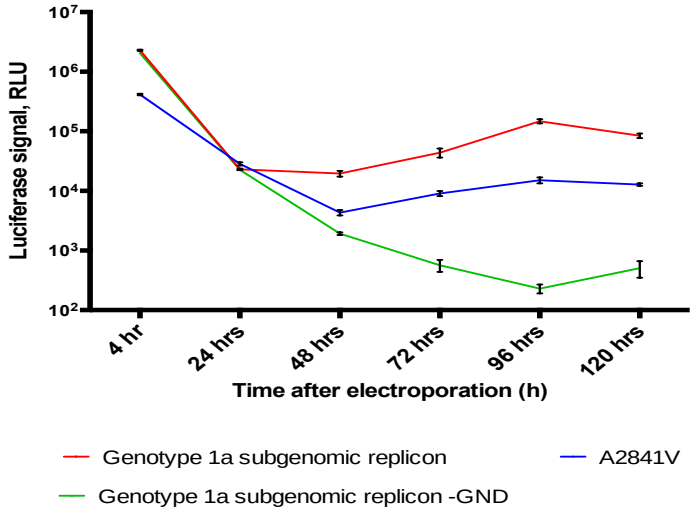
A



B



C



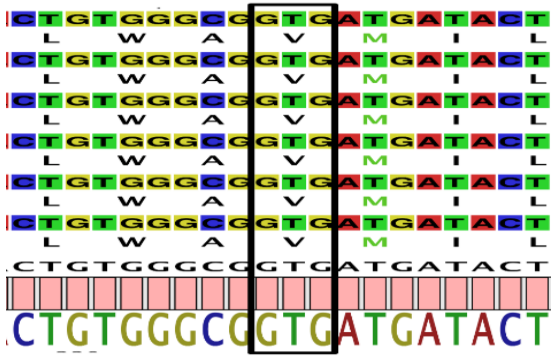
D

Time	WT (median)	A2841V (median)	P value
24 hrs.	22851	27527	0.0495
48 hrs.	19967	4087	0.0495
72 hrs.	48201	9081	0.5127
96 hrs.	153435	14195	0.0495
120 hrs.	88462	12445	0.1266

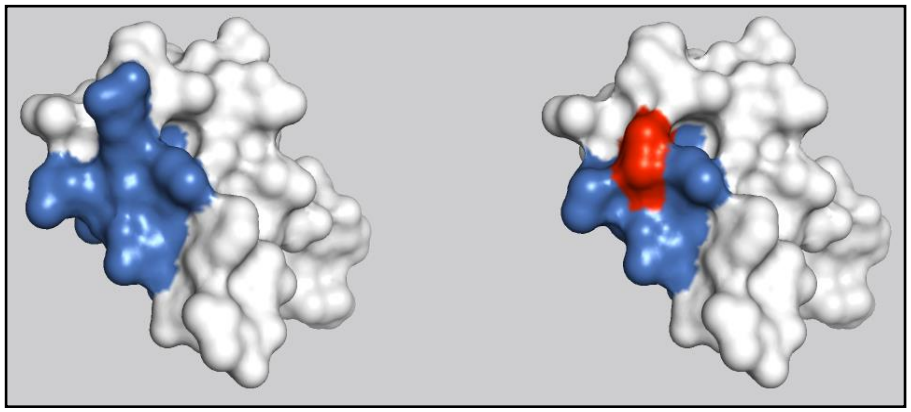
A) A single mutation (A2841V) was successfully introduced and is highlighted with a black rectangle. B) The A2841V mutation had minimal effect on predicted NS5B structure. C) RNA from the genotype 1a sub-genomic replicon containing a single mutation (A2841V) was electroporated into Huh7.5 cells. Huh7.5 were lysed at 6 time points (4, 24, 48, 72, 96 and 120 h). Post-electroporation the luciferase signal activity was measured. All assays were done in triplicate and the average value is shown. D) p value calculated using the Mann Whitney U test.

Figure 3-45: R2842V mutation introduced into the genotype 1a sub-genomic replicon.

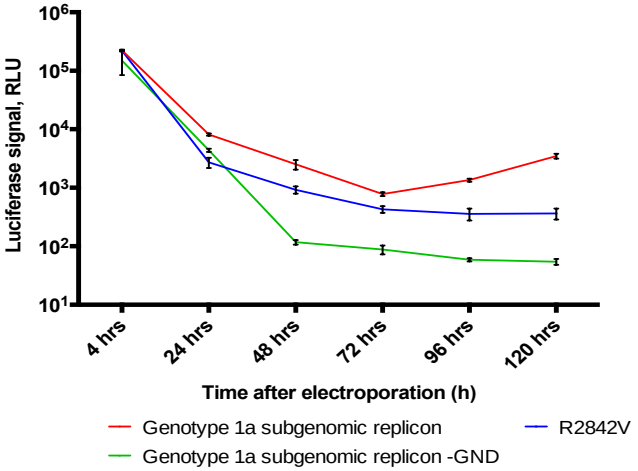
A



B



C



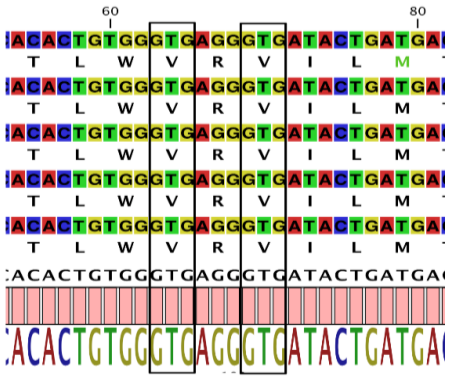
D

Time	WT (median)	R2842V (median)	P value
24 hrs.	7920	2463	0.0495
48 hrs.	2722	953	0.0495
72 hrs.	755	423	0.0495
96 hrs.	1334	314	0.0495
120 hrs.	3291	366	0.0495

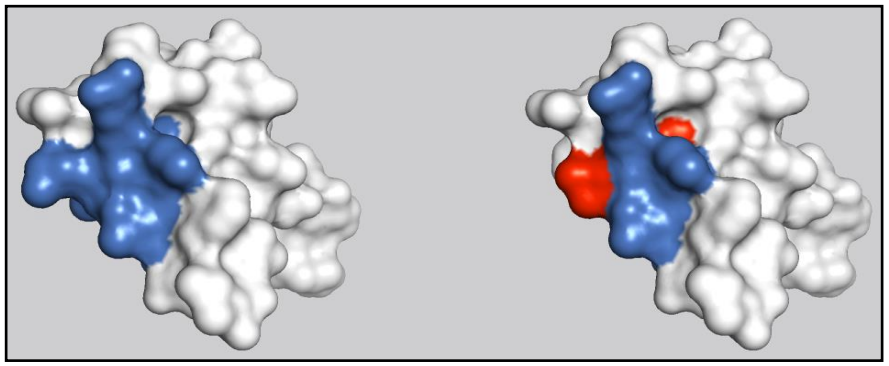
A) Single mutation (R2842V) successfully introduced and highlighted with a black rectangle. B) The R2842V mutation appeared to cause a substantial change in NS5B structure due to the substitution of arginine with a large side chain structure to valine. C) RNA from the genotype 1a sub-genomic replicon containing a single mutation (R2842V) was electroporated into Huh7.5 cells. Huh7.5 were lysed at 6 time points (4, 24, 48, 72, 96 and 120 h). Post-electroporation the luciferase signa activity was measured. All assays were done in triplicate and the average value is shown. D) p value was calculated using the Mann Whitney U test.

Figure 3-46: A2841V/M2843V mutations introduced into the genotype 1a sub-genomic replicon.

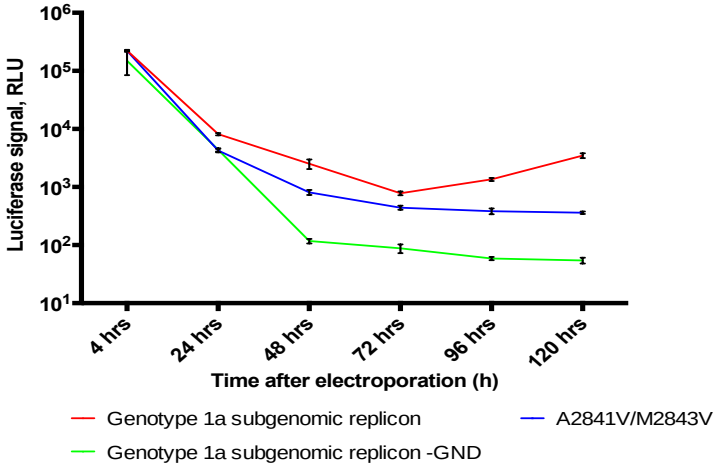
A



B



C



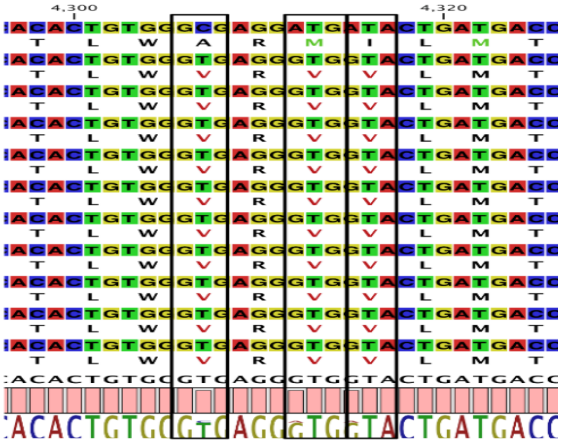
D

Time	WT (median)	A2841V/M2843V (median)	P value
24 hrs.	7920	4137	0.0495
48 hrs.	2722	849	0.0495
72 hrs.	755	419	0.0495
96 hrs.	1334	346	0.0495
120 hrs.	3291	366	0.0495

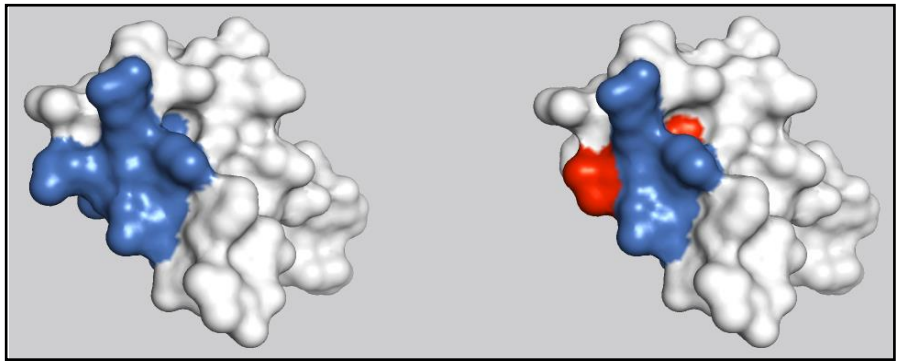
A) Double mutations (A2841V/M2843V) were successfully introduced and highlighted with a black rectangle. B) The A2841V/M2843Vmutation appeared to significantly affect NS5B structure. C) RNA from the genotype 1a sub-genomic replicon containing the double mutation (A2841V/M2843V) was electroporated into Huh7.5 cells. Huh7.5 were lysed at 6 time points (4, 24, 48, 72, 96 and 120 h). Post-electroporation luciferase signal activity was measured. All assays were done in triplicate and the average value is shown. D) p values were calculated using the Mann Whitney U test.

Figure 3-47: A2841V, M2843V and I2844V mutations introduced into the genotype 1a sub-genomic replicon.

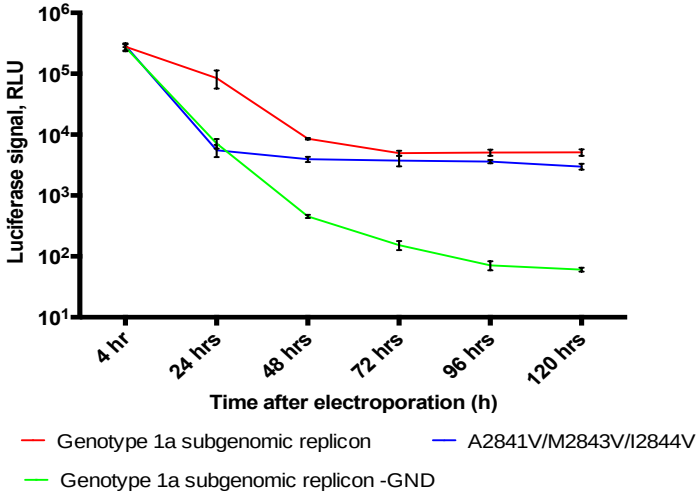
A



B



C



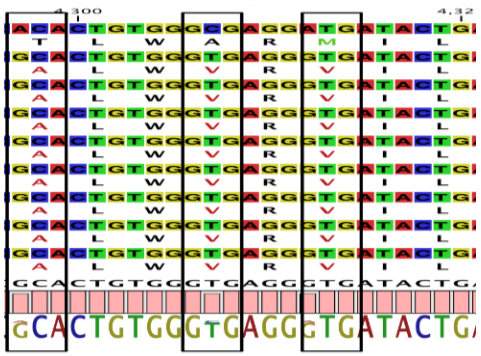
D

Time	WT (median)	A2841V/M2843V/I2844V (median)	P value
24 hrs.	89161	5531	0.0495
48 hrs.	8632	4095	0.0495
72 hrs.	4961	3482	0.1266
96 hrs.	5072	3672	0.0495
120 hrs.	5105	2812	0.0495

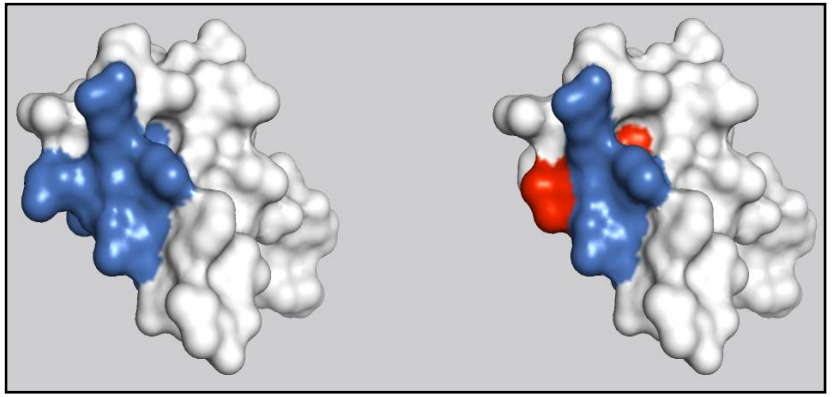
A) A triple mutation (A2841V/M2843V/I2844V) was successfully introduced and is highlighted with a black rectangle. B) Effect of A2841V/M2843V/I2843V mutations on NS5B structure; the triple mutation appeared to have only a minimal effect on predicted structure. C) RNA from the genotype 1a sub-genomic replicon containing multiple mutations (A2841V/M2843V/I414V) was electroporated into Huh7.5 cells. Huh7.5 were lysed at 6 time points (4, 24, 48, 72, 96 and 120 h). Post-electroporation the luciferase signal activity was measured. All assays were done in triplicate and the average value is shown. D) p values were calculated using the Mann Whitney U test.

Figure 3-48: A2841V, M2843V and T418V mutations introduced into the genotype 1a sub-genomic replicon.

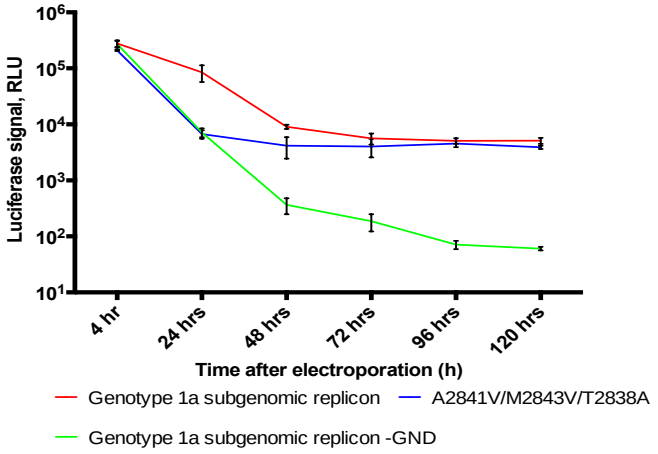
A



B



C



D

Time	WT (median)	A2841V/M2842V/T2838A (median)	P value
24 hrs.	89161	7286	0.0495
48 hrs.	8632	5132	0.0495
72 hrs.	5443	4649	0.8273
96 hrs.	5072	4285	0.2752
120 hrs.	5105	3986	0.8273

A) The triple mutation A2841V/M2843V/T418V was successfully introduced and highlighted with a black rectangle. B) The A2841V/M2843V/T2838A mutation appeared to have only a minimal effect on predicted NS5B structure. C) RNA from the genotype 1a sub-genomic replicon containing multiple mutations (A2841V/M2843V/T2838A) was electroporated into Huh7.5 cells. Huh7.5 were lysed at 6 time points (4, 24, 48, 72, 96 and 120 h). Post-electroporation the luciferase signal activity was measured. All assays were done in triplicate and the average value is shown. D) p values were calculated using the Mann Whitney U test.

In summary, the predicted structure of the triple mutants observed in patients that progressed to chronicity appeared to have been restored to be close to wild-type NS5B – this was associated with restoration of replication.

3.3 Detecting Virus Specific CD8+ T-cell responses using ELISpot

3.3.1 Subject characteristics

Fourteen patients were tested in this study using ELISpot assay technology. The cohort was divided into several groups based on their characteristics; HLA-B27+ patients who cleared HCV spontaneously (n=5) and HLA-B27+ patients who progressed to chronicity (n=4). A comparison was also made between HLA-B27+ patients (n=9) and HLA-B27- patients (n=5) and between co-infected patients (HIV/HCV) (n=9) and mono-infected patients (HCV) (n=5). Finally HLA-B27+ patients co-infected (HIV/HCV) (n=7) and HLA-B27+ mono-infected (HCV) (n=2) responses were compared. To determine the presence of specific T cell response, PBMCs were isolated, and cells were stimulated using HCV peptide pools spanning the whole HCV genome. Responses were compared using a Mann Whitney U test; data with p values less than or equal to 0.05 were considered to be statistically significant. A "broad" T cell response was defined as a response to 3 or more peptide pools while a "strong" response was considered to be a response of greater than 20 SFU/10^6 cells.

To determine the presence of specific T cell response, PBMCs were isolated, and cells were stimulated using HCV peptide pools spanning the whole HCV genome.

3.3.1.1 **Measuring production of IFN-γ in HLA-B27+ spontaneous clearers and progressors**

IFN-γ ELISPot assays were used to determine the production of IFN-γ-following stimulation of PBMCs with peptide pools supplied by BEI Resources and those designed in-house, based on prediction algorithm.

Responses were compared in HLA-B27+ patients who spontaneously cleared the HCV infection or progressed to chronicity. Significantly higher responses were found in spontaneous clearers than progressors (p=0.001). Multiple HCV peptide

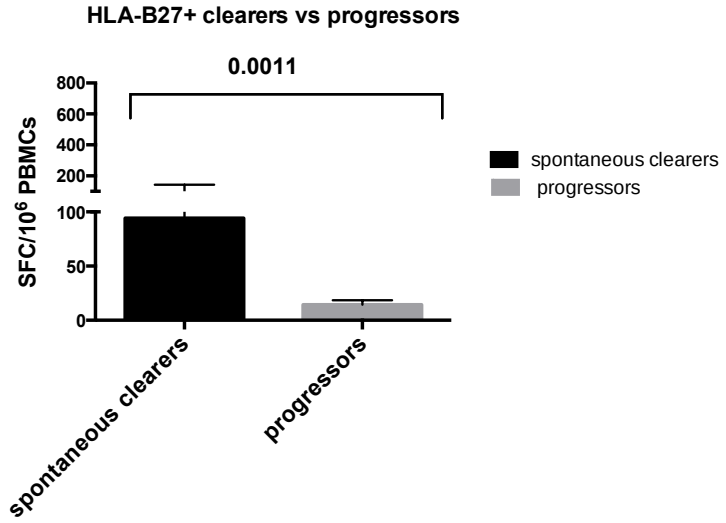
pools were significantly selected indicating a broad multi-specific response in spontaneous clearers (Figure 3-49).

3.3.1.2 **T cell responses in HLA-B27+ and HLA-B27- patients.**

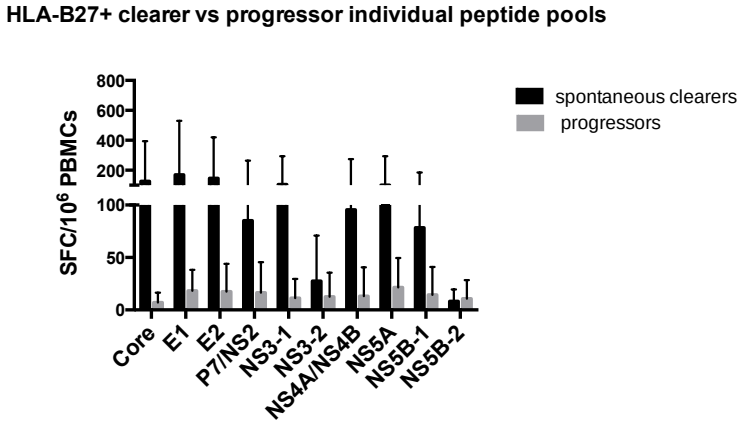
IFN-γ ELISpot production was higher in HLA-B27+ versus HLA B27- patients. Analysis of using the predicted peptide panel showed no significant difference in responses between groups (Figure 3-50).

Figure 3-49: IFN-γ production in HLA-B27+ spontaneous clearers and progressors

A



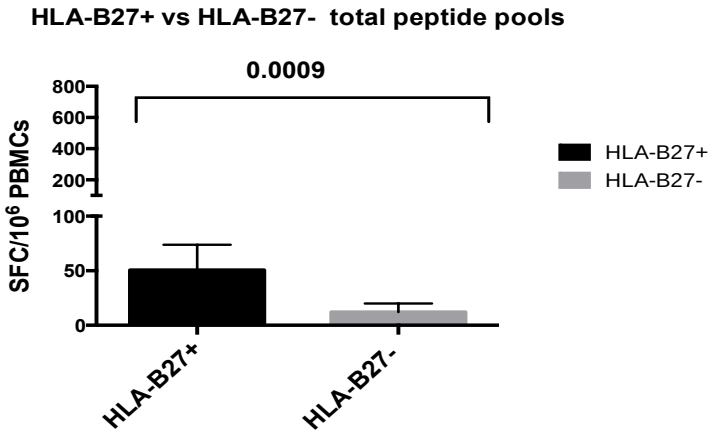
B

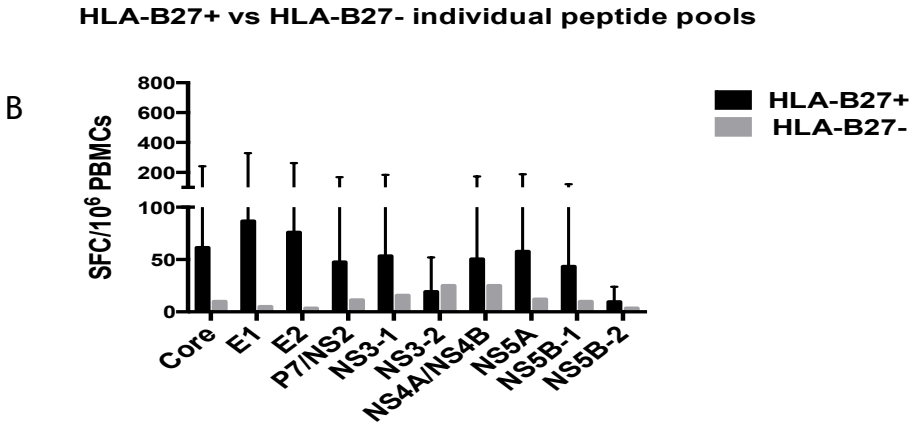


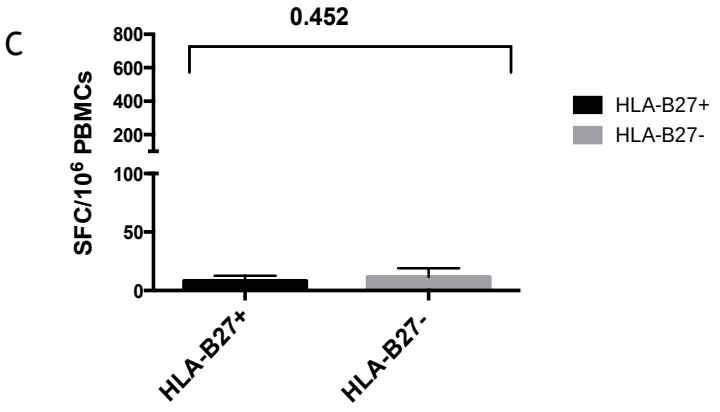
A) Total T cell responses was stronger in HLA-B27+ spontaneous clearers versus progressors (p=0.001) B) Total T cell responses to individual peptide pools.

Figure 3-50: IFN-γ production in HLA B27+ versus HLA B27- patients

A







A) Total T cell response using total peptide pools. B) Total T cell response to different individual peptide pools. C) Total T cell response between HLA-B27+ and HLA-B27- patients using predicted peptides designed in-house.

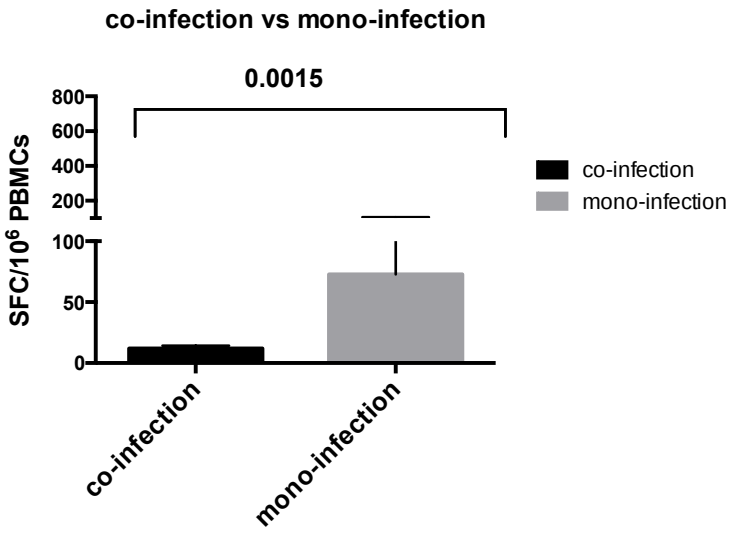
3.3.2 T- cell response in HIV/HCV co-infected and HCV mono-infected patients

IFN-γ measurement was carried out in patients (including HLA B27- control patients) with co-infection (HIV/HCV) and with monoinfection (HCV). Patients with monoinfection had higher specific T-cell responses than patients with co-infection ($p=0.0015$)(Figure 3-51).

The same comparison was made between co-infected and mono-infected HLA B27+ groups with acute HCV infection. Patients with monoinfection had higher responses ($p=0.0014$) (Figure 3-52).

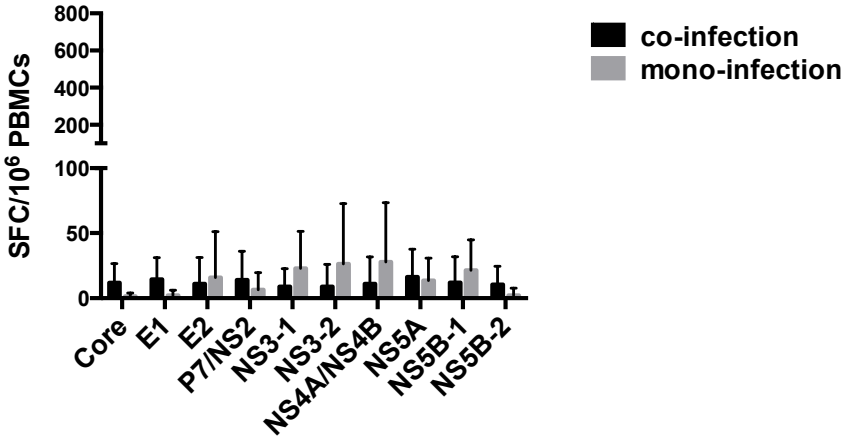
Figure 3-51: Total responses in HIV/HCV co-infected and HCV mono-infected patients

A



Individual peptide pool responses in HIV/HCV co-infected and HCV mono-infected patients

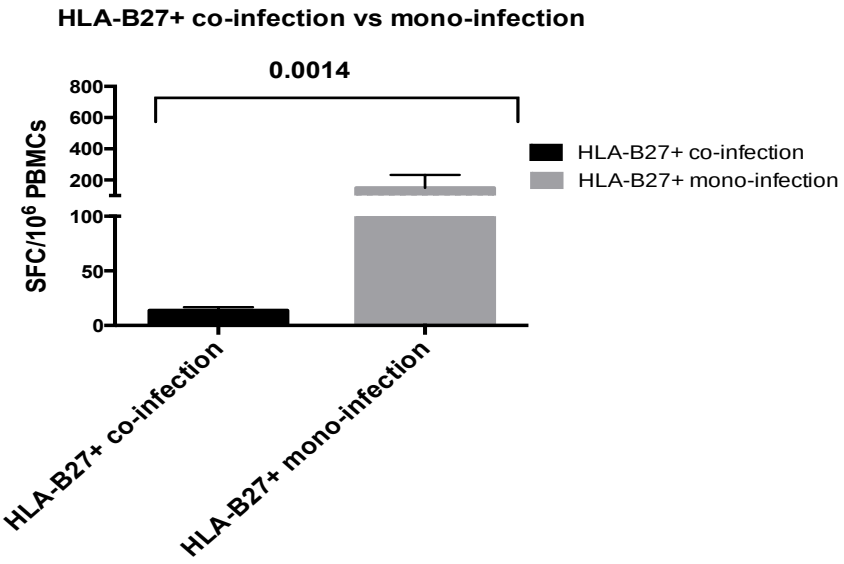
B



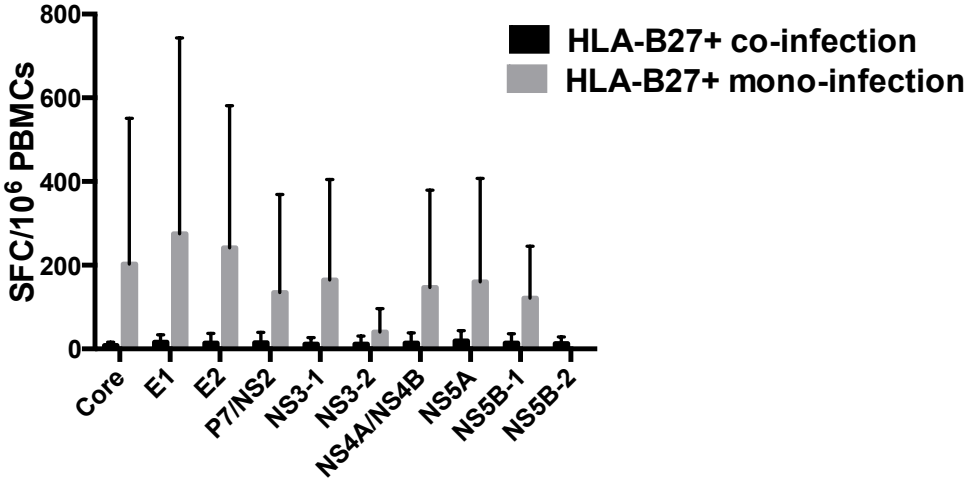
IFN-γ responses measured by ELISpot in co-infected and mono-infected patients are shown A) Reponses in HLA-B27+ patients. B) Responses measured using different peptides pools for each protein

Figure 3-52: Responses in HLA-B27+ co-infected and mono-infected patients

A



B



IFN-γ responses measured by ELISpot in HLA B27+ co-infected and mono-infected patients A) Responses in HLA-B27+ co-infected and mono-infected patients. B) Responses measured using different peptides pools for each protein

3.3.3 Individual patient responses

Individual responses in all HLA-B27+ patients were tested are shown below.

Figure 3-53: IFN-γ responses measured by ELISpot in G5 (clearer)

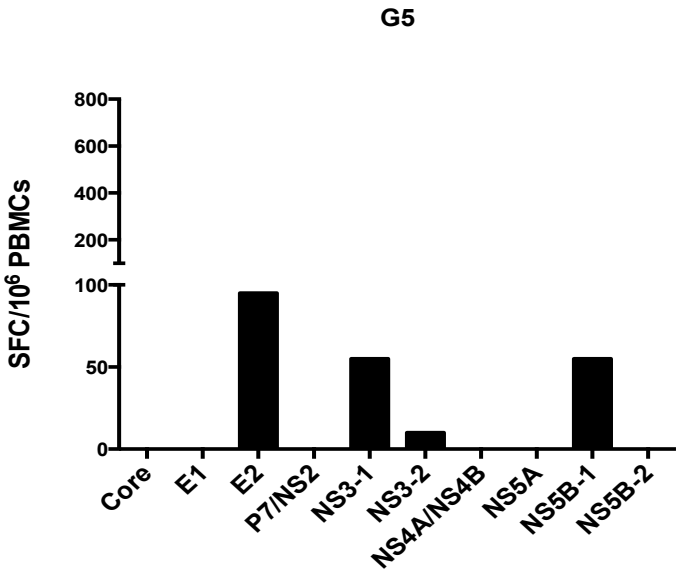


Figure 3-54: IFN-γ responses measured by ELISpot in G17 (clearer)

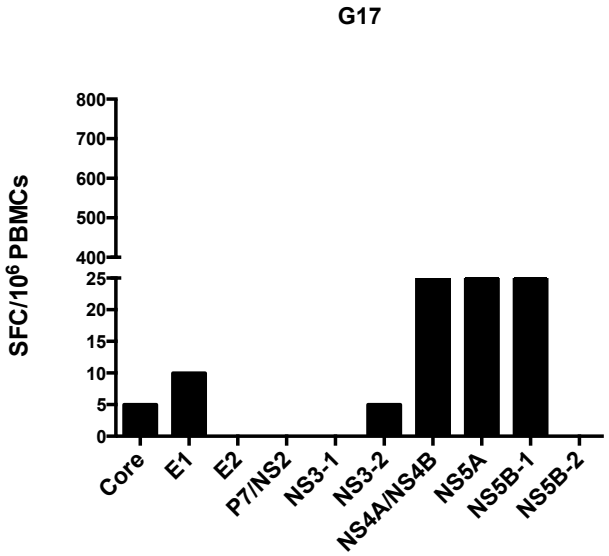


Figure 3-55: IFN-γ responses measured by ELISpot in G18 (clearer)

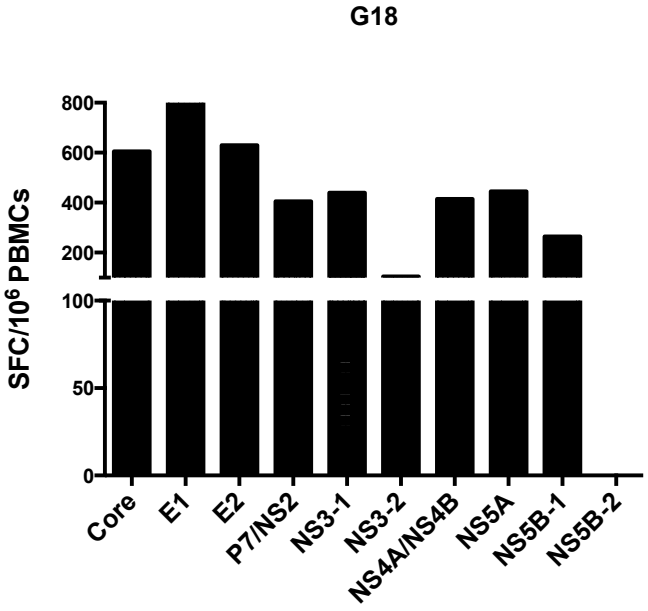


Figure 3-56: IFN-γ responses measured by ELISpot in P49 (clearer)

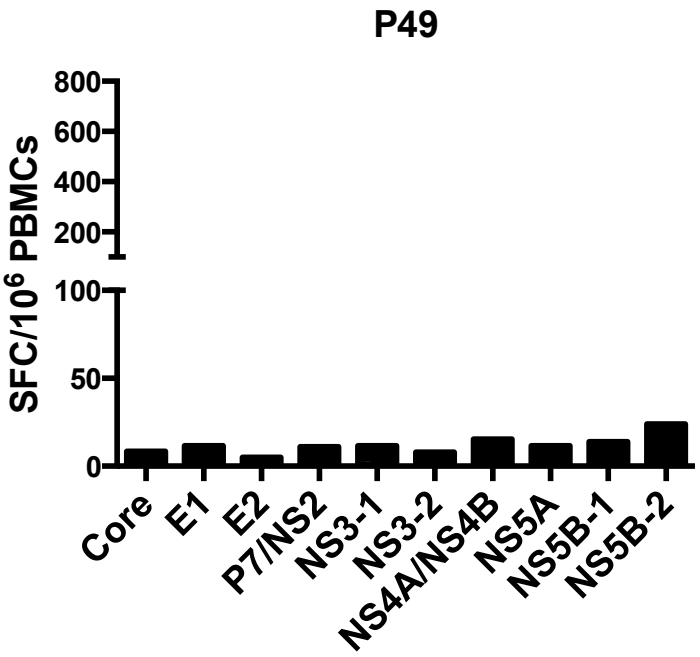


Figure 3-57: IFN-γ responses measured by ELISpot in P113 (progressor)

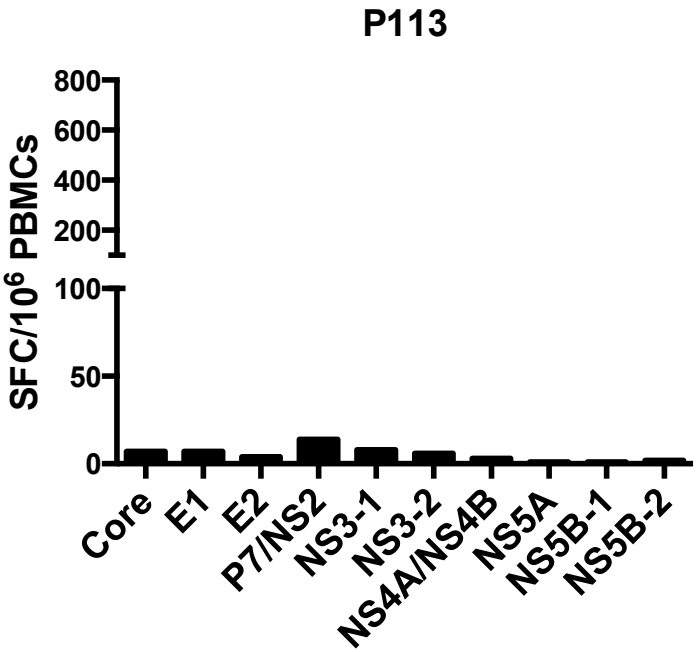


Figure 3-58: IFN-γ responses measured by ELISpot in P28 (progressor)

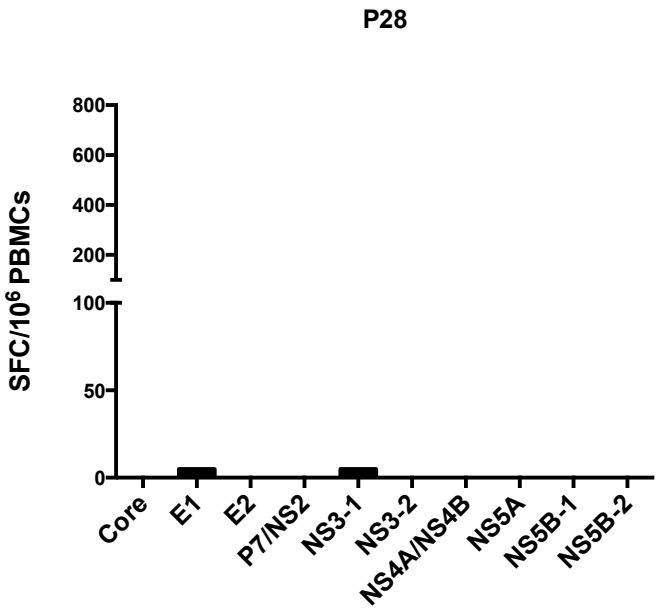
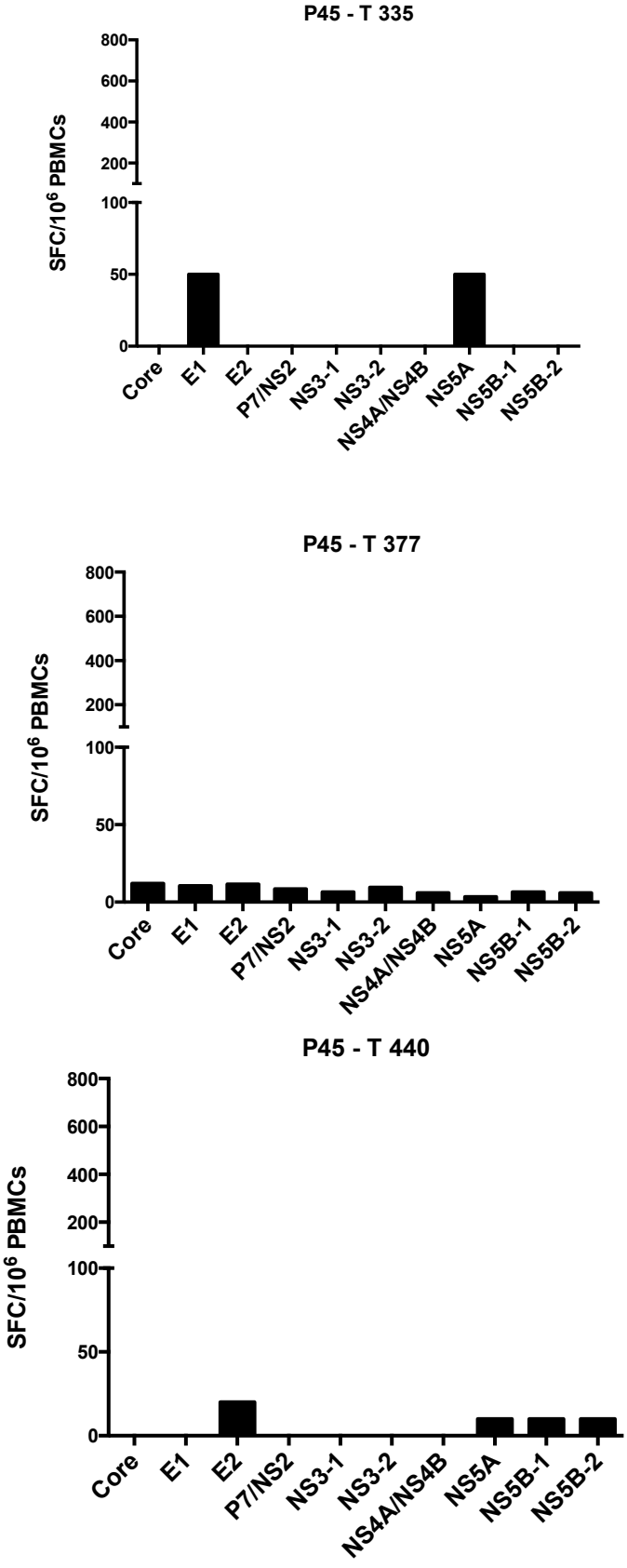


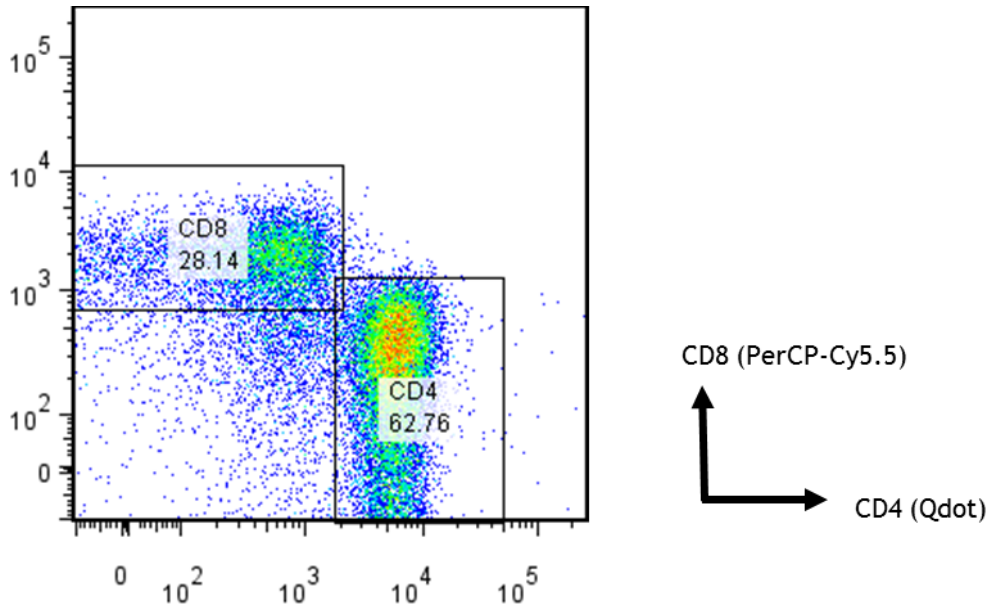
Figure 3-59: IFN-γ responses measured by ELISpot in P45 at different time points (progressor)



3.3.4 Flow cytometry

Flow cytometry was carried out in order to examine the phenotype of the T cell response in representative patients with either spontaneous clearance or progression to chronicity. We used CCR7 and CD45RA expression to divide cells into memory (effector and central), naive and effector populations. Sufficient cells to carry out flow cytometry experiments were available from single representative patients only. Samples from P28 (progressor) and G5 (clearer) were used to examine differences in phenotype in these patients. Samples were gated by lymphocyte population, CD3+ cells and CD4+ or CD8+ populations (Figure 3-60)

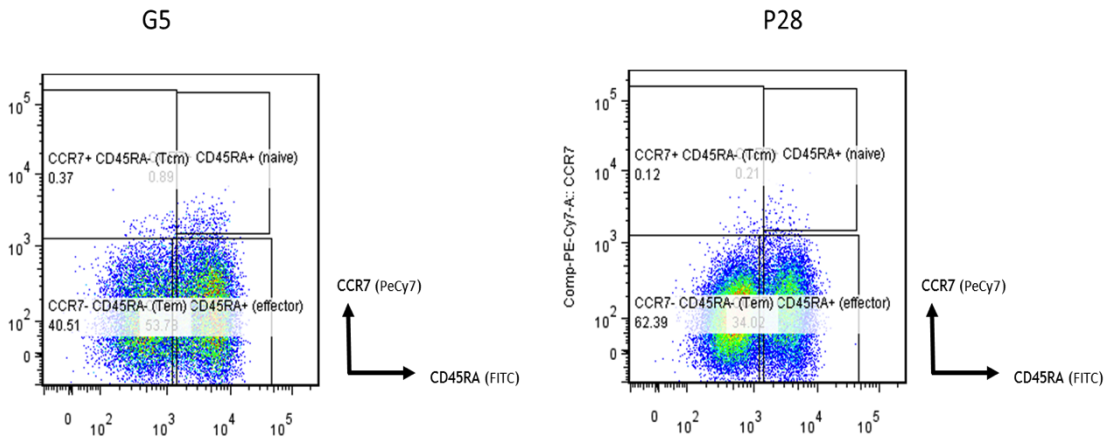
Figure 3-60: Gating CD3+ cells into CD4+ and CD8+ sub-populations



Qdot and PerCP-Cy5.5 antibodies were used to separate CD4 and CD8+ cells respectively.

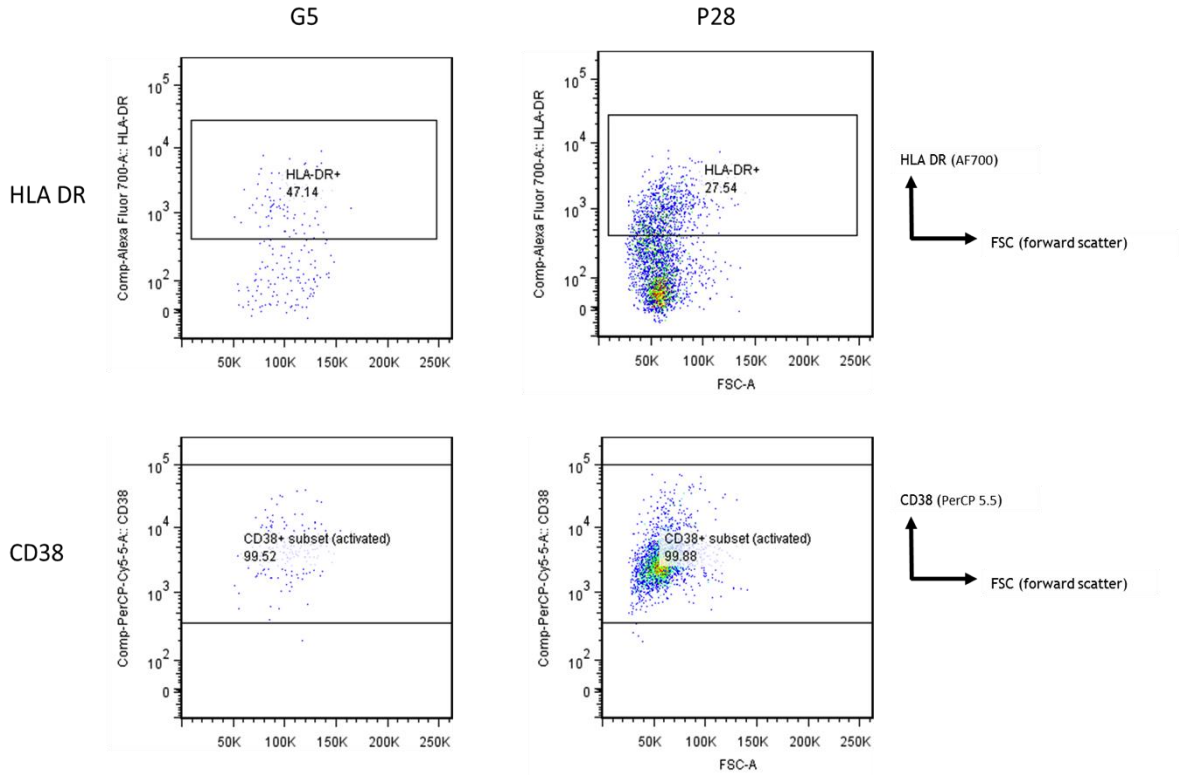
No clear difference in secretion of CD4+ or CD8+ IL2, IFN or TNF was observed between these representative patients and the phenotype of CD8+ cells was similar, although a larger proportion of effector CD8+ cells was observed in the clearer patient (G5) than the progressor (P28); Figure 3-61.

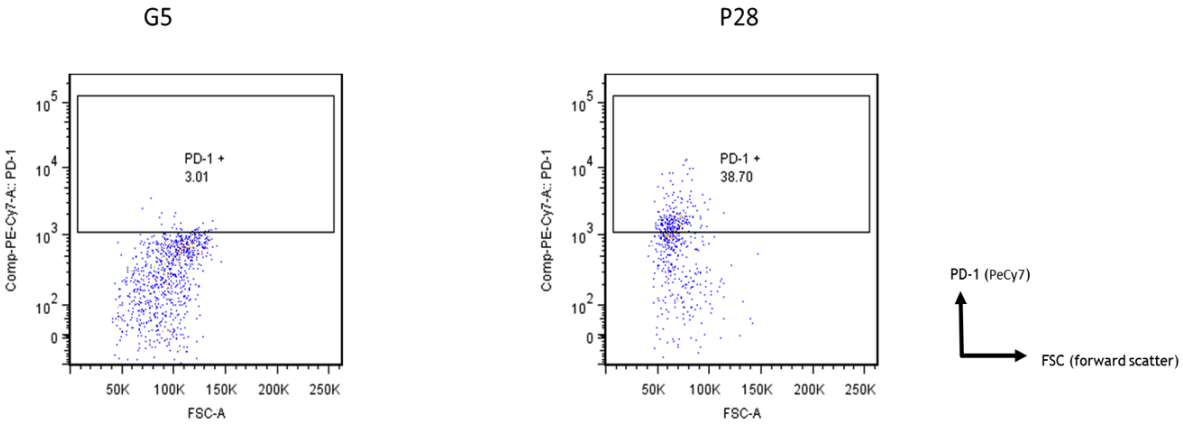
Figure 3-61: CD8+ cell populations in G5 (clearer) and P28 (progressor)



We nexct used pentamer staining to examine the phenotype of the HCV specific response directed against the NS5B2841 epitope. We examined the activation markers HLA-DR, CD38 and inhibition marker PD-1. No clear difference in the proportion of cells with NS5B2841 pentamer staining was seen in each patient and the pentamer positive cells in both patients showed an activated phenotype (CD38+) although HLA DR expression was higher in G5; Figure 3-62. Additionally, expression of PD-1 was higher in P28.

Figure 3-62: Activation and inhibition markers in patients G5 (clearer) and P28 (progressor).

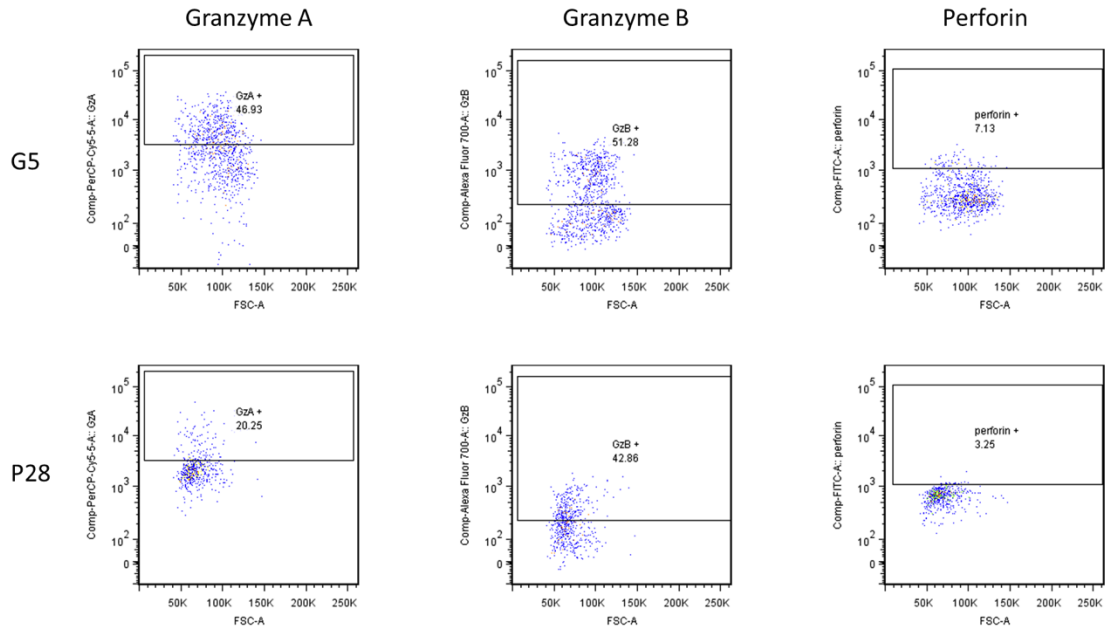




While the proportion of pentamer + CD8+ cells was similar in each patient, the proportion of granzyme A, B and perforin staining in pentamer stained cells was higher in the clearer than the progressor, suggesting a difference in functionality in cells recognising the $NS5B_{2841}$ epitope (Figure 3-63).

Finally, we looked for evidence of degranulation using granzyme A and B and perforin staining in the same patients.

Figure 3-63: Granzyme A and B and perforin staining in G5 (clearer) and P28 (progressor)



In the clearer patient (G5), higher secretion of granzymes A and B and perforin was seen, when compared with the progressor patient (P28).

A summary table is shown below outlining evidence of selection and T cell responses in the patients studied Table 3-21. A trend towards a larger number of mutations within $NS5B_{2841}$ was seen but did not reach statistical significance (the median number of mutations in clearers was 0 and 3 in the progressor group; p=0.08; Mann Whitney test). Clearance occurred in both HIV negative patients while progression occurred in 80% of coinfected patients (p=0.13; Fishers exact test).

Table 3-21: Summary of the immune response in the HLA B27+ cohort

Patient	Pattern	Selection within $NS5B_{2841-2849}$	Highest (SFU)	T cell response (ELISpot)	HIV
P49	Clearer	WT	16	Weak	Yes
G5	Clearer	WT	95	Strong	Yes
G18	Clearer	1 mutation	815	Strong	No
G17	Clearer	NA		Strong	No
P110	Progressor* (2 episodes)	3 mutations	23	NA	Yes
P45	Progressor	WT		Weak	Yes
P28	Progressor	3 mutations	5	Weak	Yes
P10	Progressor	3 mutations	NA	NA	Yes
P113	Progressor	2 mutations	14	Weak	Yes
P3	Progressor	NA		NA	Yes

*P110 was initially classified as a spontaneous clearer as he had 2 episodes of infection, both of which resolved with an intervening period between viraemic episodes of >6 months. However, the phylogeny showed a highly similar virus indicating the presence of fluctuating viraemia and progression rather than 2 episodes of clearance. The presence on relapse of 3 escape mutations within $NS5B_{2841}$ is also in keeping with progression.

Chapter 4: Discussion

Several studies have shown that host immune responses against HCV play a crucial role in controlling the infection. HCV clearance is linked to strong specific adaptive immune responses (CD4+ and CD8+ T cells), which are known to increase and accumulate in the infected liver (Ascione et al., 2007, Jacobson et al., 2010, Lechner et al., 2000c, Thimme et al., 2001a). In addition, the role of specific CD8+ T cells has been shown in an animal model (chimpanzee);depletion of these cells was associated with progression of infection (Shoukry et al., 2003a). Failure of specific CD8+ T cells responses in most HCV patients and persistence and progress to chronicity is still not very well understood (Simmonds, 2013, Neumann-Haefelin et al., 2005). More than one mechanism may lead to immune failure including the inability to make new T cells, exhaustion of CD8+ T cells functionality, or the appearance of escape mutations (Thimme et al., 2012, Sievert et al., 2011, Neumann-Haefelin et al., 2005, Rehermann and Nascimbeni, 2005). Growing evidence suggests that escape mutations from T cell responses emerge early during the acute phase of HCV infection and continue to appear in the circulation for many years (Weiner et al., 1995). Nevertheless, the evaluation of viral escape mutations during the infection with HCV is not always present . For instance, viral resolution can occur with minimum variation in the epitope before resolution. Several studies carried out on humans and chimpanzees show that not all epitopes restricted by human leukocytes class I (HLA class I) require multiple variations to produce escape mutations (Yahia, 2011, Chang et al., 2007, Erickson et al., 2001).

As the CD8+ T cells response against specific epitopes is restricted by several HLA class I alleles, it is clear that HLA plays a crucial role in the outcome of infection. A study that was carried out on women who were infected with HCV from the same single source showed that the HLA class I alleles A03, B27 and Cw01 were found more in patients who cleared the HCV infection compared with patients who progressed to chronicity. Significantly, HLA-B27 has been observed as the strongest allele that is associated with viral

clearance (Tester et al., 2005). HLA-B27 has also been found to play a significant role in HIV as it is slows disease progression, for instance, in delaying the decline of CD4 T cells and onset of symptoms and lowering the HIV viral load (Trachtenberg et al., 2003, Scherer et al., 2004). In agreement with this finding, an immunodominant HLA-B27 restricted HIV-specific CD8+ T cell epitope has been studied (Nixon et al., 1988b), and the onset of viral escape mutations within this epitope has a high impact on the HIV outcome by progression toward AIDS (Goulder et al., 1997).

4.1 The T cell mediated immune response to acute HCV

In this study, the central aim was to investigate the T cell response to acute HCV infection in patients with the HLA B27 allele who were infected or uninfected with HIV. A next generation sequencing approach was used to sequence the whole genome of HCV to look for evidence of positive selection within HLA B27-restricted epitopes, a replicon system was used to investigate the impact of mutations within the immunodominant $NS5B_{2841\text{-}2849}$ epitope and ELISpot assays and flow cytometry were employed to determine the phenotype and function of the T cell response.

The HLA B27 allele is known to be associated strongly with spontaneous clearance of HCV (around 80% of such patients spontaneously clear HCV; a reversal of the likelihood of clearance in other individuals). Within the Acute HCV UK cohort, we noted that patients with HIV had a lower likelihood of spontaneous clearance (37.5% versus 100%) and aimed to identify if this was due to disruption of the T cell response in these patients.

4.2 Failure of the T cell response in acute HCV infection

During progression to chronic infection, the CD8+ T cell responses may fail due to a weak directed response (T cell exhaustion) or a misdirected response due to immune escape. Studies carried out in humans and chimpanzees within the acute phase show that in some patients CD8+ T cells are weak or

become exhausted during the early stages of infection (Cooper et al., 1999b, Thimme et al., 2002, Thimme et al., 2001b). In chimpanzees, specific T cells may be found in the liver and in the periphery, but they do not have the ability to clear the infection (Walker, 2010). In humans, the failure of specific CD4+ T cells and CD8+ T cells against HCV infection needs to be investigated more (this has been under-studied due to a lack of patient cohorts identified in the acute phase).

Impaired priming of specific CD8+ T cells against HCV may also occur due to a low number of functional antigen presenting cells (Walker, 2010).

4.3 Viral escape

As HCV is an RNA virus with a high replication rate of an average 10^{12} virions per day (Neumann et al., 1998), changes within the genome (occurring as a result of lack of proof-reading function of the RNA polymerase) can result in rapid escape from virus-specific CD8+ T cell responses. The first descriptions of virus escape from the immune system came from chronically infected patients (Chang et al., 1997) and from a chimpanzee that was infected experimentally (Weiner et al., 1994, Erickson et al., 2001). Importantly, HCV escape mutations have more recently been found to become established within early phase of infection (acute phase) and are significantly associated with proceeding to chronicity (Erickson et al., 2001).

In various studies of patients with early HCV infection, viral escape from specific CD8+ T cell responses was not detected within patients who subsequently spontaneously cleared the infection but was detected within patients who progressed to the chronic phase (Timm et al., 2004b, Cox et al., 2005a, Cox et al., 2005b). During chronicity, mutations within CD8+ T cell epitopes were found to revert to the original sequence as T cell responses regressed (Cox et al., 2005b). In patients with dissimilar HLA types, reversion within HLA-restricted epitopes can be seen following transmission of the virus; transmission of an HLA-B8-restricted epitope to a non-HLA-B8 patient

resulted in rapid reversion of this mutation (Timm et al., 2004a).

In another study of Irish rhesus negative women infected acutely with immunoglobulin therapy, changes in amino acid sequences in HLA-restricted epitopes were seen with reversion in other women who did not carry a similar allele (Ray et al., 2005a). This phenomenon may be explained by the viral fitness cost concept, where viral escape mutations have an impact on reducing viral replication capacity (Bowen and Walker, 2005a).

Thus, immuno-dominant CD8+ T-cell responses may leave footprints in the viral sequence within the viral genome. If this does not occur at a fitness cost to the virus, such immune escape footprints may become fixed at the population level. A study carried by Thimme and colleagues in 2007 showed a particularly strong association between particular sequence variations driven by the CD8+ response in HLA-B27+ patients. A single immuno-dominant CD8+ epitope at position 421 of the NS5B protein was found in this study to be associated with a protective CD8+ response (Timm et al., 2007b, Neumann-Haefelin et al., 2006). This finding fits strongly with the hypothesis that CD8+ T-cells mediate immune pressure during acute HCV infection.

Mutations within such immuno-dominant epitopes can result in viral escape from HCV-specific CD8+ T-cell responses. Mutations within the anchor binding sites (usually positions 2 and 9 of a peptide epitope sequence) and the C-terminal amino acid can weaken peptide binding to the HLA class I molecule (such as HLA B27). Mutations located in the centre of the HLA molecule reduce the interaction with T cell receptors (TCR) (Soderholm et al., 2006). Mutations within flanking regions of the epitope may also have an impact by inhibiting proteasomal processing of the protein precursor (Seifert et al., 2004, Timm et al., 2004a, Kimura et al., 2005).

In experimentally infected chimpanzees, an impaired CD4+ T-cell response has been associated with escape from the CD8+ T-cell response and progression to chronicity (Grakoui et al., 2003b). This suggests that viral escape within specific CD8+ T-cell epitopes may occur in part as the result of

weak help from CD4+ T-cells. Another study carried out by Meyer *et al*. in 2004 showed that low diversity within the TCR repertoire could play a role in viral escape (Meyer-Olson et al., 2004). Viral escape (Boettler et al., 2005) has been shown to be strongly associated with escape from immuno-dominant HLA-restricted CD8+ responses; indicating that such class I HLA alleles have a substantial impact on the immune response (Schmidt et al., 2011).

A major limitation on viral escape is virus fitness cost, which reduces the likelihood of persistence of a specific escape mutation. An epitope within an essential area for function of a particular viral protein increases the likelihood of a protective CD8+ T cell responses; this has been shown within specific HLA-B27 and HLA-A3-restricted epitopes (Dazert et al., 2009, Fitzmaurice et al., 2011).

The HLA B27-restricted $NS5B_{2841-2849}$ epitope, situated adjacent to the active site of NS5B is particularly well-described. Mutations accrued sequentially within this epitope (cluster mutations) can diminish the HCV fitness cost by restoring viral replication. In one study, all HLA-B27+ patients infected chronically with HCV genotype 1 had clustered mutations within a single immune-dominant epitope that were strongly associated with HCV T cell escape (Dazert et al., 2009). As multiple viral immune escape mutations may take a long time to emerge within patients who have the HLA-B27 allele, the immune system gains time to react to cognate epitopes.

This observation is in line with another study of the protective HLA-A*03 allele; this allele was found to be protective in Irish women infected with HCV genotype 1b (McKiernan et al., 2004). Two separate studies carried out by Merani *et al* and the Klenerman *et al* showed that there is a strong association between this HLA class I allele and cluster mutations; these mutations were clearly observed within an HLA-A*03 restricted epitope within the NS3 protease protein.

Viral escape mutations can be found in MHC class II-restricted epitopes as well, but such mutations are far less frequently found than escape within

CD8+ MHC-class I-restricted epitopes in both humans and chimpanzees (Fleming et al., 2010). Other factors, such as impaired CD4+ T-cell function, are very likely to play a role in CD8+ epitope viral escape. Data from one study suggested that 50-70% of HCV immune escape occurs within targeted CD8+ T-cell epitopes (Neumann-Haefelin et al., 2008).

4.4 CD8+ T-cell dysfunction

Several studies have shown that viral escape is not the only reason for failure of the immune response to HCV infection; dysfunction of CD8+ T-cells also plays a significant role in progression to chronicity. Examples of T cell dysfunction include lack of production of antiviral cytokines (e.g. IFN-γ) or lack of proliferation occurring despite contact with cognate antigen (Nakamoto et al., 2009a).

A dysfunction pattern is commonly observed in HCV patients within the chronic phase and in patients within the acute phase who subsequently progress to chronicity. Such T cell dysfunction occurs in association increasing expression of inhibitory receptors such as PD-1 on CD8+ cells (Golden-Mason et al., 2007, Radziewicz et al., 2007a) and a decrease in expression of CD127 (Bengsch et al., 2007).

CD8+ T-cells within the liver that have high PD-1 expression have been found to be prone to apoptosis (Radziewicz et al., 2008). Blocking antibodies targeting PD-1 can increase proliferation of both CD127+ and PD-1+ HCV-specific CD8 T-cells (Radziewicz et al., 2007a). Another study showed that an increase in T-cells function could be elicited by blocking another inhibitory receptor (CTLA-4) (Nakamoto et al., 2009a).

This suggests that such T-cells are exhausted due to the activation of inhibitory receptor pathways (Kasprowicz et al., 2008). Several other factors may contribute to or be associated with impairment of the CD8+ T-cell

responses such a lack of CD4+ T-cell helper cell support or upregulation of T regulatory cells.

4.5 Lack of CD4+ help

CD8+ T-cells are the main effector cells against HCV infection. Strong CD8+ responses require significant contributions from CD4+ T helper cells. Studies of the mouse LCMV model have clearly shown how a significant contribution from CD4+ T-cells helps to support an effective cytotoxic CD8 T-response (Matloubian et al., 1994). Patients with chronic HCV infection commonly exhibit poor CD4+ T-cell responses that may lead to low functionality of CD8+ reponses (Semmo et al., 2005b).

4.5.1 Importance of the HLA-B27 allele and the association with spontaneous clearance of HCV

As discussed above, the HLA class I B27 allele has been associated with selection of a key HLA-B27-restricted epitope within NS5B ($NS5B_{2841-2849}$) and more recently selection within several other B27-restricted HCV epitopes. It also has an essential protective role in patients infected with HIV in association with recognition of a structurally unrelated epitope within HIV gag (Goulder and Watkins, 2008, McKiernan et al., 2004).

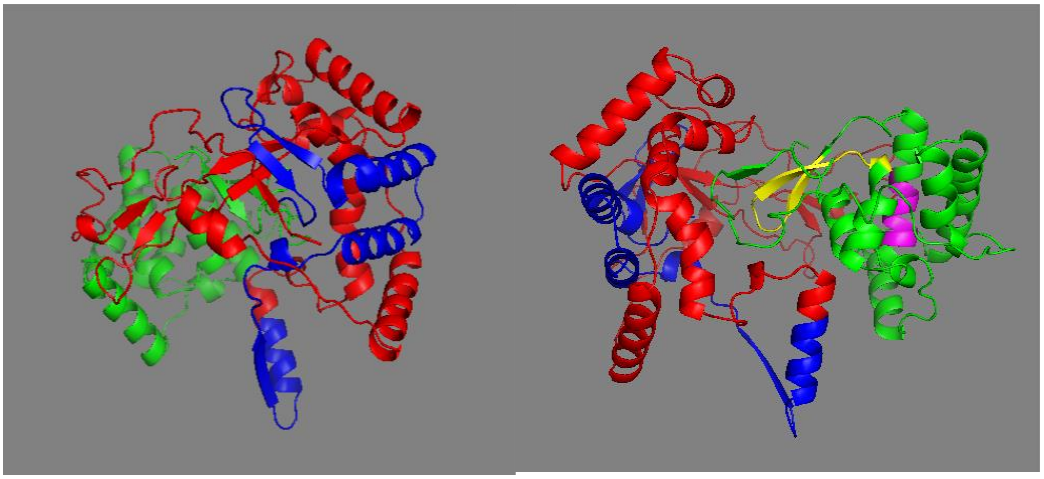
In patients with HIV infection, viral escape from the HLA-B27 restricted gag epitope has an impact on viral replication by lowering viral load, prolonging the CD4+ T-cell decline and delaying the start of AIDS symptoms (Goulder and Watkins, 2008). As has already been described, for escape from the $NS5B_{2841-2849}$ HCV epitope, escape from the B27-restricted HIV gag KK10 epitope also requires a cluster of mutations to evade the acquired immune response. A mutation noticed within this HLA-B27 restricted epitope (L268M) weakens recognition of the T cell response and this mutation can also negatively affect DC function (McMichael, 2007). Another mutation (R264K), at the main binding anchor site within the epitope, also leads to escape. This mutation often occurs in the later stages of HIV infection (McMichael, 2007).

The R264K escape mutation has an impact on viral replication fitness when it is expressed alone and is often associated with another mutation, located 90 amino acids upstream of the HLA-B27 epitope (S173A), which works as a compensatory mutation (McMichael, 2007, Schneidewind et al., 2008, Streeck et al., 2008). In summary, the HLA-B27 is associated with strong, significant, durable control of both HCV and HIV due to effective immune responses targeted against key B27-restricted epitopes. These responses may be partially abrogated by immune escape in association with compensatory mutations.

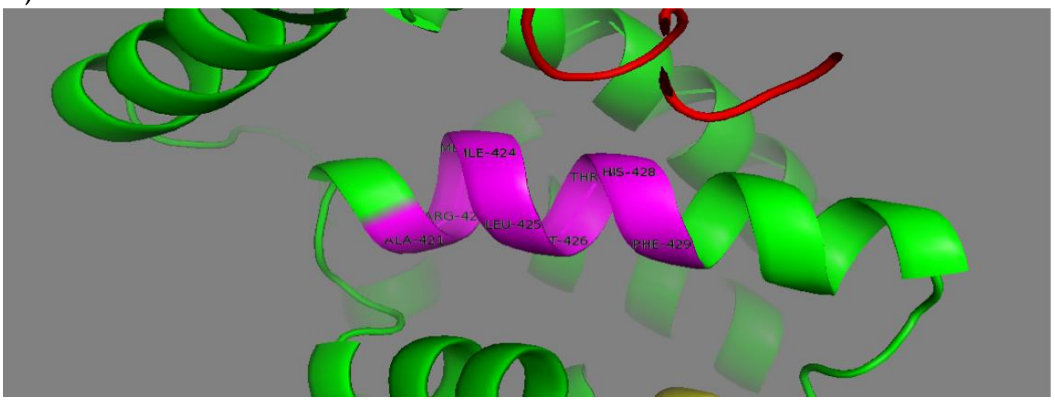
The immuno-dominant HLA-B27-restricted HCV-specific CD8+ T-cell epitope $NS5B_{2841-2849}$ of HCV (wild-type sequence ARMILMTHF) described above is present in patients infected with HCV genotype 1 (but not other HCV genotypes) and is associated with spontaneous resolution of HCV infection (McKiernan et al., 2004, Hraber et al., 2007). This HLA-B27 restricted immune response could help explain why other HCV genotypes are not cleared spontaneously as readily as other genotypes (Neumann-Haefelin et al., 2010). HLA-B27+ patients that progress to the chronic phase classically display cluster mutations indicating a high level of selection pressure within this epitope (Neumann-Haefelin et al., 2006).

Figure 4-1: Structure of NS5B

A) B)



C)



(A) NS5B (front) displayed and coloured according to individual domains using Pymol (red-fingers; blue-palm; green-thumb). (B) NS5B (back). The HLA B27-restricted $NS5B_{2841-2849}$ epitope (upper right and lower panels) is shown in pink. It is located at the thumb domain and close to the finger domain (C) Close-up of the epitope region with sequence positions.

In this thesis, multiple mutations within the immunodominant $NS5B_{2841-2849}$ epitope were found to be present in HLA B27+ patients with genotype 1a acute HCV. In keeping with the studies described above, escape within this epitope was seen in patients with HCV that subsequently progressed to chronicity and interestingly in one patient (P110) that displayed spontaneous clearance of HCV on two occasions.

The predicted structure of the mutations observed in this patient was not hugely altered however, despite the presence of multiple clustered mutations (Figures 3-23 and 3-26) .

Mutations observed *in vivo* were introduced into a genotype 1a replicon system in order to assess viral fitness and to provide a model reflective of the genotype 1a sequence observed in patients in this cohort (previous studies have all employed a genotype 1b replicon system). The majority of epitope mutations observed in this study were within TCR contact residues rather than anchor sites; this has been observed before in other studies. Although not observed in this study, mutations were introduced within HLA-B27 binding anchor sites in order to assess the impact this would have on viral replication (under the hypothesis that these do not persist due to poor replicative fitness). Such mutations were associated with reduced replication.

As in other studies, multiple clustered viral escape variants were observed in patients with progression to chronicity, highly suggestive of successful viral escape from the specific CD8+ T-cell response as observed in other studies (Dazert et al., 2009). The accumulation of mutations appeared to occur sequentially during early infection. In theory, the HCV immune response could be primed by including this conserved region in a vaccine (Timm et al., 2007a).

4.5.2 Mutations detected within HLA-B27 epitopes using NGS

Deep sequencing generates a vast amount of sequence data, with more than 10 million nucleotides of the viral genome produced in each sequencing run. Low-frequency mutations may be visualised with higher depth than ever before and variants that enable escape from T-cell immune pressure can be identified (Bull et al., 2011a, Plauzolles et al., 2013). In this study, we aimed to investigate the role of HLA-B27 in defining outcomes in early HCV infection in a cohort of individuals with or without HIV co-infection (Thomson et al., 2010).

Advances in next-generation sequencing techniques allowed us to sequence full HCV genomes in the majority of patients, even those with low viral loads at the time of sampling (one genome was obtained from a patient with a viral load of 50 IU/ml). Two approaches were used; metagenomic and target-enrichment-based sequencing.

Target enrichment was found to be a more efficient and sensitive method than metagenomic sequencing and genomes detected using both methods were found to be identical, indicating that the purification of HCV with target probes did not result in biased sampling.

Known HLA-B27 restricted CD8+ T-cell epitopes within the HCV genome were examined to investigate the natural history of accumulation of mutations within these epitopes and variation across the entire genome was also studied using measurement of pairwise distance and dN/dS ratios. In the majority of patients, a gradual accumulation of mutations over time occurred within a well-described immuno-dominant HLA-B27-restricted epitope ($NS5B_{2841-2849}$) in evolving progressors, showing allele-specific selection pressure (Neumann-Haefelin et al., 2006, Timm et al., 2007a). Such mutations are associated with CD8+ T-cell viral escape and have been described in HLA B27+ patients with chronic HCV, although accumulation of these mutations and their early accumulation in acutely infected patients has not been previously described.

We examined other mutations recently described to occur in HLA B27+ patients but did not find significant immune selection within these in evolving progressor patients or spontaneous clearers.

We next aimed to identify novel epitopes using a bioinformatic method based on known binding of the HLA B27+ molecule to amino acid sequences and the presence or absence of essential contact sites e.g. to metal ions or other amino acids using structural pdb data (in collaboration with Dr Sreenu Vattipally).

Although the $NS5B_{2841-2849}$) epitope was correctly identified using this method, other predicted epitopes did not result in functional T cell responses by ELISpot analysis. However, the presence of another HLA B27-restricted epitope was implicated by a significant dN/dS signal within the core of HCV. Peptide mapping would help to narrow down the location of this epitope but would require a large number of cells – further studies to examine this finding further in similar patients are indicated.

Immunological analysis using class I pentameric complexes has shown that a single mutation in the $NS5B_{2841-2849}$ epitope slightly decreases T-cell binding and the release of interferon gamma (IFNγ) but that multiple (more than one mutation) clustered mutations result in the elimination of T-cell recognition (Dazert et al., 2009).

We used Illumina deep sequencing to identify variation within these HLA-B27 epitopes over time during acute HCV infection in order to associate these with spontaneous clearance or progression to chronicity. NGS identified one patient with mixed infection with other genotypes; P28 had mixed infection with genotypes 1a and 4d. In this patient, mixed infection did not substantially affect the number of mutations detected identified but variation in replication of co-dominant strains could in theory affect viral escape in other patients.

The majority of patients from the progressor group developed mutations within the $NS5B_{2841}$ epitope over time; we hypothesized that this was due to the effect of HIV and an impaired CD8+ T-cell response against HCV and aimed to investigate this further by measuring T cell function using IFN ELISpot assays.

The majority of patients with spontaneous clearance of HCV had 0-1 mutations within all previously described HLA-B27 epitopes. One patient who had a late spontaneous clearance of HCV more than a year after infection (P110) showed evidence of clustered mutations within a single epitope ($NS5B_{2841-2849}$). However, this patient later develop recurrence of infection

and was reclassified as a progressor patient.

Viral persistence in the absence of T-cell escape may reflect a failure of the CD8+ T-cell response. As discussed above, this may occur as a result of an insufficient CD4+ T-helper cell response (especially in the context of co-infection with HIV) or of upregulation of inhibitory markers such as PD-1 expression that are associated with T-cell exhaustion (Dazert et al., 2009). We aimed to investigate the mechanism of persistence in those patients with no evidence of positive selection in known or unknown epitopes using ELISpot and flow cytometry when sample availability allowed.

Multiple clustered mutations of (two or more) amino-acids were found to be associated with markedly reduced viral replication. This supports our observation that wild-type variants (and variants with single amino acid substitutions) decline and are replaced by fitter triple mutants as the virus establishes persistence. These mutations may not emerge in patients who do not raise an effective T-cell mediated immune response; this may indeed be more likely in patients co-infected with HIV as the CD4+ T-helper cell response may be compromised (Grakoui et al., 2003a, Tester et al., 2005, Timm et al., 2004b).

Stratifying patients by HLA type has not been previously possible in most studies as HCV patients during the acute phase are usually asymptomatic, so most studies have been of 10-20 patients at a time and HLA-B27 only occurs with a percentage frequency of approximately 8%. Furthermore, HCV is only persistent in around 30% of HLA-B27+ patients (Gonzalez-Galarza et al., 2011); thus a follow-up of this group of patients during their evolving progression has never been reported until now. We found in this study that while both HLA B27+ HIV uninfected patients spontaneously cleared infection in keeping with other studies, the majority of HIV-infected patients exhibited persistence. A previous study showed no variations in the HLA-B27-restricted NS5B epitope in patients that had acute but resolving HCV infection. This supports the concept that the absence of viral escape can help to protect patients. We generally observed single mutations in the epitope in patients

during spontaneous clearance – this was likely to have been insufficient to result in immune escape and may additionally have been detrimental to viral replication.

Adaptation to T-cell immune pressure is characteristic of infection with HCV. However, while HCV has a high mutation rate, the viral genome appears to show restricted plasticity, as evidenced in certain regions by an accumulation of silent synonymous (rather than non-synonymous) changes. While inaccurate replication has advantages, the possibility of error catastrophe is ever-present and may result in the production of non-viable or replication-deficient viral strains. In this study, the majority of sites studied using dN/dS ratios showed evidence of purifying (negative) selection although a single significantly positively selected codon was identified in HLA B27+ patients.

It is likely that the CD8+ T-cell immune pressure that arises in HLA-B27 patients in reaction to a single NS5B epitope is sufficient to clear the virus in most patients infected with HCV before multiple mutations can occur in TCR residues. However, unlike the majority of HIV-uninfected patients, most of the patients in our HIV-positive cohort proceeded to chronic infection. The mechanism for failure of the immune response in these patients was most likely to be the immune escape from the CD8+ T-cell response (as evidenced by the gradual accumulation of mutations within the $NS5B_{2841\text{-}2849}$ epitope). This is likely to have been worsened by a dysfunctional CD4+ T-cell response due to HIV co-infection. Flow cytometry in representative patients revealed that CD8+ cells that stained with the $NS5B_{2841\text{-}2849}$ epitope (pentamer positive cells) had higher HLA-DR and lower PD-1 expression in a spontaneous clearer patient than a progressor indicating an activated profile. In addition, the spontaneous clearer had higher expression of functional CD8+ proteins granzymes A and B and perforin. Thus although the epitope was recognised by CD8+ cells in the progressor patient, these had a less activated phenotype.

4.5.3 Functional analysis of observed mutations in the $NS5B_{2841-2849}$ epitope

In this series of experiments, we aimed to study whether single or clustered mutations observed *in vivo* within or outwith the highly conserved $NS5B_{2841-2849}$ epitope (Figure 3-43) have an impact on viral replication (fitness cost). Mutations observed *in vivo* were introduced into a subgenomic replicon (Figure 3-42).

The replication kinetics of mutant variants were compared with wild-type HCV genotype 1a replicon (WT 1a), a wild-type genotype 1a replicon containing GND (1a GND; this is a replication-deficient subgenomic replicon incorporating a GDD to GND mutation within NS5B), the genotype 2a JFH-1 subgenomic replicon (pSGR-luc-JFH1) and the JFH-1 subgenomic replicon containing GND (pSGR-luc-JHF1-GND).

4.5.3.1 **Single mutations**

A g1a replicon mutant containing the single mutation A2841V replicated poorly, with a reduction in replication that was seven-fold lower than WT (1a). Previous studies utilising site-directed mutagenesis of a genotype 1b HCV replicon showed that a single mutation led to replication at a similar level to the wild type. Additionally, a single R2842V mutation (an HLA B27 anchor site) showed a 7-fold decline in luciferase activity compared to the sub-genomic replicon indicating that changes at this site are associated with a significant viral fitness cost.

Our results differ from previous work; this may reflect the use of a different replicon model based on genotype 1a rather than 1b. Moreover, we selected mutants that were observed *in vivo* while other studies were based on hypothetical sequential mutations during evolving progression to chronicity during the acute phase, which had not been previously examined.

4.5.3.2 **Multiple mutations**

Next, double mutations were tested; the A2841V/M2843V replicon resulted in a nine-fold lower replication compared to wild-type replicon. Patients with triple mutations or double mutants with an additional variant site outside the HLA-B27 epitope (T2838A) showed restored replication. Triple mutations were introduced sequentially in addition to the other two variations, such as, A2841V/M2843V/I2844V. Replication capacity based on luciferase activity was increased compared with single or double mutation variants –around 3-fold lower than WT replicon.

A mutation (T2838A), commonly found outside the HLA-B27 epitope in HLA B27+ evolving progressor patients, was found to restore the replication capacity to a similar level compared to the genotype 1a sub-genomic replicon.

In patients infected with HIV, multiple mutations within the HLA-B27 $NS5B_{2841\text{-}2849}$ epitope occurred frequently and were associated with progression to chronicity. These mutations emerged late and may indicate that immunosuppression associated with HIV may result in impaired CD8+ responses (in particular lack of CD4+ support as evidenced by lack of IL-2 production in such patients).

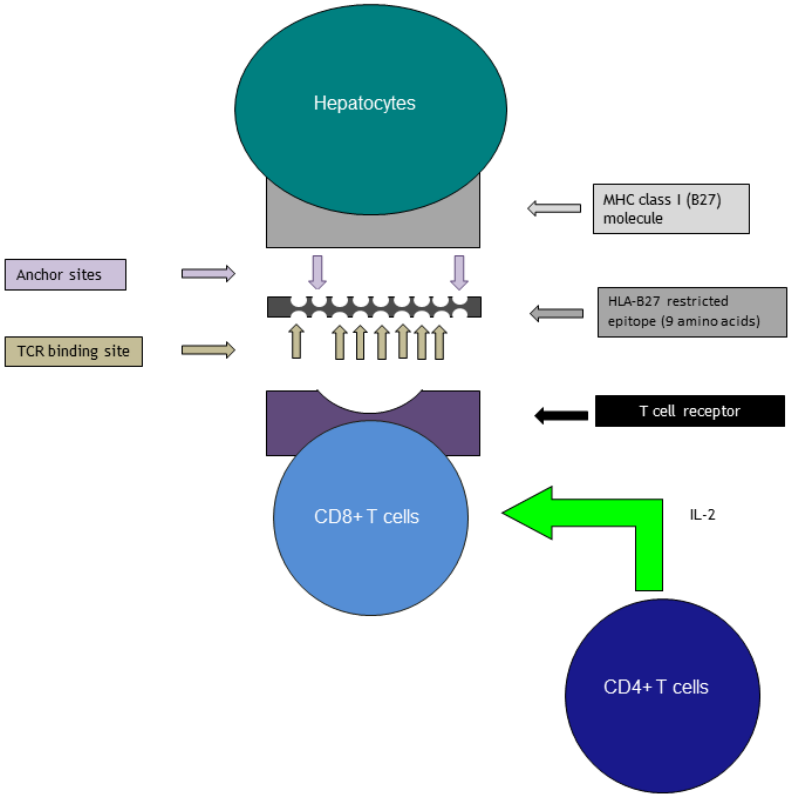
In the B27+-directed HIV immune response, escape from immuno-dominant responses also requires clustered mutations (Flicek and Birney, 2009, Grüner et al., 2000, Metzker, 2010). Mutations occuring within anchor binding sites within the dominant HCV epitope can result in immune escape but may be associated with a fitness cost that is too high.

Previous work has shown that escape can occur quickly when the second position arginine that first within the HLA B pocket is mutated (Dohm et al., 2008, Fishman and Branch, 2009, Hillier et al., 2008a). Our data suggest that the acquisition of mutations results in immune escape occurring as a result of reduced T-cell receptor binding rather than of HLA binding, likely due to the

impaired fitness cost of a mutation within this anchor position (Table 3-13).

The potential impact of variations within the NS5B region can be further analysed by considering the structural features within the HLA-B27 restricted epitope (Figure 4-1). The distinctive shape of the Hepatitis C virus NS5B RNA-dependent RNA polymerase results from an extended interaction between the two subdomains (the fingers and thumb). This link between these two subdomains is facilitated by an insertion inside the fingers subdomain. The insertion inside the fingers subdomains form two loops that pack against the thumb subdomain. Residues located between two motifs (motif A and B) create two amphipathic α-helices, which are packaged in an antiparallel arrangement next to a B helix. The third subdomain of the NS5B (thumb) includes residues from 371-528; these residues form seven different α-helices and a ß-loop (Lesburg et al., 1999).The $NS5B_{2841-2849}$ epitope is positioned within the helix Q of NS5B. This helix is functionally critical for the interaction of the two subdomains, the thumb and fingers of NS5B, and flanks the two pockets that contain the NNI binding sites A and B. Some of the HLA-B27-restricted epitope residues such as A2841, I2844, and M2846 are completely enclosed within the thumb domain in the hydrophobic core.

Figure 4-2: Model illustrating presentation of class I epitopes to CD8+ cells



These amino-acids can be exchanged with other amino-acids that have an equal type and size (hydrophobic amino-acids) within the hydrophobic core without resulting in a major structural impact, which explains the observation that these substitutions appear to have only a slight effect on viral fitness.

In contrast, substitutions of residues R2842, M2843, H2848, and F2849, which line pockets A and B at the thumb surface, would have a much more marked structural effect; this may explain the stronger effect on viral replication and the high degree of conservation at these sites. One residue has considerable impact (T2847), as it is also located within the thumb domain in the hydrophobic core; this amino acid reported as being an essential amino acid in the stabilization of this region. Substitution of this amino acid with another amino acid would be highly likely to have an impact on the rearrangement of this critical region (NS5B) (Dazert et al., 2009).

4.5.4 CD8+ T-cell responses in evolving spontaneous clearance and progression to chronicity.

This is the first study examining HLA B27+ restricted T-cell responses during acute HCV infection within an HIV-HCV co-infected cohort. Our results show that patients who cleared HCV spontaneously have a strong T-cell response as evidenced by ELISpot. In contrast, patients with evolving progression to chronicity display weaker T cell responses and this was significantly more likely in patients with HIV.

We measured the immune response in both evolving spontaneous clearance and progression to chronicity using ELISpot assays following stimulation with overlapping peptide pools spanning the whole HCV genome. Patients who cleared HCV had significantly stronger IFN-γ responses; in keeping with findings from other studies (Heim, 2013, Neumann-Haefelin and Thimme, 2011) whereas patients who progressed to chronicity showed weak responses. Core, NS3, and NS5B peptide pools elicited the highest IFN-γ responses.

Peptides predicted to elicit protective immune responses in HLA B27+ patients were also tested. While peptides spanning the HCV genome were found to be significantly stronger in HLA-B27+ patients versus HLA-B27- ones, the predicted peptide set showed no significant difference between groups.

Our study showed the importance of this epitope in stimulation of T-cell immune response in acute HCV infection. Clearly, a specific definition of immunodominant HCV epitope across HCV genotypes is essential, and more work needs to be carried to develop a new vaccine. This epitope in addition to other epitopes across the HCV genome might be used to prevent the disease, or in infected patients can be used as prophylactic and a therapeutic hepatitis C virus vaccine to stimulate the adaptive immune response and eliminate the HCV infection.

This study is limited by two major restrictions; firstly the number of patients available to study the impact of the adaptive immune response in acute HCV and secondly, the number of samples available from patients at different time points. These restrictions have occurred because acute HCV infection is asymptomatic and not usually noticed by the patient and because patients with acute HCV are in vulnerable groups such as PWID who do not attend follow-up regularly. While the sample size is small, this is the largest acute HCV cohort ever studied for immune response targeting HLA-B27 restricted epitopes. This sample size may affect the statistical significance of the results; the conclusions of this study need to be considered as a precursor for further study of the same cohort with larger number of patients. Study also of other protective alleles such as HLA B57 may further strengthen the findings. The next generation sequencing work would have been strengthened by carrying out sequencing in duplicate or even triplicate. However such an approach would be extremely expensive and was not possible due to the amount of sample available for each timepoint. During the course opf the thesis, the laboratory has improved methods available for analysis and limitation of cross-contamination – currently, known RNA/DNA sequences are being put into each well during all stages of sample preparation; this allows

for detection of low-level contamination. Further work with this cohort, would benefit from this approach. The T cell work in this study was based on the use of heterologous peptides; the use of autologous peptides might have increasd detection of strong T cell responses but this approach is also expensive.

4.5.5 Conclusions and future work

In summary, in this study, patients with the HLA-B27 allele had a high rate of spontaneous clearance of HCV infection as observed in other studies (McKiernan et al., 2004). However, HIV-infected individuals had a lower than expected clearance rate with evidence of slow evolution of escape mutations within a previously described immuno-dominant epitope $NS5B_{2841-2849}$ or of weak T cell responses associated with no evidence of positive selection.

Other HLA-B27 restricted epitopes within the NS5B, p7 and NS3 proteins were not strongly associated with immune escape. Variation within the HLA-B27 epitope was associated with a stronger T-cell response. Our results therefore indicate that an HLA-B27 restricted CD8+ T-cell response in HIV infected individuals may be defective, most likely by impaired CD4 help for CD8+ cells.

Earlier studies indicate that protective responses may be sustained. In chimpanzee and human subjects, responses can be detected for as long as seven years following HCV recovery (Lechner et al., 2000b, Cooper et al., 1999b, Shoukry et al., 2003b, Cox et al., 2005a, Lauer et al., 2002). One study has shown that specific CD8+ T-cell responses can persist for decades following clearance of HCV (Takaki et al., 2000).

Variation also occurred (to a lesser extent) in other HLA-B27 restricted epitopes within the HCV genome (p7, NS3, and NS5B) suggesting escape in these epitopes also. Two patients (P28 and P45) showed clustered mutations

within an HLA-B27 restricted p7 epitope while no variations occurred within $NS5B_{2841-2849}$ epitope. One patient (P113) had mutations within the $NS5B_{2820-2828}$ epitope and one mutation within the $p7_{780-788}$ epitope. This data is in accordance with other published data showing that escape mutations from CD8+ T-cell responses happen as a result of clustered mutations within restricted immuno-dominant epitopes (Timm et al., 2004b).

Associations between the expression of other alleles (HLA-B7, HLA-B8 HLA-B25, and HLA-B37) and mutations within restricted epitopes which are presented by these alleles have also been described (Timm et al., 2004a, Ray et al., 2005b). Such associations highlight the importance of HLA- restricted T cell selection pressure and how it is associated with HCV evolution within hosts and at a population level. It is likely that the main factor determining the outcome of infection is whether variation in an epitope can result in a reduction in virus fitness; for instance, mutations within the genome affecting certain conserved amino-acids can lead to a loss of viral enzymatic functions and even change virus structure (Altman and Feinberg, 2004).

Interestingly, the NS5B protein is generally conserved among HLA-B27 negative patients. Other immunological factors can play a significant role in the disease outcome and in viral escape from CD8+ T-cell responses such as diversity within the TCR (Meyer-Olson et al., 2004) and a lack of strong CD4+ T-cell help. Chimpanzee models show an inadequate control of HCV replication via memory CD8+ T cells in the absence of strong support from CD4+ T-cells, resulting in viral escape from specific CD8+ T-cell responses (Grakoui et al., 2003a).

The HLA-B27 protective role in varied virus infections (both HIV and HCV) is not fully understood. Various mechanisms have been proposed, including the molecular characteristics of peptides presented by HLA-B27, differences in binding capacity of different forms of HLA-B27 (including heavy chain homodimers with variable tertiary structure), the failure of KIR3DL1 as a B27 ligand, resulting in increased NK cell stimulation or linkage disequilibrium with other immune gene responses (López de Castro, 2005,

Stewart-Jones et al., 2005).

To sum up, our results confirm an association between HLA-B27 restricted CD8+ T-cell responses and resolution of HCV infection in acutely infected patients. In addition, our results indicate that this association is reduced in patients with HIV co-infection in association with weaker T cell responses and escape within HLA-B27 restricted epitopes even in a cohort of patients with relatively preserved total CD4 cell counts. An accumulation of multiple clustered mutations within HLA-B27 restricted epitopes is required for viral escape from the CD8+ T-response. A summary of our findings is shown in Table 3-21.

A new compensatory mutation was observed outwith the previously described HLA-B27 restricted $NS5B_{2841-2849}$ epitope that restores viral replication. Additionally, a positively selected codon within core may form part of a previously undescribed epitope and further functional work is indicated to confirm this. All these data provide evidence that the outcome of HCV infection is controlled by highly specific CD8+ T-cell responses that are restricted by protective MHC class I alleles such as the HLA-B27 molecule.

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